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Universidad del País Vasco
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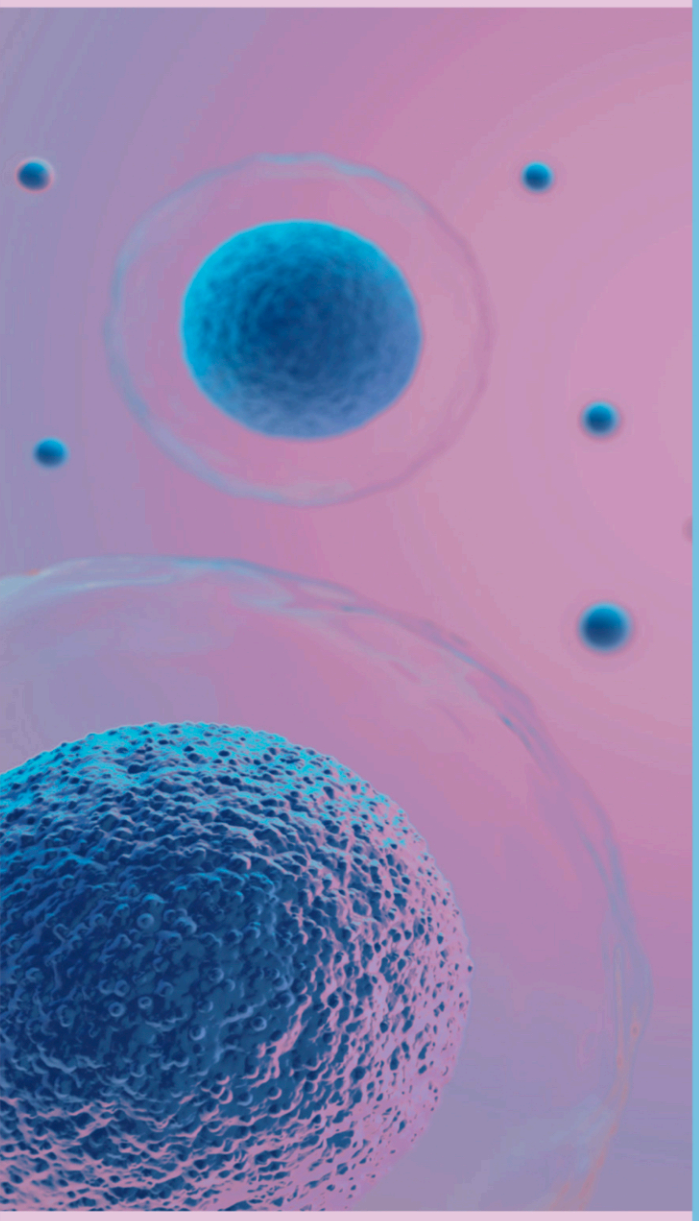

MikroRNAen potentziala B Zelula Handiko Linfoma Hedatsuaren diagnostiko, sailkapen eta pronostikoan

Doktorego tesia

Ane Larrabeiti Etxebarria

Leioa 2021

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Tesis doctoral / Doktorego tesia

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Nire bi izartxuei, Eneko eta Malen

Ama, zuretzat.

Esker onak

Quiero aprovechar estas líneas para agradecer el apoyo de todas las personas que han hecho posible esta tesis y que han estado a mi lado durante esta etapa.

En primer lugar, a África, gracias por brindarme la oportunidad de realizar la tesis y haber confiado en mí para este proyecto.

Eli, muchas gracias por tu gran apoyo y sobre todo por toda la paciencia que has tenido conmigo. Ha sido un camino duro en el que a pesar de todo el trabajo que tenías siempre has estado dispuesta a ayudarme y tirar para que esta tesis saliera adelante. Angela, desde la distancia has conseguido trasmitirme tu gran positividad y motivarme para no tirar la toalla.

¡Muchísimas gracias a las dos! Indudablemente sois unas maravillosas investigadoras y bellísimas personas que trasmitís pasión con vuestro trabajo. Siempre estaré agradecida por vuestro apoyo.

Agradecer a Maitane, Borja, Nerea e Idoia, por formar parte del equipo, guiarme y ayudarme a dar forma a este trabajo.

Santi, en ti encontré la motivación para embarcarme en este proyecto y siempre has estado ahí, me has entendido y ayudado.

Eskerrik asko nire familiari, bereziki, Ama, Eneko eta Malentxu. Amatxu gracias por estar siempre a mi lado, creer en mi incondicionalmente y ayudarme a superar todos los baches que me he encontrado. Eneko eskerrik asko zure pazientziagaitik, zu barik hau ez litzateke posible izango. Durante estos años me has animado y en los momentos más difíciles me has hecho sacar fuerzas de donde creía que no las tenía. Zuri Malen, gure izarra. Llegaste al final de este proyecto iluminando nuestras vidas. Nunca olvidaré esas horas de trabajo contigo en el pecho como un koalita. Jesus, zuri ere mila esker, urte hauetan zehar hain ondo zaintzeagaitik.

Eskerrik asko denoi, bihotz-bihotzez!

Argitalpenak

Tesi honetako lana ondorengo argitalpenetan jaso da:

Systematic Review of the Potential of MicroRNAs in Diffuse Large B Cell Lymphoma. Larrabeiti-Etxebarria A, Lopez-Santillan M, Santos-Zorrozua B, Lopez-Lopez E, Garcia-Orad A. Cancers (Basel). 2019 Jan 26;11(2). pii: E144.

Potential of microRNAs in the diagnosis, classification and prognosis of Diffuse Large B Cell Lymphoma. In preparation.

Gainera, tesi hau garatu bitartean, artikulu hauek ere argitaratu ditut:

Circulating miRNAs as biomarkers in diffuse large B-cell lymphoma: a systematic review. Lopez-Santillan M, Larrabeiti-Etxebarria A, Arzuaga-Mendez J, Lopez-Lopez E, Garcia-Orad A. Oncotarget. 2018 Apr 27;9(32):22850-22861.

Laburdura	**Azalpena**
aaIPI	Adinaren araberako IPIa
AAL	leuzemia akutu linfoblastikoa
ACVR2B	2B motako aktibina-hartzailea
adib	adibidez
allo-HSCT	Zelula ama hematopoietikoen trasplante alogenikoa
AML	ziren leuzemia mieloide akutua
auto-HSCT	zelula ama hematopoietikoen trasplante autologoa
B-CLL	B zelula linfatikoko leuzemia kronikoa
BBZG	Beste bat zehaztu gabe; not otherwise specified
BEAM	BCNU, etoposidoa, zitarabina eta melfalan
CLL	leuzemia linfatiko kronikoa
CLL	leuzemia linfatiko kronikoa
Cox PH	arrisku proportzionalen Cox-en metodoa
CR	erantzun osoa
CR	erantzun osoa
CRG	Erregulazio Genomikoaren Zentroa
CTCL	azaleko T-zelula linfoman
CTCL	azaleko T-zelula linfoman
CTL	T linfozito zitotoxikoA
DE	adierazpen diferentziala
DEG	Adierazpen diferentziala duten geneak
EBB	Epstein Barr Birusa
ECOG	Eastern Cooperative Oncology Group
EEM	erabateko erredukzio metabolikoa
EHL	Ez-Hodgkin linfoma
EET	erabateko erantzun tasak
EMT	trantsizio epitelial mesenkimalean
ENCODE	ADN Elementuen Entziklopedia
EO	Erremisio osoa
EP	Erremisio partziala
ET	erradiazio terapia
ez-CR	erantzun osorik ez
ez-ZGB edo ABZ	aktibatutako B zelula mota
FFPE	formalinan finkatutako eta parafinan sartuta
GAP	Gene adierazpen profila
GELTAMO	Espainiako Linfoma Taldea
GEO	*Gene Expression Omnibus*
GGP	giza genoma proiektua
GIB	giza immunodefizientziaren birusa
GO	Gene Ontologia
HCC	gibeleko minbizia
HCC	minbizi hepatozelularra
HIF1α	hipoxia induzitzen duen faktore 1α
HM	hezur muina
IHK	Immunohistokimika

IPI	Indize Pronostiko Internazionalaren
JZ	jatorrizko zelula
LDH	laktato deshidrogenasa
logFC	fold change
MALT	eremu marginaleko linfoma
MOE	Munduko Osasun Erakundea
MTI	mikroRNA-itu interakzio
MTX	metotrexato
NCCN	The National Comprehensive Cancer Network
ncRNA	RNA ez-kodifikatzaileak
NGS	hurrengo belaunaldiko sekuentziazioa
NK	zelula hiltzaile natural
not CR	ez erantzun osoa
NSZ	Nerbio sistema zentrala
ONA	Osagai nagusien analisia
OS	biziraupen globala
p adj	Positibo faltsuen aurkitze-tasa
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PCA	Osagai nagusizko analisia-Principal component analysis
PEP-C	prednisona, etoposidoa, prokarbazina-ziklofosfamida
PFS	aurrerapenik gabeko biziraupena
PPI	proteina-proteina interakzioen sarea
R	erituximab
R-ACVBP	erituximab, doxorubizina, ziklofosfamida, bidesina, bleomizina eta prednisona
R-CHOP	Erituximab, ziklofosfamida, doxorubizina, binkristina eta prednisona
R-DHAP	erituximab-dexametasona, zitosina arabinosidoa eta zisplatinoa
R-GDP	erituximab, genzitabina, zisplatinoa, eta dexametasona
R-GemOx	erituximab-genzitabina, oxaliplatinoa
R-ICE	erituximab, ifosfamida, karboplatinoa eta etoposidoa
R-ICE	erituximab-ifosfamida, karboplatinoa eta etoposidoa
R-miniCHOP	dosia murriztutako R-CHOP immunokimioterapia
RISC	RNA-isiltasuna eragiten duen konplexua
RT-qPCR	alderantzizko transkripzioaren PCR kuantitatiboa
STRING	Search Tool for the Retrieval of Interacting Genes
TGF-β	β hazkuntza-faktorea
XPO5	Exportine5
ZG	Zentro germinala
ZGB	zentro germinaleko B zelula mota
ZHBLH	B-zelula handiko linfoma hedatsua

Laburpena

B-zelula handiko linfoma hedatsua (BZHLH) helduetan gertatzen den neoplasia linfatiko arruntena da, ez-Hodgkin linfoma (EHL) kasuen % 30-40a izanik. BZHLH-k morfologia, genetika eta portaera biologikoari buruzko oinarri aldagarriak ditu, pazienteen artean heterogeneotasuna sortzen dutenak. Pazienteen sailkapenerako eta kudeaketarako tresna berriak garatu diren arren, horietako % 40k gaixotasun errefraktarioa dute oraindik edo berriro gaixotu egiten dira. Gainera, gaixotasun honen patogenesiari buruzko faktore anitz ez daude argituta, beraz, biomarkatzaile berrien identifikazioa beharrezkoa da. Ildo hori jarraituz, berriki egindako ikerketek adierazten dute mikroRNAak minbiziaren biomarkatzaile baliagarriak direla, baita garrantzitsuak ere gaixotasunaren garapenean. Hala ere, BZHLHri dagokionez, orain arte, ez dago funtsezko daturik. Horregaitik, lan honetako helburu nagusiak hauek izan ziren: BZHLH diagnostikorako, sailkapenerako, pronostikorako eta tratamendurako erabilgarriak diren mikroRNA multzo bat zehaztea, baita gaixotasunaren sorrerako mikroRNA ekintza-mekanismoa ezagutzea ere. Helburu horiek lortzeko, literaturan jadanik deskribatutako mikroRNAk gure BZHLHko populazioan biomarkatzaile fidagarriak ote ziren aztertu genuen. Lau mikroRNAz osatutako sinadura bat identifikatu genuen, BZHLH diagnostikoa egitean erregulazioan aldaketak aurkezten dituztenak. Hala ere, beste ikerketa lanetan analizatu dituzten mikroRNA kopuru murriztua dela eta eta haien emaitza kontraesangarriak ez dute ondorio sendorik ekartzen eta beraz, ezin daiteke gaixotasunaren sailkapena, tratamenduaren erantzuna eta pronostiko sinadura identifikatu. Ondoren, gure BZHLHren kohortean dauden mikroRNAa guztiak analizatu genituen RNA txikien sekuentziazioa erabiliz, eta, hurbilketa honekin, diagnostikoa, sailkapena, tratamenduaren erantzuna eta pronostikorako sinadura berriak zehaztu ahal izan genituen. Azkenik, mikroRNA-mRNA interakzioa-sarearen analisia erabiliz, erregulazioa aldatuta duten mikroRNAen ekintza mekanismoa aztertu genuen, zeina BZHLHren patogenesiarekin zerikusia izan lezake. Laburbilduz, gure ikerketak ohartarazten du mikroRNAk paper garrantzitsua joka dezaketela biomarkatzaile gisa BZHLHren diagnostikoan, sailkapenean, tratamenduan eta pronostikoan, baita gaixotasunaren patogenesian ere. Gainera, Next Generation Sequencing balio handiko teknika da eta gutxi ikertutako mikroRNAk aztertzea ahalbidetzen du.

Index

SARRERA

B ZELULA HANDIKO LINFOMA HEDATSUA

Sarrera

B-zelula handiko linfoma hedatsua (BZHLH) helduetan gertatzen den neoplasia linfatiko arruntena da, ez-Hodgkin linfoma (EHL) kasuen % 30-40a izanik (1). ZHBLH erabat heterogeneoa da morfologia, genetika eta portaera biologikoari dagokienez, eta horrek tratamenduarekiko erantzun eta biziraupen desberdintasun handiak dakartza pazienteen artean (2). Nahiz eta desberdintasun hauek, ZHBLH paziente gehienak kimioterapia erregimen estandarrarekin tratatzen dira, eta horrek erremisio osoa (EO) ahalbidetzen du % 75-80en kasuetan. Hala eta guztiz ere, pazienteen % 40ek ez dute tratamenduari erantzuten edo birgaixotzea aurkezten dute (3). Gainera, paziente hauek bigarren mailako kimioterapia eskemei erantzun eskasa aurkezten dute eta horrek hilkortasun kausa nagusia izaten jarraitzen du gaur egun (4). Ondorioz, biomarkatzaile fidagarriak eta tratamendurako itu berriak ikertzea da BZHLHren erronketako bat paziente hauen biziraupena hobetu ahal izateko.

Definizioa

BZHLH, ez-Hodgkin linfoma agresiboan B zelula linfoide handiek hazkuntza patroi hedatua aurkezten dute. Bere ezaugarri nagusien artean, B zelulen nukleoek makrofagoen nukleoaren tamaina (edo handiagoa) edo linfozitoen nukleoaren bikoitza aurkezten dute (5).

BZHLHren diagnostikoa nahiko hetereogeneoa da, morfologia, genetika eta portaera biologikoari dagokionez. Gaur egun, Munduko Osasun Erakundearen (MOE) laugarren edizioaren arabera diagnostiko maila berezia osatzen du (1. taula) (5).

1. taula: B-zelula handiko linfoma hedatsuaren sailkapena (5)

B-zelula handiko linfoma hedatsua, BBZG
B- zelula handiko beste linfoma azpimotak
T-zelula/histiozitoetan aberats diren B-zelula linfoma
NSZko lehen mailako B-zelula linfoma
Larruazaleko lehen mailako B-zelula handiko linfoma hedatsua
B-zelula handiko linfoma hedatsua EBB-positiboa, BBZG
Inflamazio kronikoarekin erlazionatutako B-zelula handiko linfoma hedatsua
Granulomatosi linfomatoidea
B-zelula handiko linfoma IRF4 berrantolaketarekin
Lehen mailako B-zelula handiko linfoma mediastinikoa (timikoa)
B-zelula handiko linfoma intrabaskularra
B-zelula handiko linfoma ALK-positiboa
Lehen mailako isurtze linfoma
Gradu altuko B-zelula linfoma
Gradu altuko B-zelula linfoma MYC eta BCL2 edota BCL6 berrantolaketarekin
Gradu altuko B-zelula linfoma, BBZG
B-zelula linfoma, sailkaezina
B-zelula linfoma, sailkaezina, B-cell lymphoma, unclassiflable, B-zelula handiko linfoma eta Hodgkin lymphoma klasikoaren arteko ezaugarriekin

Laburdurak: BBZG: Beste bat zehaztu gabe; not otherwise specified; NSZ: Nerbio Sistema Zentrala; EBB: Epstein Barr Birusa

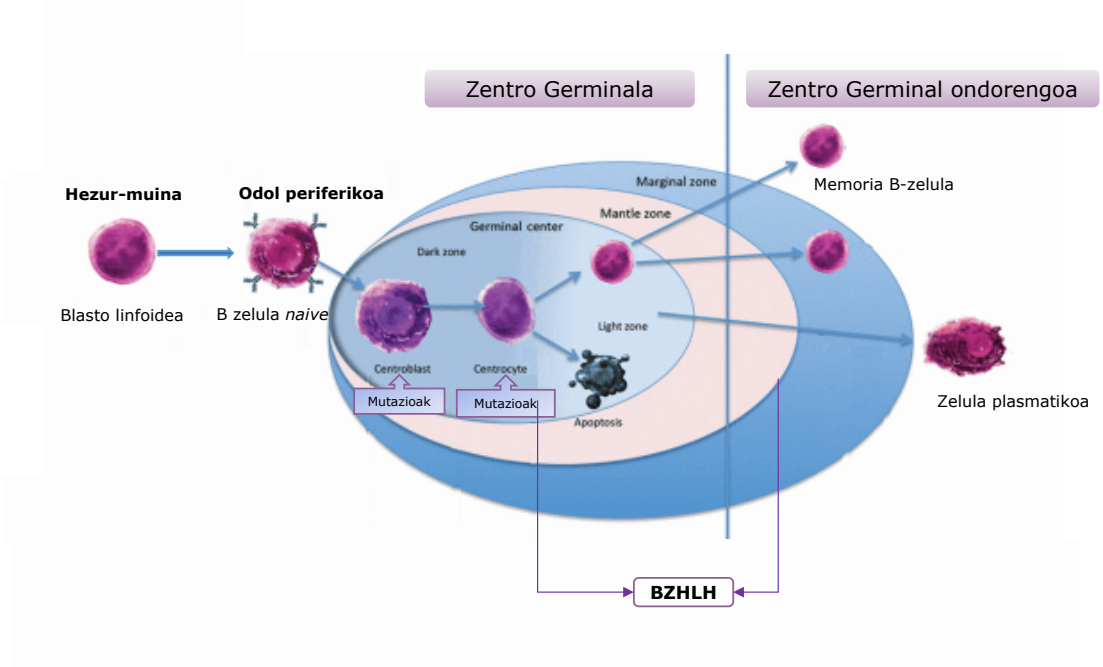
Hala ere, zenbait kasutan, heterogenizitate biologikoa dela eta ez dago sailkapenerako irizpide onartu eta argirik (6). BZHLH kasu hauek BBZG bezala sailkatzen dira. B linfoma ohikoena BZHLH denez, neoplasia linfoide hau ikertzea beharrezkoa da linfoma mota hauen biologia eta patogenesia hobeto ulertzeko eta bide batez, estrategia terapeutiko berriak garatzeko. Hori dela eta, gure lana bide honetan zentratzen da.

Epidemiologia

BZHLH helduaroko gaixotasuna da, gehien bat hirurogeita-hamargarren hamarkadan agertzen dena, nahiz eta, haur eta gazteetan garatu daitekeen ere. Gainera, gizonezkoetan emakumeetan baino arruntagoa da. BZHLH, BBZG EHL motarik ohikoena da; guztizkoaren %25-30 osatuz zehazki mendebaldeko herrialdetan eta ugariagoa garapen bidean dauden herrialdeetan. Europan, BZHLH intzidentzia gordina 3,92/100.000/urtekoa da, eta hori adinean gora egin ahala modu esangarrian areagotzen da (7).

Etiologia

BZHLH B-zelula helduetan sortzen da desberdintze-fase ezberdinetan. Zenbait alterazio genetikoek B-zelulen aldaketak sustatzen dituzte gene adierazpenean eraginez eta eraldaketa neoplasikoa bultzatuz (5) (1. irudia).



1. irudia: B linfozito normalen garapen diagrama eta B-zelula handiko linfoma hedatsuaren patogenesia (Basicmedical Key-tik moldatua (https://basicmedicalkey.com/cutaneous-b-cell-immunobiology/))

B linfozitoen heltze-prozesua hezur-muinean bertan gertatzen da non B zelula aitzindaria eraldatu eta B zelula helduan bihurtzen den. B linfozito helduak hezur muinetik atera eta linfa organo sekundarioetara barreiatzen dira (ehun linfoide sekundarioen gune interfolikularrean) antigenoa ezagutzeko zehazki (8).

Folikulu sekundarioen zentro germinalean (ZG) zentroblastoak zentrozitoetan bihurtzen dira ZG alde argira igarotzen diren heinean. ZGn, B zelulak errekonbinazio somatikoa eta hipermutazio somatikoa jasaten dute. BZHLH eta gainontzeko ez-Hodgkin linfoma gehienetan, immunoglobulinen gene berrantolatuek hipermutazio somatikoaren ezaugarri diren mutazioak jasaten dituzte. Antigorputzen dibertsitate mekanismo hau, organo linfoide periferikoen zentro germinalean gertatzen da normalean (9). Hori dela eta, BZHLZ ZGko B zeluletatik edo ondorengo desberdintze fasetatik garatu daiteke (10).

BZHLH *de novo* zein maila baxuko B zeluletako linfoma mota askoren eraldaketaren bidez agertu daiteke. Besteak beste leuzemia linfatiko kronikoa (adib. Richter´s eraldaketa), linfoma linfoplasmazitikoa, linfoma folikularra, eremu marginaleko linfoma (MALT) eta eremu marginaleko linfoma esplenikoa (11,12).

Diagnosia

BZHLHaren diagnostikoa analisi morfologiko, immunohistokimiko, zitogenetiko eta molekularretan oinarritzen da. Diagnostikoa linfomak erasotutako organo edota gongoil linfatikoaren biopsia bidezko azterketa anatomopatologikoaren bitartez burutzen da. Honek gongoil linfatikoen ezaugarriak eta ikerketa fenotipiko eta molekularrak burutzeko materiala ahalbidetzen du (12).

Biopsiak fixatu gabe laborategira bidaltzea da aukerarik aproposena, fluxu zitometria ikerketak burutu ahal izateko eta kalitate handiko DNA eta RNA erauzi ahal izateko. Biopsia kirurgikoa arriskutsua den pazienteetan orratz lodi bitartezko biopsia burutu daiteke (12,13).

Ezaugarri kliniko eta patologikoak

Kasu gehienak gongoil linfatikoetan gertatzen dira eta kasuen %40an gongoil kanpoko guneetan esaso egiten du tumore primario moduan. Gongoil kanpoko kasuak traktu gastrointestinalean gertatzen dira maizenik, baina edozein organotan ager daiteke, besteak beste, larruazala, NSZ, hezur-muina (HM), listu guruina, birika, giltzurruna eta gibela. Hezur muineko inplikazioa kasu guztien %11tik %27ra gertatzen da, eta odol periferikoan kasu gutxitan agertzen da (14,15).

BZHLH duten pazienteek azkar hedatzen den masa sintomatikoa aurkezten dute gongoilean bertan edo gongoiletik at (leku bakar edo ugaritan gehienetan lepo edo abdomenean) (16).

Gutxi gorabehera, BZHLH duten %60ak gaixotasun berantiarra aurkezten dute (III edo IV estadioa normalean) eta %40ak, aldiz, gaixotasun lokalizatua aukezten dute (17).

Paziente gehienak asintomatikoak dira baina sintomak agertzen direnean inplikatutako organoaren oso menpekoak dira. "B" sintoma sistemikoak (adib. sukarra, pisua galtzea, blai egiten duen gaueko izerdia) gaixoen %30 inguruan hautematen dira, eta laktato-dehidrogenasak (LDH) gora egiten du gutxienez kasuen erdietan (18).

Morfologikoki, gongoil linfatikoek zelula linfatiko handien hedapen zabala erakusten dute eta kasu gehienetan arkitektura zelularra erabat galdu egiten dute. Tamaina ertaineko zelulak gailentzen diren kasuetan ikerketa bereziak burutzea beharrezkoa da hezur-muin kanpoko leuzemiak, Burkitt linfoma edota mantu zeluletako linfoma barianteak baztertu ahal izateko (5).

Sailkapena

BZHLH, BBZG morfologiaren, ezaugarri molekularren eta alterazio genetikoen arabera sailkatzen da (2. taula)(5).

2. Taula: BZHLH, BBZG azpimotak MOEren 2017ko sailkapenaren arabera (5).

B-zelula handiko linfoma hedatsua, BBZG
Aldaera morfologikoak
Zentroblastikoa
Immunoblastikoa
Anaplastikoa
Beste aldaera arraroak
Azpimota molekularrak
Zentro germinaleko B-zelula
Aktibatutako B-zelula

I-Aldaera morfologikoak

Ezaugarri morfologikoak oinarritzat hartuz, hiru aldaera morfologiko arruntak deskribatu dira (zentroblastikoa, immunoblastikoa eta anaplasikoa) eta zenbait aldaera ezhoikoak. Aldaera zentroblastikoa motarik arruntena da eta zentroblasto agerpenak karakterizatzen du. Zentroblastoek tamaina ertaineko edo handiko zelula linfatikoak dira kromatina fina aurkezten dutenak nukleoan. Hala ere, kasu gehienetan, tumoreak polimorfikoak dira, zentroblasto eta immunoblasto (<% 90) nahastea aurkezten dutelarik. Immunoblastoak aldaera, %90 baino immunoblasto gehiagoz osatuta dago. Immunoblastoek nukleo handi, obalatu edo biribila dute, erdigunean kokatutako nukleolo bar eta zitoplasma basofiliko handi samarra. Beste alde batetik, bariante anaplasikoa nukleo pleomorfikoa duten zelula handiengatik bereizten da (5).

Morfologian oinarritzen diren sailkapenak porrot handia izan dute emaitzak errepikaezinak baitira patologoen artean eta horrek elkarren arteko desadostasun diagnostikoak dakartza (15).

II-Azpimota molekularrak

Zelula handien B motako linfomaren sailkapen molekularra, **"Jatorrizko zelula"** identifikatuz oinarritzen da. "Jatorrizko zelula" kontzeptua, linfomak ezaugarri kliniko, fenotipiko edo genetikoen arabera antza gehien duen zelula ez linfoidearekin erlazionatzean datza. "Jatorrizko zelula" hauetatik azpimotak identifikatzen dira.

II.a. Gene adierazpen profila (GAP)

GAP, "*gold standar*"tzat hartzen da azpimota molekularrak esleitzeko (10). Horrela, BZHLH bi azpimota molekular nagusitan bana daiteke: zentro germinaleko B zelula motakoak (ZGB) eta aktibatutako B zelula motakoak (ABZ edo ez-ZGB). ZGB eta ez-ZGB azpimotak ezberdinak dira kromosoma aldaketetan, seinaleztapen bideen aktibazioan eta biziraupenean (19). Gutxi gorabehera, kasuen % 10-15 ezin sailkatu gabe geratzen dira (11).

BZHLH bi azpimotak ehunka gene ezberdinen adierazpen desberdinagaitik bereizten dira ehun izoztuan RNA mikroarraiekin aztertuz (10). Gene horiek azpimota bakoitza B zelularen desberdintze eta aktibazio fasearekin erlazionatzen dute (10).

Hala ere, GAP ez dago eskuragarri ohiko proba kliniko gisa, eta linfoma ehun izoztuak ez daude beti eskuragarri ikertu ahal izateko. Horrela, jatorriko zelularen determinazioa immunohistokimika (IHK) algoritmoak onartzen dira.

II.b. Immunohistokimika

Biomarkatzaile panel txikiak erabiltzen dituzten zenbait algoritmo immunofenotipikoak garatu izan dira, ikerkuntza molekularrei esker dagoen informazio esangarriena ohiko plataforma klinikoa bihurtu ahal izateko, besteak beste, Colomo (20), Hans (21), Choi (22), Muris (23) eta Tally (24) algoritmoak (2. irudia). Algoritmo horietako bi immunoterapia aroa baino lehenago diseinatu zituzten (Colomo eta Hans, 2.A irudia eta 2.B irudia) eta beste hirurak erituximabarekin tratatutako pazienteen kohorteetan garatuak izan ziren (Muris, Choi eta Tally, 2.C, 2.D eta 2.E irudiak). Erabilitako markatzaileak, alde batetik, ZGB markatzaileak kontuan hartzen dituzte, esate baterako CD10, BCL6, GCET1, eta LMO2 eta beste alde batetik, zentro postgerminalarekin (ez-ZGB) erlazionatutakoak, hala nola MUM1/IRF4 eta FOXP1.

IHK bitartezko sailkapenaren lehen saiakeretako bat Colomo algoritmoa izan zen, zeinek ZGB eta ez-ZGB fenotipoak ezarri zituen hiru markatzaileen arabera (MUM1/IRF4, CD10 eta BCL6) 2.A irudian zehazten den moduan, baina algoritmo honek ez ditu ondorio klinikorik aurreikusten (20).

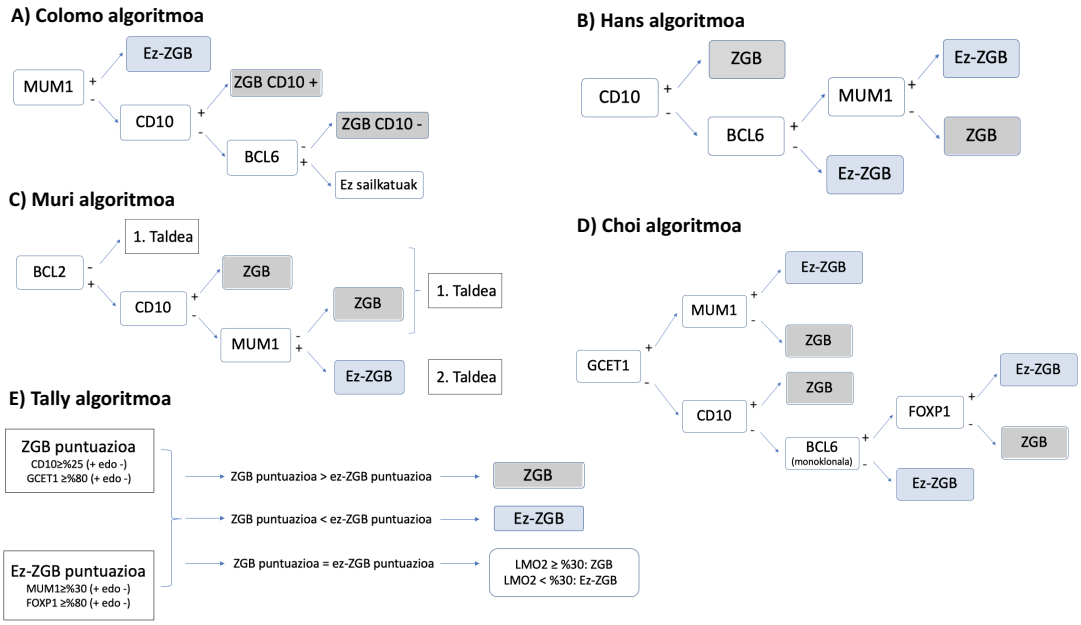
2004. urtean Hans *et al.,* ZGB eta ez-ZGB azpimotak bereizteko hiru markatzaileen adierazpenean oinarritutako algoritmo ohikena porposatu zuten: CD10, BCL6, eta IRF4/MUM1 (2.B irudia), GAParekin alderatuz %85etik %93rako zehaztasuna duena (12,21).

Proposaturiko metodo gehigarrien artean Muris algoritmoa dago, BCL2, CD10 eta MUM1 kontuan hartzen dituenak ZGB edo ez-ZGB fenotipoan sailkatzeko. Ondoren, BCL2 immunotindaketa bi talde pronostiko ezberdinetan (1 eta 2) bereizteko erabili zen (23).

Bestalde, beste bi adierazle gehigarri gehitu zizkioten Hans algoritmoari Choi *et al*.; FOXP1 eta GCET1. FOXP1 adierazpena germinal zentroko fenotipoa galdu duten BZHLH kasuen % 20tan deskribatu da (22) eta GCET1 ZGB motarekin oso erlazionatuta dagoela (25).

Azkenik, Tally algoritmoa, Meyer *et al.,* proposatu zuten 2011an eta LMO2 erabiltzen du germinal zentroko markatzaile bezala, Choi algoritmoaren BCL6aren ordez (24). Algoritmo

honek, ZGB markatzaile (GCET1 eta CD10) eta ez-ZGB markatzaile (FOXP1 eta MUM1/IRF4) kopuru berdina jasotzen du, ZGB edo ez-ZGB fenotipoa zehazteko (26).



2. irudia: Azpimota molekularrak sailkatzeko immunohistokimika ereduetan oinarritutako algoritmoak (A) Colomo´s algoritmoa, (B) Hans' algoritmoa, (C) Muri´s algoritmoa, (D) Choi´s algoritmoa, eta (E) Tally´s algoritmoa.

Hala ere, ondorio klinikoak aurreikusterakoan desadostasunak daude sailkapen ezberdinen artean, beraz, oraindik ez dago BZHLH sailkatzeko metodo egokirik eta gaur egun, GAP bitartezko sailkapena aproposena izaten jarraitzen du.

Jakina da IHK algoritmoek ez dituztela identifikatzen arestian aipatutako GAP bitartez sailkatu gabeko tumoreen %10-15a. Izan ere, emaitzak errepikatzeko arazoak ditu eta ez dago uniformeki aitortuta pronostikoa iragartzeko erabilgarritasuna duenik (1).

II.c. Jatorrizko zelula zehazteko beste teknika batzuk

Duela gutxi, BZHLH JZ GAP metodoak erabiliz identifikatzea lortu zuten formalinan finkatutako eta parafinan sartutako ehunetan sinpleagoa den gene adierazpen sinadura digital baten bitartez (Lymph2Cx) nanostring plataforma bat erabiliz (27).

Formalinan finkatutako eta parafinan dauden ehunetatik ateratako RNA kuantifikazioan oinarritutako metodo berriagoek GAP konbentzionalarekiko emaitza bateratuak ematen dituzte, laborategien artean errepikagarriak dira eta zelularen jatorrizko sailkapenaren eragin pronostikoa antzematen dute (28). Hala ere, metodo hauek ez daude eskuragarri laborategi gehienentzat, baina egungo algoritmoen alternatiba izan daitezke (1).

Arriskuen ebaluazioa eta tratamendu aukerak

Espainian BZHLH tratatzeko erabiltzen den protokoloak Espainiako Linfoma Taldearen (GELTAMO) ildoak jarraitzen ditu. Protokolo honek, hainbat bertsio ditu eta beraien artean alde txikiak aurkitzen dira. Gidaren azken gomendioak aurrerago deskribatzen dira (12).

Patologia honen ezaugarri nagusietako bat jokaera kliniko eta tratamendu kimioterapikoaren erantzun hetereogeneoa denez, gaur egungo tratamendu estrategiak arrisku faktoreen arabera estratifikatzean oinarritzen dira. Arrisku estratifikazio hauek, besteak beste, gaixotasunaren hedapena (estadio mugatua edo gaixotasun aurreratua), pazientearen adina eta Indize Pronostiko Internazionalaren (IPI) puntuazioa dira.

Arriskuen ebaluazioa

Ez-Hodgkin linfoma erasokorra duten pazienteen estadiajea Ann Arbor sailkapenarekin zehazten da (29). Sailkapen hau Hodgkin gaixotasunerako garatu zen hasiera batean eta ez da hain zehatza ez-Hodgkin linfoma duten pazienteen azpitalde pronostikoak identifikatzeko (30).

Ann Arbor sailkapenerako beharrezkoa da, besteak beste, Eastern Cooperative Oncology Group (ECOG) egoera funtzionala, B sintomen presentzia, azterketa fisiko osoa linfadenopatia edo biszeromegalia dagoen enfasi zehatza emanez, eta honako odol azterketa hauek: odoleko zelulen kontaketa, analisi biokimikoa, laktako deshidrogenasa (LDH), beta2 mikroglobulina, uratoa, gibel eta giltzurrun funtzioa, proteinograma, B hepatitisaren serologia (HBs antigenoa, anti-HBc antigorputzak), C hepatitis eta giza immunodefizientziaren birusa (GIB) (12).

IPIa BZHLH pazienteen pronostikoa zehazteko eskala erabiliena da eta parametro klinikoetan oinarritzen da. Adierazle horrek gaixoak estratifikatzen ditu bost faktoreren arabera, irizpide bakoitza betetzekotan puntu bana emanez: adina, estadioa, gongoil kanpoko infiltrazio agerpena, egoera orokorra eta LDH (3. taula). IPIk pazienteak 4 taldetan banatzen ditu 5 urteko biziraupen globala (OS) %26 eta %73 artean aurkituz. IPI puntuazio baxu batek (0-2) biziraupen global hobea duten pazienteak identifikatzen ditu IPI puntuazio altua duten pazienteekin alderatuz (3-5). Adinaren araberako IPIa (aaIPI) 60 urte baino gutxiagoko pazienteekin erabiltzen da eta puntuazioa kalkulatzeko, IPI arruntan erabiltzen diren faktoreak erabiltzen dira, adina eta gongoil kanpo erasandako eremuak izan ezik (31).

3. taula: Indize Pronostiko Internazionala (IPI) eta adinaren araberako IPI (aa-IPI) (6,31).

Arrisku faktorea	Puntuak	Arrisku taldea	5 urteko BG (%)
IPI			
Adina			
≤ 60 urte	0		
> 60 urte	1		
Ann Arbor estadioa		• Baxua (0 edo 1)	73
I edo II	0		
III edo IV	1	• Tarteko baxua (2)	51
ECOG performace status			
0 edo 1	0	• Tarteko altua (3)	43
≥ 2	1		
Serum LDH		• Altua (4 edo 5)	26
≤ 1 x normala	0		
> 1 x normala	1		
Afektazio extranodala			
≤ 1	0		
> 1	1		
aaIPI (≤ 60 urte)			
Ann Arbor estadioa			
I edo II	0	• Baxua (0)	83
III edo IV	1		
ECOG performace status		• Tarteko baxua (1)	69
0 edo 1	0		
≥ 2	1	• Tarteko altua (2)	46
Serum LDH			
≤ 1 x normala	0	• Altua (3)	32
> 1 x normala	1		

Arrisku handiko gaixoak hobeto sailkatzeko, batez ere pronostiko txarrena duten pazienteentzat, beste indize batzuk garatu dira, hala nola R-IPI, NCCN-IPI eta GELTAMO-IPI.

1990eko hamarkadaren amaieraz geroztik, R-CHOP eta "R-CHOP *like*" erregimen arruntetara erituximab (R) gehitu zen eta horrek arrisku talde guztien biziraupen hobekuntza ekarri zuen (32). Hori dela eta, IPIa erituximabarekin tratatzen diren pazienteetan berriro ebaluatu egin zen eta pronostikoa zehazteko erabilgarritasuna mantentzen zuela frogatu zen. R-IPIak hiru pronostiko talde ezberdin identifikatzen ditu: pronostiko oso ona dutenak (4 urteko biziraupen globala (OS) %94), ona (4 urteko OS %79), eta pronostiko txarra dutenak (4 urteko OS %55) (33).

Duela gutxi, The National Comprehensive Cancer Network (NCCN)-IPI eta GELTAMO-IPI garatu izan dira BZHLH pazienteen arriskua hobeto aurresan ahal izateko. NCCN-IPIk, IPIak erabiltzen dituen 5 aldagai berdinak kontuan hartzen ditu, baina, horrez gain, adina kategoriatan banatzen du, laktako deshidrogenasa normalizatua erabiltzen du eta leku zehatzetako afektazio extranodala kontuan hartzen du. IPIarekin alderatuz, NCCN-IPIk arrisku handiko taldea hobeto bereiztea lortzen du 5 urteko %33ko OSrekin, hala ere, pazienteen %8 eta %14 bitartean baino ez da agertzen pronostiko txarreko gaixotasuna, eta inplikazio extranodalaren presentzia

identifikatzen du (34). GELTAMO-IPIak beta-2 mikroglobulinaren gorakada kontuan hartzen du eta arrisku altuko pazienteak hobeto sailkatzen ditu (5 urteko OS %39 vs. %49) (35).

Tratamendu aukerak

GELTAMO tratamendu-protokoloa duela gutxi argitaratutako jarraibideetatik jasotzen da(12):

I) Lehen lerroko tratamendua

Lehenengo lerroko tratamendua gaixotasunaren hedapena (estadio mugatua edo hedatua), pazientearen adina eta IPI puntuazioaren arabera estratifikatu daiteke.

Ia) Gaixotasun mugatua (Ann Arbor I-II)

Erituximab, ziklofosfamida, doxorubizina, binkristina eta prednisona (R-CHOP) kimioterapia konbinatua 21 egunero erabiltzen da BZHLHdun paziente guztientzat lehen lerroko tratamendu gisa. Gaixotasun mugatua eta masa tamaina txikia (<7 cm) duten pazienteak erituximab-CHOP (R-CHOP) 4 ziklorekin tratatzen dira eta arrisku faktoreak aurkezten dituzten pazienteak beste R-CHOP bi ziklo gehiagorekin tratatzen dira. R-CHOP 4 ziklo jaso ondoren erabateko erredukzio metabolikoa (EEM) lortzen ez duten pazienteetan beste R-CHOP bi ziklo eta erradiazio terapia (ET) gomendatzen da (36).

Masa handiko eta estadio II-ko gaixotasuna duten pazieentek, masa txikikoa eta estadio II-koa dutenek baino pronostiko laburragoa dute, eta R-CHOP×6 + RT gomendatzen da paziente talde honentzat. Zoritzarrez, erradiazio terapia gehitu arren, masa handiaren presentziak pronostiko txarreko faktorea izaten jarraitzen du eta biziraupen tasak masa txikia aurkezten duten gaixoenak baino okerragoak izaten jarraitzen dute (37).

Ib) Gaixotasun hedatua (Ann Arbor III-IV)

- 60–80 urte bitarteko gaixoak

Paziente hauen tratamendu estandarra R-CHOP 8 ziklokoa da 21 egunetik behin. Nahiz eta "R-CHOP *like*" erregimenei bortezomiba gehitu izana ezta erituximab obinutuzumabagatik (II motatako anti-CD20 antigorputza) ordezkatzeak ere, ez du biziraupena hobetu (38,39). Gongoil kanpoko lesioak edota tamaina handiko gaixotasuna tratatzeko erradioterapia erabiltzea eztabaidatsua da (40).

- Arrisku baxu edo ertaineko pazienteak (IPI puntuaketa, 0–2) eta <60 urte

Paziente hauen tratamendu estandarra ere R-CHOP 6-8 ziklo dira 21 egunetik behin (41,42). Masa handia aurkezten duten pazienteek erradioterapia jasotzen dure. BZHLH duten paziente gazte eta adinaren araberako (aa)-IPI=1 duten tratamendu erregimen intentsiboena jasotzen dute: R-ACVBP (erituximab, doxorubizina, ziklofosfamida, bidesina, bleomizina eta prednisona)/metotrexato (MTX)/R-ICE (erituximab, ifosfamida, karboplatinoa eta etoposidoa) eta honen ondoren, zelula ama hematopoietikoen trasplante autologoa egiten da (auto-HSCT), biziraupena handitzen duena baina toxikotasun gehiagorekin lotuta dagoena (43,44).

- Arrisku altuko pazienteak (IPI puntuaketa, 3–5) eta <60 urte

Paziente talde honentzako tratamendu estandarrik ez dagoen bitartean, normalean R-CHOP 6 eta 8 zikloekin tratatzen da 21 egunetik behin. DA-EPOCH-Ran oinarritutako erregimena aukera egokia izan daiteke arrisku altuko pazienteetan (IPI: 3–5; aaIPI: 2–3) (45) [dosi-murriztua(etoposidoa, prednisona, binkristina, ziklofosfamida, doxorubizina-erituximab)] (46). Zenbait ikerketek dosi altuko kimioterapia osteko zelula ama hematopoietikoen trasplante autologa ikertu izan dute (47), baina emaitza kontraesankorrak direla eta, ezin da gaixo guztientzat gomendatu.

- Komorbilitatedun pazienteak edo >80 urte

Ez dago tratamendu estandarrik 80 urtetik gorako gaixoentzat eta ebaluazio geriatrikoa burutzea gomendatzen da paziente "*fit*"-ak identifikatzeko. Erituximab eta doxorubizina daraman kimioterapiaren konbinazioak erremisio osoa eragiten du eta biziraupena hobetzen du. Hori dela eta, R-CHOP moduko tratamendu konbentzionalak erabiltzea gomendatzen da, edo dosia murriztutako immunokimioterapia (R-miniCHOP) (48). Patologia kardiobaskularrak dituzten gaixoetan adriamizina alde batera utz daiteke (R-COP), edo doxorubizina liposomatua edo mitoxantrona, etoposido edo gemzitabina bezalako farmakoengaitik ordezkatu daiteke (49–51).

II) Bigarren lerroko eta ondorengo terapiak

Pazienteen % 30-40k gaixotasun errefraktarioa du edo berriro gaixotu egiten dira lehenengo lerroko tratamendua jaso ondoren eta, ondorioz, erreskate terapia behar izango dute.

IIa) Dosi altuko kimioterapia HSCT hautagaietan

Salbamendu immunokimioterapia komorbilitaterik ez dituzten paziente gazteen artean aukerazko tratamendua da (<60-70 urte). Kimioterapiari sentikorrak diren pazienteetan, tratamenduarekiko erantzuna kimioterapia dosi altuekin eta auto-HSCT-arekin sendotu behar zaie (52). Hala ere, ez dago erregimenik besteen gainero nagusitasunik erakutsi duenik; R-DHAP (erituximab-dexametasona, zitosina arabinosidoa eta zisplatinoa), R-ICE (erituximab-ifosfamida, karboplatinoa eta etoposidoa) (53) edo R-GDP (erituximab, genzitabina, zisplatinoa, eta dexametasona) antzeko efikazia azaldu dute (54). Hala ere, azaldutako tratamenduen edozein erregimenekin lortutako emaitzak oso eskasak dira, PFS %20 ingurukoa delarik (55). Beraz, gaixo hauentzako aukerarik honena farmako berriak dituzten entsegu klinikoetan sartzea da (12).

Trasplantea egiteko garaian pronostiko faktore nagusia linfoma estatusa da, PET/CT bitartez zehazten dena, EEM lortzen duten pazienteek biziraupen hobeagoa dute erremisio partziala (EP) lortzen dutenak baino (PFS: %72–%87 vs. %18–%49) (56,57). BEAM (BCNU, etoposidoa, zitarabina eta melfalan) egokitze erregimena Europan gehien erabiltzen dena da (12). Zelula ama hematopoietikoen trasplante alogenikoa (allo-HSCT) eraginkorra izan daiteke tratamendu lerro anitzdun ondoren berrerortze edo gaixotasun aurrera egitea duten pazienteetan. Izan ere aukera egokia izan daiteke auto-HSCT izan duten pazienteentzat eta arrisku handiko faktoreak aurkezten dituztenentzat, hala nola lehengo mailako gaixotasun errefraktarioa (58,59).

IIb) auto-HSCT ez hautagiak

Gaixo talde hauetan ez dago tratamendu erregimen estandarrik ezarrita eta erantzun iraunkorrak ez dira ohikoak.

R-GemOx (errituximab-genzitabina, oxaliplatinoa) tratamenduarekin lortzen diren erabateko erantzun tasak (EET) %60 ingurukoak dira eta urte bateko OS %45koa delarik eta 12 hilabeteko PFSa %25 ingurukoa (60). Hori dela eta, posiblea den kasu guztietan pazienteak entsegu klinikoetan parte hartu behar dute (12).

III) Hirugarren lerroko eta geroko terapia lerroak

Hirugarren lerroko terapia aukerazkoa da erreskate tratamendura erantzun ez duten pazienteentzat. Zenbait kasutan, gaixo hauek aurretik erabili ez diren bigarren lerroko tratamenduak jaso ahal dituzte. Aurretik tratamendu 3 lerro jaso dituzten pazienteetan, pixantronak %40ko erabateko erantzun tasak erakutsi ditu monoterapian (61). Lenalidomida monoterapian edo errituximabarekin konbinatuta efikazia erakutsi du egoera honeran, %30eko erabateko erantzun tasa lortuz, iraupen laburreko erantzun erlatiboarekin (62,63). Bestalde, ez dago entsegu klinikoko daturik beste farmakoak monoterapian erabiltzeko (gemzitabina, oxaliplatinoa, etoposidoa, mitoxantrona, vinorelbina) egokienak zehazten duenik. Efikazia datuak eskuragarri daude bi tratamenduekin: zelecoxib, metotrexatoa eta ziklofosfamida, %30eko EET eragiten duena eta PEP-C (prednisona, etoposidoa, prokarbazina-ziklofosfamida) erregimena, 9 gaixoetatik 3retan erantzuna eragiten duelarik, toxizitate altuarekin (64). Paziente hauek entsegu klinikoetan parte hartzea gomendatzen da ahal den kasu guztietan (12).

Pronostikoaren estratifikaziorako proposamen berriak

Gaixoen % 30-40ak, errefraktarioak dira edo berriro gaixotu egiten dira eta hauek biziraupen txarra eta tratamendu aukera mugatuak dituztela kontuan hartuta, linfomaren biologia eta markatzaile biologiko berriak ikertzeak, BZHLH duten pazienteen pronostikoa hobetzeko ikerketak garatzea ahalbidetu du.

Laborategiko zenbait **parametro**, hala nola, albumina serikoa eta β2M IPI faktoreekin konbinatu dira (35,65). Parametro hauek beste parametro kliniko batzuekin konbinatu dira (66) edota laborategi parametroekin (67), eta baita IHKrekin (68) pronostikoa hobeto iragarri ahal izateko.

Bestalde, **gene adierazpenean** oinarritutako zenbait sinadura identifikatu dira biziraupena iragarri ahal izateko BZHLH gaixoetan. Linfoma/leuzemia profil molekularraren poiektuak pronostikoa iragartzeko 17 gene (69) eta 3 osagaiez osatutako sinadura proposatu zuten (70). Lossos *et al.,* 6 genetan oinarritutako eredua proposatu zuten (71). Alizadeh *et al*., eredu hau sinplifikatu zuten 2 gene kontuan hartzen dituen eredua garatuz arriskua aurreikusteko (72). Nahiz eta geneetan oinarritutako iragarleek diskriminazio gaitasun ona izan, bakarrik erabiltzen direnean IPIak BZHLH duten pazienteen emaitza klinikoaren iragarle fidagarria izaten jarraitzen du (73).

GAP bitartez zehaztutako jatorrizko zelula fenotipoa pronostiko faktore garrantzitsua da baita ere (74). ZGB eta ez-ZGb motako BZHLH OS eta EFS ezberdintasun esangarriekin lotuta daude. Nahiz eta paziente guztien batez besteko 5 urteko biziraupena %52koa izan, ZGB motako

pazienteen %76k bizirik jarraitzen dute bost urte ondoren, aktibatutako B zelula mota duten pazienteen %16arekin alderatuz. Horrela, bi linfoma mota hauen arteko alde molekularrak portaera klinikoan ezberdintzasunak eragiten ditu, ZGB eta ez-ZGB gaixotasun ezberdinak bezala kontuan hartu behar direla iradokiz (14).

Beste alde batetik, translokazioak kontuan hartu gabe, BZHLHan *MYC* eta *BCL2* adierazpenak biziraupen globala eta gaixotasunik gabeko biziraupen laburragoa iragartzen dute (75,76).

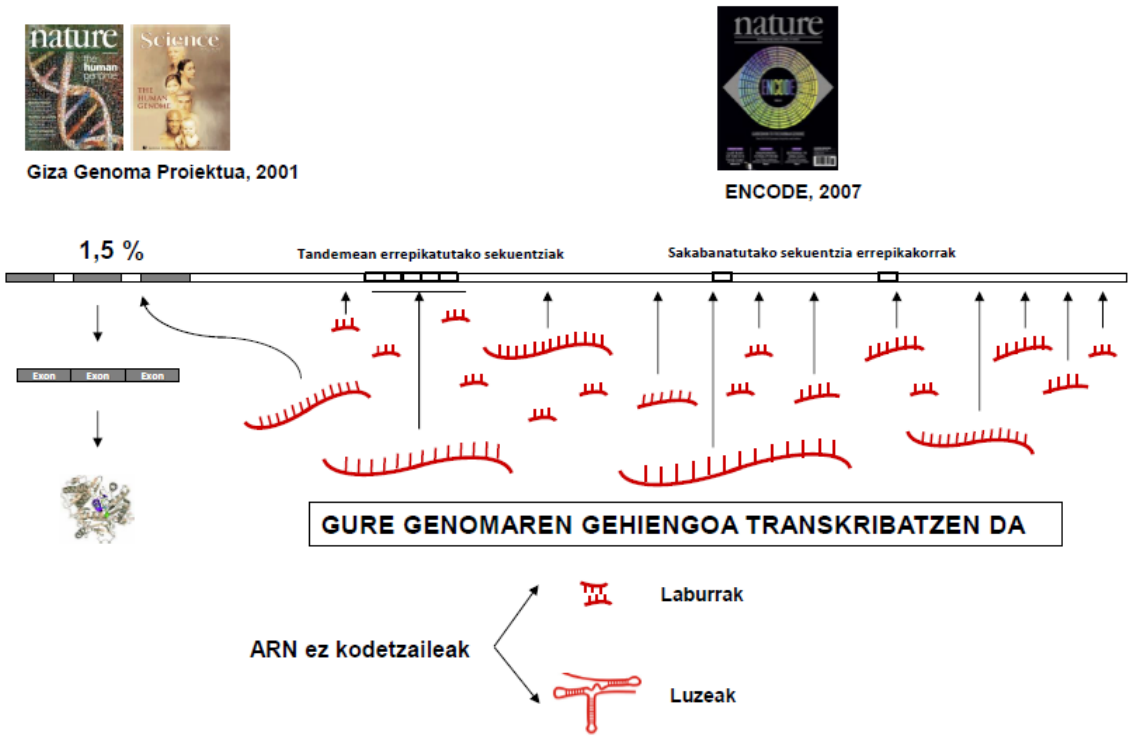
Berrikiago, hurrengo belaunaldiko sekuentziazioa (ingelesez, "*Next Generation Sequencing*" NGS) azterketek mutazio somatiko komunak identifikatu dituzte BZHLH azpitaldetan, baita ZGB eta ez-ZGB azpimotetan ezberdinak diren alterazioen profil zehatza. BZHLH bi azpimotetan ohikoak diren mutazio somatioen artean *TP53* inaktibatzen duten mutazioan, immunozaintzan erlazionatutako geneak (*B2M*, *CD58*), erregulatzaile epigenetioen alterazioak (*CREBBP/EP300*, *KMT2D/C* [*MLL2/3*], *MEF2B*), eta *BCL6*aren aktibazio onkogenikoak aurkitzen dira. ZGB azpimotak histona metil transferasan *EZH2* aldaketak aurkezten ditu maiz, *BCL2* translokazioak, eta zelularen mugikortasunaren erregulatzailean *GNA13* mutazioak. Aldiz, ez-ZGB BZHLH geneetan aurkezten dituzte mutazioak (*MYD88*, *CD79A*, *CARD11*, *TNFAIP3*) B-zelula errezeptorea/Toll-like errezeptorea aktibatuz eta NF-κB bidezidorra aktibatuz. Hala ere, mutazio hauen inplikazio klinikoak ez dira erabat ulertzen (1).

Eredu asko pronostikoa egiaztatzeko baliagarriak direla esan den arren, ez dago paziente errefraktarioak edo berriro gaixotuko direla iragartzen duen eredurik. Beraz, faktore hauen erabilera mugatua da praktika klinikoan eta momentu honetan markatzaile hauetan oinarritutako erabaki terapeutikoak entsegu klinikoetara mugatzen dira.

Hortaz, tratamenduarekiko erresistentzia modu goiztiarrean identifikatzea minbiziaren terapia arrakastatsu baterako oso garrantzitsua da eta hori dela eta, markatzaile terapeutiko berrien ikerkuntza funtzeskoa da.

GUNE EZ-KODETZAILEAK

Giza Genoma Proiektuak (GGP) 2001ean proteinak kodifikatzen dituzten geneen kopurua 20.000-25.000 ingurukoa dela frogatu zuen, genoma osoaren %1,5arekin bat datorrena gutxi gorabehera eta proportzioa %2ra heltzen da itzuli gabe dauden eskualdeak kontuan hartzen badira (77).



3. irudia: Gure genomaren transkripzioa

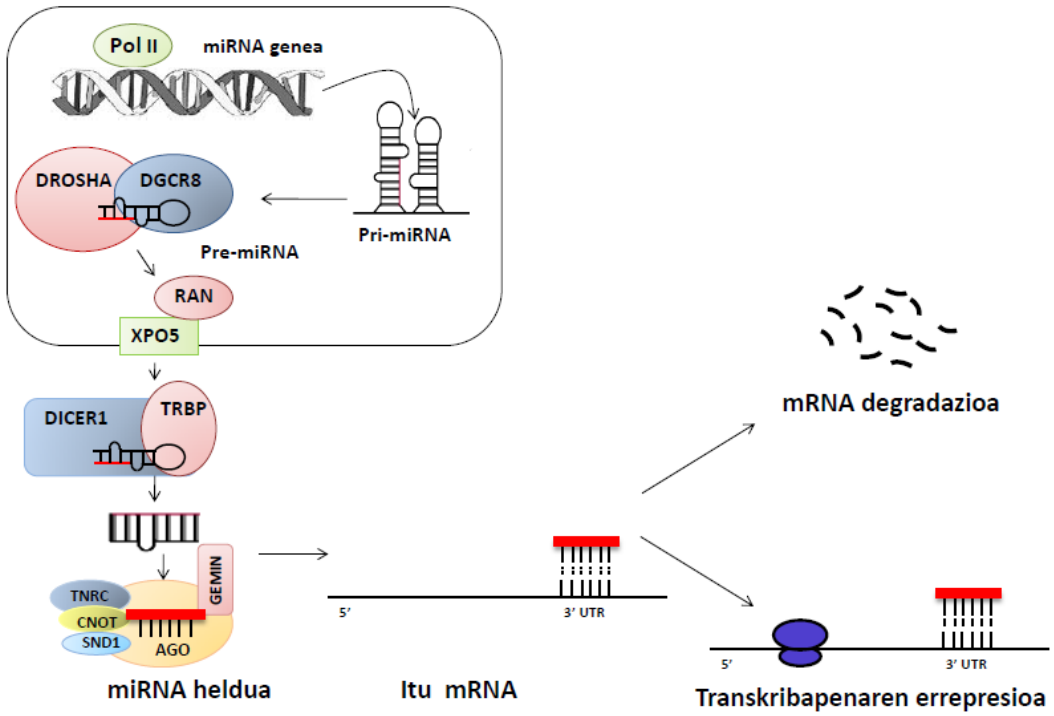
Hala ere, giza genomaren sekuentzia jaso zenean, erronka berriak proposatu ziren GGPak sortutako datuak interpretatzeko, eta ondoren ENCODE (ADN Elementuen Entziklopedia) izeneko proiektua abiarazi zen. Proiektu honen helburu nagusia, giza genoman kodifikatutako elementu funtzional guztiak biltzen zituen katalogo oso bat prestatzea izan zen eta lortutako ondorio nagusietako bat gure genomaren %80a proteinak kodifikatzen ez dituzten elementu gisa transkribatuta dagoela izan zen. Hasiera batean "zabor DNA" kontsideratua izan zen arren, proteinak kodifikatzen ez duten alde handi bat funtzionala dela zehaztu zen. Elementu hauen RNA ez-kodifikatzaileak dira (ncRNA) (78).

NcRNAk bere luzeragatik sailkatzen dira. RNA ez-kodifikatzaile txikien artean, mikroRNAk dira ikertuenak eta minbiziarekin duten harremana baieztatuta dago (79) (3.irudia).

MikroRNAk

MikroRNAk 18-22 nukleotidoko luzerako harizpi bakarreko RNA familia handia osatzen dute eta gene adierazpenaren erregulatzaile giltzarri gisa agertu dira transkripzio osteko mailan, geneen isiltzea eragiten dutelarik (80). MikroRNAk genomaren kokapen ezberdinetatik transkribatzen dira RNA polimerasa II-aren bidez pri-mikroRNA izeneko transkribatu primario luze batzuetara (dsDNA, 300-5000 bp). Pri-mikroRNA hauek, ezaugarri moduan, 30-40 nukleotidoko luzera duten harizpi bikoitzeko RNA (dsRNA) zentral bat dute, lakio terminal bat eta elkarri kontrajarritako harizpi bakarreko bi RNA (ssRNA) zati ditu. Pri-mikroRNA hauek nukleoan prozesatzen dira DROSHA RNasa eta dsRNA lotzeko domeinuak dituen DGCR8 konplexuaren bitartez. DsRNA sekuentziak bere egitura sekundarioa eta proteina prozesatzaileei atxikitzeko gaitasuna zehazten du (81).

Pri-mikroRNA prozesamendua eta gero, lortutako molekula txikiago hauek (70 nukleotiko ingurukoak) pre-mikroRNA bezala ezagutzen dira. Pre-mikroRNAk Exportine5 (XPO5) eta RAN GTPasa (82,83) proteinen bitartez nukleotik zitoplasmara garraiatzen dira. Zitoplasman, Dicer (84,85) eta TARBP2 entzimen bitartez pre-mikroRNAk prozesatzen dira, zeintzuek lakioa deusezten duten, mikroRNA duplex bezala ezagutzen den dsRNA molekula sortuz (86). Mikro-RNA duplex-a harizpi bakarreko mikroRNA heldua sortzeko banatzen da. MikroRNA duplex-etik aukeratutako arizpia RISC (RNA-isiltasuna eragiten duen konplexua) izenaz ezagutzen den proteina anitzen konplexura erantsi egiten da, EIF2C1 (AGO1), EIF2C2 (AGO2), SND1, GEMIN3, GEMIN4 eta CCR-NOT geneez osatzen dena (87). MicroRNA heldua RISC konplexuaren bitartez RNA mezularietara garraiatua izaten da, erregulazioaren azken itua (88). MikroRNA mRNA-ren 3´ UTR eremuetan dauden base osagarriei lotzen zaio (4. irudia).



4.irudia. MikroRNAren biogenesia eta ekintza mekanismoa.
Iturria: Ryan *et al*.,-etik egokitua 2010 (Ryan, Robles, eta Harris 2010)(89).

MikroRNAk ituen ezagutzarako balio duen 7 bp-tako sekuentzia bereizgarri bat dute, "seed" eremua bezala ezagutzen dena. MikroRNA-k "seed" sekuentzia honi osagarria den itu sekuentzia baten arteko lotura espezifikoari esker jarduten du. Erregulazio mekanismoa mikroRNA-mRNA osagarritasun graduaren araberakoa da. Esate baterako, osagarritasuna erabatekoa denean mRNA-ren degradazio zuzena eragiten da eta baseen parekatzea ez denean erabatekoa, proteinen itzulpenaren blokeo edo inhibizioa gertatzen da (90).

MikroRNA "seed" sekuentziarekin osagarriak direnez, mikroRNAak "seed" eremuaren osagarriak diren mRNA-en zenbait itu deuseztu ditzakete edo itzulpena inhibitu dezakate. Hala ere, posible da mikroRNA anitzek gene bakar bat erregulatzea.

Erregulazio mekanismo honen bitartez, mikroRNAek giza geneen %50 baino gehiago erregulatu ditzakete, minbiziarekin lotutako geneak ere aurkitzen direlatik (91). Hala ere, mikroRNA-en kopuru aldaketak mikroRNAk gidatutako gene erregulazioan eragina izan dezake. Alabaina, mikroRNA bidezko geneen erregulazioan mikroRNAen maila edo kantitateak eragin dezake. Ondorioz, mikroRNAen adierazpenak minbiziaren diagnostiko, sailkapen eta pronostikoaren iragarle potentziala erakutsi du.

MikroRNA adierazpena giza minbizietan

MikroRNA profila eta sekuentziazio sakonei esker mikroRNA adierazpena giza minbizian erregulazioa aldatuta dagoela baieztatu da. Erregulazio aldaketa hauek hainbat mekanismoren bidez gertatzen da, besteak beste, mikroRNA geneen anplifikazioa edo ezabatzea, mikroRNAen kontrol transkripzional anormala, deregulatutako aldaketa epigenetikoak eta mikroRNA biogenesi makinarian akatsak. Ondorioz, erregulazioa aldaketak dituzten mikroRNAk minbiziaren bereizgarri diren hainbat mekanismoetan eragina dutela frogatu da, besteak beste, seinale proliferatiboak eutsiz, hazkuntza ezabatzaileak saihestuz, zelulen heriotza eutsiz, inbasioa eta metastasia aktibatuz eta angiogenesia eraginez (92).

MikroRNA adierazpenaren sinadurak tumore zelulak ehun normaletatik bereiztea, minbizi mota ezberdinak zehaztasun handiz bereiztea eta gutxi desberdindutako tumoreak jatorrizko ehunagatik identifikatzea ahalbidetzen du (93,94). Azpimarragarria da mikroRNAek onkogene edo tumore ezabatzaile gisa jardun dezaketela baldintza jakin batzuetan. Gainera, mikroRNA sinadura ezberdinek minbizi mota gehienak pronostiko talde ezberdinetan sailkatzea ahalbidetu dezakete.

<u>MikroRNA tumore ezabatzaileak</u>

Minbizian mikroRNAen parte hartzea iradokitzen duen lehen ikerketa Croce doktorearen taldeak burutu zuen, kromosoma eremu batean (13q14) tumore ezabatzaile geneak aurkitzen saiatzen zirelarik, B zelula linfatikoko leuzemia kronikoa (B-CLL) duten pazienteetan maiz delezioa aurkezten duena. Proteina bat kodetzen duen tumore ezabatzailea den gene kanoniko bat aurkitu beharrean, tumore ezabatzaile bezala jarduten duten bi mikroRNA aurkitu zituzten, miR-15 eta miR-16-1. Emaitza honen arabera, mikroRNAk giza minbiziaren patogenesiarekin erlazionatuta daudelaren lehenengo froga lortu zen (95). Lehen azterketa honen ondoren, mikroRNA ugari aurkitu dira azpi-erregulatuta minbizian.

Minbizian azpi-erregulatuta dauden mikroRNAen artean, tumore funtzio ezabatzailea betetzen dutenen artean, miR-34 familiak (miR-34a, miR-34b eta miR-34c) funtsezko arreta jaso du. MikroRNA hauek beheranzko erregulazioa aukeztu dute birika, bular eta beste hainbat minbizietan (96,97). Familiako hiru kide hauek p53 tumore ezabatzailea erregulatzen dute transkripzionalki DNA kalte erantzunaren bitartean (98). Ikerketek iradokitzen dute p53 tumoreek erantzun immunea zuzenean modulatzen dutela PDL1 erregulatuz miR-34aren bitartez (99).

Bestalde, hainbat ikerketek mikroRNAren let-7 familiak galdu edo azpi erregulazioa dutela frogatu dute hainbat minbizietan, hala nola, bular, birika edo prostatako minbizietan (100–103). Let-7 adierazpena galtzearen inplikazio funtzionalak ez daude guztiz argituta baina tumore

progresio arintzea eragiten du RAS familiako proteinak inplikatuta dauden seinale sareen asaldurak direla eta (104).

Gainera, obarioko minbiziari dagokionez, mRNA-en itu potzentzialen adierazpenaren sare integratuaren analisiak miR-506 obario ehunetan azpi erregulazioa duela azaldu egin du. Gainera, miR-506 metastasia bultzatu egiten du (105). Bular eta obarioko minbizietan, miR-520 beheranzko erregulazioa aurkezten du eta tumore ezabatzaile gisa jarduten duela dirudi, *TGFBR2* azpi-adierazpena erregulatuz, zeinak metastasia eragin dezakeen TGF seinaleztapenaren proteina errezeptore bat kodifizatuz (106,107).

Minbizian azpi-erregulatuta dagoen beste mikroRNA familia miR-200 da, tumore metastasia eta angiogenesiarekin erlazionatuta dauden proteinen adierazpena modulatzen duena. Haien ituek eta funtzio biologikoek trantsizio epitelial mesenkimalean (EMT) parte hartzen duten faktoreak barne hartzen dituzte (108,109).

MikroRNA onkogenikoak

Onkogeneak diren mikroRNAk ere identifikatu egin dira, tumore ezabatzaileen kontrako funtzioa dutenak.

Esate baterako, ikerketa ugarik frogatu dute miR-21ak paper antiapoptotikoa duela eta gain-erregulatuta dagoela tumoreetan, ehun normalekin alderatuz (110–112). Hain zuzen ere, 17q23.2 eskualde kromosomikoaren anplifikazioa hauteman da, miR-21 barne hartzen duena, bular, birika, gibel, obario eta prostata minbizian (113).

Tumore-sustatzailea den beste mikroRNAen artean, miR-155 aurkitu dezakegu, linfoma eta beste hainbat tumore solido motatan onkomiR garrantzitsu gisa jarduten duena (113–117), eta miR-210, hipoxia induzitzen duen faktore 1α (HIF1α)-ren itu nagusienetarikoa bezala deskribatu dena, minbizi zelulen biziraupena sustatzen baitu (118).

MiR-17-92 klustera, miR-17, miR-18a, miR-19a, miR-20a, miR-19b eta miR-92a osatzen dutena, transkripzionalki gorantza erregulatuta dago hainbat gaitzetan (119). Asoziazio hau, *MYC* bultzatutako bere gene errezeptorearen, *MIR17HG,* gain-erregulazio transkripzionalagatik gertatzen dela aurkitu zen. MiR-17-92 mota askotariko rolak ditu: adibidez, BIM proteina pro-apoptotikoa azpi-erregulatzen du (*BCL2L11* bezala ere ezagutua) B linfozitoetan, B linfozitoen apoptosi gutxiera eraginez (120).

Bularreko minbizi metastasikoa, glioblastoma eta melanoma bezalako beste minbizi mota batzuk miR-10b-ren goranzko erregulazioa azaldu dute (121–123), eta gibeleko minbizian (minbizi hepatozelularra (HCC) ere deitzen dena) gorantzazko erregulazioa duen mikroRNA esangarriena miR-221 da, tumore ezabatzaile diren mRNA ituak dituena, hala nola, *p27KIP1* (*CDKN1B* bezala ere ezaguna), *PTEN* eta 3 metaloproteinasen ehun inhibitzaileak (124,125).

MikroRNAk pronostiko eta iragarle biomarkatzaile gisa

Tumore ezabatzaile edo onkogene gisa duten paperaz gain, zenbait ikerketa taldek mikroRNAen potentziala deskribatu dute pronostiko markatzaile bezala eta minbiziaren eboluzioa iragarri ahal izateko. Adibidez, miR-155 goranzko adierazpena eta let-7a azpi-adierazpena birikietako

minbizian eboluzio txarra iragarri ahal dute (126). Era berean, miR-191 eta miR-193a maila altuak biziraupen laburragoarekin erlazionatuta daude (127). Minbizi gastrikoan, 7 mikroRNAko sinadura biziraupen globala eta gaixotasunik gabeko biziraupena aurreikus ditzake fidagarritasun handiarekin (128).

Nahiz eta biziraupena iragartzea garrantzitsua izan daitekeen zentzu orokorrago batean, terapia espezifikoei erantzuteko iragarpenak askoz balio kliniko handiagoa du. Zenbait mikroRNA gain-adierazita egon daitezke farmakoen erresistentzia kasuetan, hala nola miR-19 eta miR-21 bularreko minbizian eta miR-221/222 klusterra biriketako eta gibeleko minbizian (124,129,130).

Beste batzuk, miR-130a eta miR-298 adibidez, azpi-erregulazioa aurkezten dute, mikroRNAk prozesu honetan duten paper biologiko garrantzitsua iradokiz (131,132). Beste kasu batzuetan, mikroRNAek tratamenduen erresistentzian duten eragina ehun espezifikoa da. Izan ere, miR-27a zeharka lotuta dago obarioko minbiziarekin, eta zuzenean lotuta egon daiteke leuzemiaren tratamenduarekiko erresistentzian (133).

Guzti hau kontuan izanda, mikroRNA profil garapenaren ikerketak martxan daude prognosia eta tratamenduaren erantzuna aurreikusi ahal izateko. Biomarkatzaile iragarle gisa erabiltzetik aparte, mikroRNA adierazpenaren eta terapia espezifikoen erantzunaren arteko korrelazioak haien tratamendu adjubante potentzial esperantzagarria ere proposatu du, nahiz eta hipotesi hau batez ere *in vitro* ikerketetan oinarritzen den (134). MikroRNAk, mRNA anitz itu moduan izateko duten gaitasun handia dela eta, molekula horiek hautagai terapeutiko (microRNA mimics) edo itu terapeutiko (antimiRs) gisa erabiltzeko interesgarri bihurtzen ditu (135).

MikroRNAk gaitz hematologikoetan

MikroRNAen adierazpen anormala B zelulako neoplasmetan ere ohikoa da, B zelula linfoma barne. Jakina da mikroRNAen bitartezko gene erregulazioan araudi zehatzak behar direla zelula hematopoietikoak modu egokian garatu ahal izateko, eta honen etenaldiak zelulen transformazio gaiztoa dakar. MikroRNAk erregulazio-rol kritikoak ditu sistema immunologikoan, zelula amen mantenimenduan, zelularen funtzio immunologikoetan, B-zelularen heldutasuna eta immunoglobulina ekoizpenean (136).

Leuzemiaren biomarkatzaile gisa proposaturiko mikroRNAei dagokienez, emaitza sendoak behatu dira miR-155ren gain-adierazpenean leuzemia akutu linfoblastikoan (ALL), leuzemia linfatiko kronikoan (CLL), azaleko T-zelula linfoman (CTCL), B-zelula handiko linfoma hedatsuan (BZHLH) eta beste hainbat gaixotasun hematologikoetan (137–139). Gainera, miR-150 adierazpen murriztua aurkitu da ALL duten paziente pediatrikoetan osasuntsu dauden pazienteekin aderatuta (140), eta zenbait mikroRNA, hala nola, miR-720, miR-1246, miR-451, miR-1915, miR-1308 eta miR-638, modu esangarrian adierazita aurkitu dira mieloma anitzan paziente osasuntsuekin alderatuz baita ere (141).

Horrez gain, mikroRNA adierazpenak leuzemia moten artean desberdintzeko erabili dira. Adibidez, miR-128a, miR-128b, let-7b eta miR-223 ALL eta AML bereizteko gai zirela aurkitu zen (142).

Azkenik, mikroRNAk pronostikoarekin lotu dira baita ere gaitz hematologikoetan. Adibidez, miR-146a maila altuak pronostiko onarekin erlazionatu ziren AMLan (143), ostera miR-122 gain-adierazpena pronostiko txarrarekin erlazionatuta dagoela baieztatu da (144).

Hori guztia kontuan hartuta, BZHLH duten pazienteen ehun laginen mikroRNAen adierazpen-mailaren ebaluazioa gaixotasunaren diagnostikoa eta karakterizazioa egiteko erabili daiteke, minbiziaren prognosia iragartzeko eta terapia aukeratzeko edo erantzunaren markatzaile gisa. Hala ere, orain arte adierazitako datuak bateraezinak dira. Adibidez, miR-150-5p BZHLHko lau ikerketetan azpi-erregulatuta aurkitu zen (145–148), beste ikerketa batean gainadierazita aurkitu zen bitartean (149) beste ikerketa baten adierazpena aldaketa gabe behatu zen (150). Era berean, miR-222-3p pronostiko onarekin asoziatu zen lau ikerketetan (151–154) eta ez zen asoziaziorik aurkitu beste lau ikerketetan (147,150,155,156). Horregatik, ikerketa gehiago behar dira mikroRNAen aplikazioa sakoago aztertu ahal izateko, BZHLHko diagnostiko klinikorako, sailkapenerako edo pronostikorako markatzaile espezifiko eta sentikor gisa erabili ahal izateko.

HIPOTESIA ETA HELBURUAK

HIPOTESIA

BZHLHren mekanismo patologikoak are gehiago ulertu eta erituximab oinarritzat duen kimioimmunoterapia gehitu den arren, tratamenduak eraginkortasun gutxiago du pazienteen % 30-40arengan gaitzak heterogeneotasun handia duelako eta farmakoekiko erresistentzia azkarra agertzen delako. Biziraupen tasak hobetzeko, sakonago ulertu behar dira BZHLHren patogenesia eta farmakoekiko erresistentzia eragiten duten mekanismoak.

Azkenaldian, minbizi-zelulen portaera biologikoan eta farmakoekiko erresistentzian mikroRNA molekulek dituzten eraginei buruzko informazio garrantzitsu ugari agertu da.

Horren harira, BZHLHren jatorrian, azpimoten arteko ezberdintasunetan eta tratamenduari emandako erantzunean mikroRNA molekulek ere parte hartzen dutela proposatzen dugu lan honetan. Hori hala dela frogatuz gero, BZHLHren diagnostikorako, sailkapenerako, pronostikorako eta tratamenduari emandako erantzuna neurtzeko biomarkatzaile gisa erabili ahal izango lirateke mikroRNAk.

HELBURUAK

Lan honen helburu nagusiak alde batetik, BZHLH diagnostikatzeko, sailkatzeko, pronostikoa egiteko eta tratamenduari emandako erantzuna neurtzeko balioko duen mikroRNA multzoa zehaztea, eta, bestetik, erregulazioan alterazioak dituzten mikroRNA molekulek gaitzaren jatorrian duten ekintza-mekanismoa argitzea.

Helburu nagusi horiek lortzeko, helburu espezifiko hauek ezarri ditugu:

1. BZHLHren diagnostikoarekin, sailkapenarekin, tratamenduari emandako erantzunarekin eta pronostikoarekin erlazionatuta egon daitezkeen mikroRNAen xedea zein den zehaztea:
    a. BZLHLren biomarkatzaile gisa aurretiaz proposatu diren mikroRNAk identifikatzea berrikuspen sistematiko bat eginez.
    b. Identifikatutako mikroRNAen erabilgarritasuna GELTALMO gidalerroak jarraiki tratatutako eta diagnostikatutako kohorte berrian biomarkatzaile gisa balioestea.
2. BZHLHen biomarkatzaile gisa erabili daitekeen mikroRNA molekulen sinadura berria definitzea; horretarako, anotazioa duten mikroRNA guztien azterketa egitea RNA txikien sekuentziazioa erabiliz.
3. Erregulazioan alterazioak dituzten mikroRNA molekulek BZHLHn duten ekintza-mekanismoa identifikatzea, mikroRNA-mRNA interakzio-sarearen analisia erabiliz.

MATERIALAK ETA METODOAK

BERRIKUSPEN SISTEMATIKOA

Bilaketa-estrategia

Bilaketa sistematikoa egin zen [((“Non-coding RNA”) OR (“miRNA” OR “microRNA” OR “miR”) OR (“exosome”) OR (“extracellular vesicle”) OR (“secretome”)) AND (“Diffuse large B cell lymphoma” OR “DLBCL”)] terminoekin, PubMed datu-basea erabilita (https://www.ncbi.nlm.nih.gov/pubmed/); 2020ko martxora arte argitaratutako artikuluak hartu ziren kontuan.

Inklusio- eta esklusio-irizpideak

Gure inklusio irizpideak betetzeko giza populazioetan mikroRNAk BZHLHren tumore-ehunean duten adierazpena diagnostiko gisa, azpimoten arabera sailkatzeko, tratamenduari emandako erantzuna aurreikusteko edo pronostikoaren biomarkatzaile gisa mikroRNAk ebaluatu zituzten azterketa original eta independenteak izan behar zuten. Ezaugarri hauek dituzten ikerketak baztertu ziren: jatorrizko datuak (berrikuspenak, meta-analisiak, gutunak eta iruzkinak) ez dituzten artikuluak, kasu-txostenak, laburpenak, ingelesez argitaratu ez diren artikuluak, gizakiak ez ziren beste espezietako mikroRNA molekulen datuak ez zituzten ikerketak eta BZHLH ez diren gaitzei buruzko artikuluak. Testuak guztiz aztertu ondoren, baztertu egin ziren, halaber, beste gaitz batzuk ere barnean hartzen zituzten artikuluak, zirkulazioan dauden mikroRNAk aztertu zituztenak, BZHLH ez-primarioan arreta jartzen zutenak, diagnostikoan, azpimotetan, tratamenduei emandako erantzunean edo pronostikoan mikroRNA molekulek zuten funtzioa ebaluatzen ez zutenak edo mikroRNA molekulen adierazpena aztertzen ez zutenak. Identifikatutako lanetan zeuden erreferentziak berrikusi egin ziren, irizpideekin bat egiten zuten ikerketa gehigarriak aurkitzeko. Bi ikertzailek egin zuten hautaketa-prozesua, modu idenpendentean, eta bat ez etortzeak kontsentsu bidez ebatzi ziren.

Datuak erauztea

Informazio hau erauzi zen azterketa bakoitzetik: argitalpen-urtea, aztertutako ehun-lagin mota, aztertutako populazioaren ezaugarriak, metodologia, aztertutako mikroRNA kopurua eta modu esangarrian adierazitako mikroRNA molekulen zerrenda. Bi ikerketetan edo gehiagotan estatistikoki adierazgarriak ziren eta emaitza esangarriak zituzten mikroRNAk soilik hautatu ziren.

MIKRORNA MOLEKULEN ADIERAZPENAREN ANALISIA

Azterketako populazioa

BZHLH diagnostikoa zuten eta R-CHOP edo antzeko kimioterapia-erregimenekin tratatutako 78 paziente helduren eta 17 kontrolen laginak formalinan finkatutako eta parafinan sartutakoak (FFPE) erabili ziren azterketan. Euskadiko Ikerkuntza Klinikoko Batzorde Etikoak onartu zuen azterketa (P2016121). Parte-hartzaile bakoitzak baimen informatua sinatu zuen, eta Helsinkiko Hitzarmeneko zehaztutasunak jarraituz egin zen azterketa.

BZHLH diagnostikatu zitzaien momentuko 78 pazienteren tumore aleetatik eskuratu ziren laginak (epe luzeko erremisio totala zuten 50 paziente, gaitz errefraktarioa zuten 12 paziente

eta azkenik, diagnostikatu eta 10 urteren epean berriro gaixotu ziren 16 paziente). 1999 eta 2018 urteen bitartean eskuratu ziren laginak Espainiako erreferentziako hiru ospitaletako Hematologia Unitateetatik (Gurutzetako Unibertsitate Ospitalea, Donostiako Unibertsitate Ospitalea eta Arabako Unibertsitate Ospitalea). Kontrol laginak, hau da, BZHLHrik ez duten pertsonak, gongoil ez-tumoraletatik hartu ziren Arabako Unibertsitate Ospitalean.

4. taula: Diagnostikoko BZHLH pazienteen ezaugarri demografikoak eta klinikoak (n=78).

Datu klinikoak	BZHLH pazienteak (n=78)	Kontrolak (n=17)
Adina		
Batezbesteko adina (tartea)	58,66 (21-81)	67,76 (31-86)
≥60	43	4
<60	34	13
NA	1	0
Sexua		
Gizonezkoa	41	9
Emakumezkoa	37	8
Estadioa		
I	7	
II	11	
III	23	
IV	27	
NA*	10	
B sintomak		
Bai	31	
Ez	31	
NA*	18	
IPI puntuazioa		
Arrisku baxua (0-1)	15	
Arrisku baxu edo ertaina (2)	13	
Arrisku eratin edo altua (3)	15	
Arrisku altua(4-5)	20	
NA*	15	
IHK azpimotak edo molekularrak		
ZGB	32	
Ez-ZGB	21	
NA*	25	
Terapia erantzuna		
CR	50	
Ez CR	28	
LDH		
Normala	23	
Handituta	40	
NA*	17	
β2-MG, β 2 mikroglobulina		
Normala	22	
Handituta	39	
NA*	17	
5 urteko PFS		
Batezbestekoa, hilabeteak	39,68	
5 urteko OS		
Batezbestekoa (tartea), hilabeteak	46,63	

Laburdurak: CR, erantzun osoa; BZHLH, B-zelula handien linfoma hedatsua; ZGB, zentro germinaleko B zelula mota; IHK, immunohistokimika; IPI, Indize Pronostiko Internazionala; β2-MG, β 2 mikroglobulina; LDH, laktato deshidrogenasa; PFS, aurrerapenik gabeko biziraupena; OS, biziraupen globala; NA, ez eskuragarri; *Hainbat aldagaik balio falta dute, pazienteen historio klinikoetako informazio faltagaitik.

Bi ikertzaile independentek jaso zituzten datu demografikoak eta klinikoak pazienteen historia klinikoetatik. Adina, sexua, fase klinikoa (I-IV), B sintomak (bai/ez), IPI kalifikazioa (0-5), azpimotak (ZGB edo ez-ZGB), tratamenduari emandako erantzuna —erantzun osoa (CR), erantzun osorik ez (ez CR)—, LDH kontzentrazioa (normala edo altua), β-2 mikroglobulina kontzentrazioa (normala edo altua), tratamenduaren emaitza, aurrerapenik gabeko biziraupena (PFS) eta biziraupen orokorra bost urtera (4. taula). Kontrol-pazienteengandik ere eskuratu zen informazio demografikoa (4. taula).

Laginen prozesamendua eta RNA erauzketa prozesua

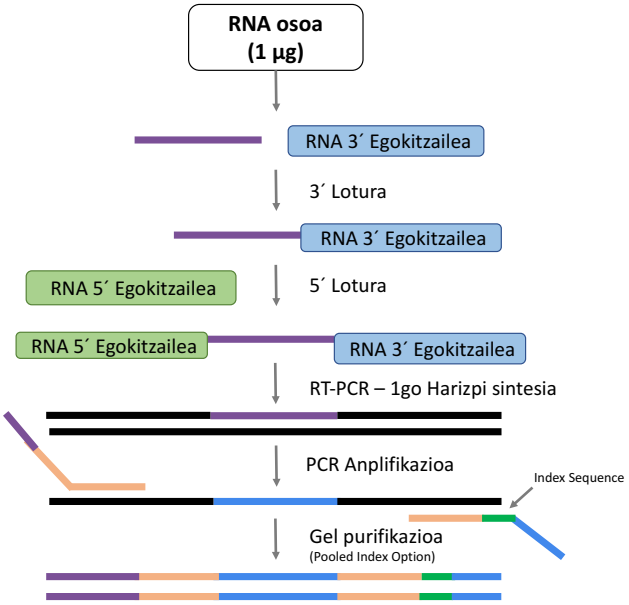
Euskal Biobankuak (www.biobancovasco.org) eman zituen azterketan parte hartu zuten pazienteen laginak eta datuak, eta lanketa-prozedura estandarrei jarraituz landu ziren.

RNA erauzteko, zutabetan oinarritutako erauzketa-metodoak erabili ziren, zeinetan RNA molekula txikiak eta handiak batera bakartzen baitira *miRNeasy FFPE Kit*-a erabilita (Qiagen, Hilden, Germany).

RNA txikien sekuentzien liburutegia prestatzea eta sekuentziatzea

TruSeq small RNA Sample Prep Kit-a (ref. RS-200-0012, Illumina) erabili zen, fabrikatzailearen protokoloari jarraituz. RNA osoaren 1 µg erabili zen. Lehendabizi, 3' egokitzaileak eta, gero, 5' egokitzaileak lotu zitzaizkion RNAri. Ondoren, cDNA sintetizatu zen alderantzizko transkriptasa (SuperScript II, ref. 18064-014, Invitrogen) eta 3' RNA egokitzailearen osagarria den "*primer*" edo abiarazle espezifiko bat erabilita (RNA RT Primerra). cDNA are gehiago anplifikatu zen PCR bidez, kit-ean zeuden egokitzaile indexatuak erabilita. Azkenik, tamainaren arabera hautatu ziren liburutegiak, *% 6ko Novex® TBE Gel*-ak erabilita (ref. EC6265BOX, Life Technologies). 18-36 bp-ko tamaina zuten txertatze-sekuentziak ebaki ziren geletik, eta DNA molekulak prezipitatu eta eluitu egin ziren 10 µl EB erabilita.

Agilent DNA High Sensitivity txipak erabili ziren lortutako liburutegiak aztertzeko, hau da, kantitatea zenbatesteko eta tamainaren araberako banaketa egiaztatzeko. Ondoren, qPCR erabiliz kuantifikatu ziren; horretarako, *KAPA Library Quantification Kit*-a (ref. KK4835, KapaBiosystems) erabili zen Illumina-ren *cBot*-arekin anplifikatu aurretik. Berrogeita hamar nukleotidotako eta irakurketa bakarreko sekuentziez osatu zen liburutegia, Illumina-ren *HiSeq 2500* sistema erabilita. Sekuentziazioaren sakonera 10 milioi irakurketakoa izan zen lagineko, gutxi gorabehera. Erregulazio Genomikoaren Zentroan (CRG) egin zen prozesu osoa.



5. irudia: Truseq RNA lagin prestaketa (Truseq laginak prestatzeko gidatik egokitua). https://support.illumina.com

Azterketa bioinformatikoa eta mikroRNAren adierazpen diferentziala

Skewer erabilita, irakurketak ebaki egin ziren RNA txiki egokitzaileak zeuden tokietan, irakurketa iragaziak lortuz. Hau da, 15 base baino laburragoak eta 40 base baino luzeagoak ziren sekuentzia guztiak kendu ziren. Gainerakoak erreferentziako genomarekin lerrokatu ziren (GRCh38) *ShortStack* erabilita, eta *htseq-count* erabiliz aztertu ziren lortutako lerrokatzeak, gene bakoitzeko etiketa kopurua zehazteko. Gencode partzuergoko v27 anotazioa erabili zen. Azkenik, mikroRNA geneetara murriztu zen kontaketa-matrizea.

Kondizio guztietan egindako irakurketen batura kontuan hartuta 10 irakurketa edo gutxiago zituzten RNA txiki guztiak baztertu ziren eta gainerako geneak *DESeq2*-ren adierazpen diferentzialaren (DE) analisirako erabili ziren. *DESeq2*-ek irakurketa-kontaketak normalizatu eta transformatu egiten ditu, dispertsioak zenbatesten du eta Wald proba egiten du DE gene nabarmenak detektatzeko. Positibo faltsuen aurkitze-tasa (p adj) erabiliz doitu ziren p-balioak.

Osagai nagusizko analisia (ingelesez, "*Principal component analysis*", PCA) egin zen, datu multzo handien dimentsioa txikitzeko eta adierazpen-profilean oinarrituta berez agertu ziren lagin klaseak aztertzeko. Horren bidez, hasierako datu multzoaren proiekzioa egin daiteke bata bestearekiko independenteak diren ardatzen arteko eremu murriztuan (osagai nagusiak), eta, hala, sistemaren bariantza osoaren zati nabarmena azaltzen du. MikroRNA guztien adierazpenak kontuan hartuta, laginak bariantza horren arabera multzokatzen diren adierazpena eskura dezakegu. Transformazio logaritmikoaren bidez normalizatutako balioak erabili ziren azterketarako. Bariantzaren osagairik handienak X ardatzean ezartzen dira, eta bigarren handienak Y ardatzean. R-ren *prcomp* paketea erabiliz egin zen PCA, eta irakurketa-kontaketa transformatuak aztertzeko erabili zen.

Biziraupenaren analisia

Biziraupenaren analisiak egin ziren, epe luzeko erremisioa zuten eta gaixotasuna berriro garatu zuten pazienteen diagnostikoko laginetan adierazpen diferentziala zuten mikroRNA horiek PFS OS duten eragina zenbatesteko (doitutako p-balioa < 0,05; $\log_2$ aldaketa > 2 edo < –2). Erregulazioan aldaketak duten mikroRNA molekulen adierazpenaren mediana kontuan hartu zen pazienteak adierazpen gutxiko edo adierazpen handiko taldeetan sartzeko (miR-4444, miR-449c-5p, miR-3681-5p, miR-3928-3p, miR-449b-5p, miR-370-3p eta miR-4424 pronostiko onaren biomarkatzaileak; miR-133a-3p, miR-208b-3p eta miR-205-5p pronostiko txarraren biomarkatzaileak). Kaplan-Meyer analisia erabili zen biziraupen-kurbak definitzeko, eta Log-Rank testa esangarritasuna ebaluatzeko. Aldagai anitzeko analisia egiteko, azpimota eta IPI egoera ere kontuan hartu genituen arrisku proportzionalen Cox-en metodoa (Cox PH) erabilita. *Survival R* paketea erabiliz egin ziren kalkulu guztiak. Biziraupenarekin lotura adierazgarria kontsideratu zen p-balioa < 0,05 zuten kasuetan.

mikroRNA-mRNA interakzio-sarea

MikroRNA molekulen adierazpenari buruz ditugun datu propioak (BZHLH duten pazienteak vs kontrolak) *Gene Expression Omnibus* (GEO) datu-basean (157) dauden mRNA molekulen adierazpen-datuekin elkartu eta mikroRNA-mRNA interakzio-sare bat sortu genuen, BZHLH garatzeko mekanismoa argitzeko.

Itu-geneak identifikatzea

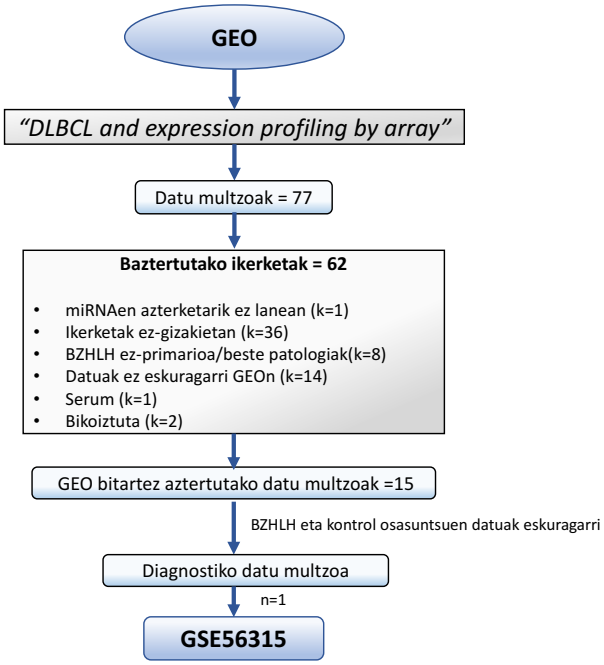
Diagnostikoa egiterakoan erregulazioan aldaketak zituzten mikroRNA molekulen itu-geneak identifikatu ziren (BZHLHdun pazienteak *vs* kontrolak). MiRTarBase (158)(http://mirtarbase.cuhk.edu.cn/php/index.php) erabili zen esperimentuz baliozkotutako mikroRNA-mRNA itu-interakzioak bilatzeko. Hirurehun eta hirurogei mila mikroRNA-itu interakzio (MTI) ditu jasota MiRTrBasek; literatura egokia aztertuta biltzen dira guztiak eskuz. Oro har, bildutako MTIak era esperimentalean baliozkotzen dira adierazle-azterketak (ingelesez, *"reporter assay", "Western blot"-ak, "microarray*") eta hurrengo belaunaldiko sekuentziazio-esperimentuak (ingelesez, *"Next generation sequencing"*) erabilita. qRT-PCR, ELISA, immunohistokimika, "Westen blot" eta adierazle-azterketa bidez baliozkotutako ituak dira mikroRNA baten eta haren ituen arteko zuzeneko interakzioa (balizko interakzio) erakusteko metodo fidagarrienak. "Microarray" edo hurrengo belaunaldiko sekuentziazioak, berriz, mikroRNA eta haien ituen arteko harreman ez-zuzenak eskaintzen dituzte (interakzio ahulak). Azterketa honetarako mikroRNA-mRNA balizko interakzioak soilik hautatu ziren.

GEO errepositorioko datu-baseak

GEO errepositorioa erabili zen informazio klinikoa duten BZHLH tumoreen eta kontrol osasuntsuen geneen adierazpen-datuak (mRNA) bilatzeko. Informazio genetiko osoa bilatzeko, "*DLBCL and expression profiling by array*" (BZHLH eta adierazpenaren profilak bilatzea array bidez) terminoak erabili genituen, hasiera-data mugagabea eta amaiera-data 2020ko iraila zuten

ikerketa-argitalpenak identifikatzeko. 77 datu multzo aztertu genituen eta sartzeko irizpideak betetzen zituzten 15 artikuluetatik batek bakarrik zituen kontrol-datuak; gure analisian sartu genuen azterketa hori (6. irudia)._MikroRNA eta mRNA molekulen adierazpen bateratuaren sarearen analisiaren lan-fluxua 7. irudian ikus daiteke.

GPL570 plataformako "microarray" datu multzo bat ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array) deskargatu zen GEOtik (http://www.ncbi.nlm.nih.gov/geo/), kontrolarekin alderatuz BZHLHn adierazpen diferentziala duten geneak aztertzeko. Adierazpen-profilak gongoilen 55 BZHLH lagin eta 33 lagin osasuntsu zituen, Aalborg-eko Unibertsitate Ospitaletik bidalitakoak (159,160).



6. irudia: Datu hautapenaren fluxu-diagrama.

Adierazpen diferentziala duten geneak (DEGak) identifikatzea

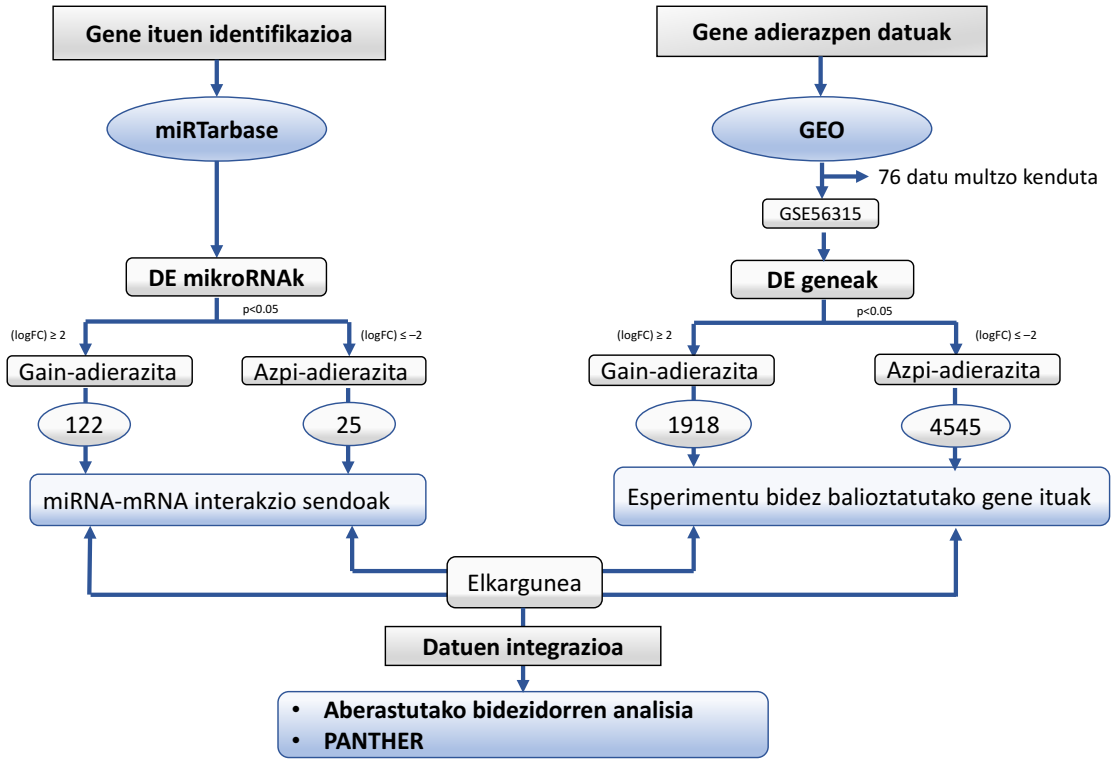
GEO serieetako lagin sorta bi edo gehiago alderatzeko aukera ematen duen web-tresna interaktiboa da GEO2R. Alderaketa horren bidez, kondizio esperimentaletan adierazpen diferentziala duten geneak identifika daitezke. Alderaketaren emaitza geneak adierazkortasunaren arabera antolatutako taula batean adierazi dira. Halaber, grafikoki adierazi da adierazpen diferentziala duten geneak ikusarazteko eta datu multzoen kalitatea ebaluatzeko. Datu multzoak paziente eta kontrolen kategorietan sailkatu ondoren, parametro lehenetsiekin egin zen azterketa. Inklusio-irizpide hauek ezarri genituen DEGetarako: erregulazioa handituta duten geneen $\log_2$ aldaketak edo "*fold change*" (logFC) ≥ 2 balioa izan behar du eta doitutako p-balioak < 0,05 izan behar du; azpi-erregulazioa duten geneek, aldiz, logFC ≤ –2 eta doitutako p-balioa < 0,05 izan behar dute.

Datuak bateratzea

Desregulazioa aurkezten duten mikroRNA molekulen ituak, esperimentu bidez baliozkotutakoak, adierazpen diferentziala duten mRNA molekulekin gainjarri ziren (azken horiek GEO datu-baseetatik eskuratutakoak) (7. irudian ikus daiteke). Adibide gisa, gain-adierazitako mikroRNAen itu-geneak eta azpi-adierazitako geneak gainjarri ziren (GEO datuetatik eskuratutakoak), mikroRNA molekulen ekintza-mekanismoari jarraituz. Azpi- zein gain-adierazitako geneen arteko ebakiduraren mapa egin zen Venn paketea erabilita (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Analisi bioinformatikoa eta aurreikuspen funtzionala

Gainjarritako geneekin azterketa hauek egin ziren: Gene Ontologiaren (GO) anotazio funtzionalak, PANTHER (Protein ANalysis THrough Evolutionary Relationships) sailkapen-sistema eta seinale-bideen aberaste-analisiak (Consensus Pathway database http://cpdb.molgen.mpg.de/). miR-205ren DEGen interakzioak aztertzeko, STRING (Search Tool for the Retrieval of Interacting Genes/http://string.embl.de/) datu-basera igo genituen DEGak.

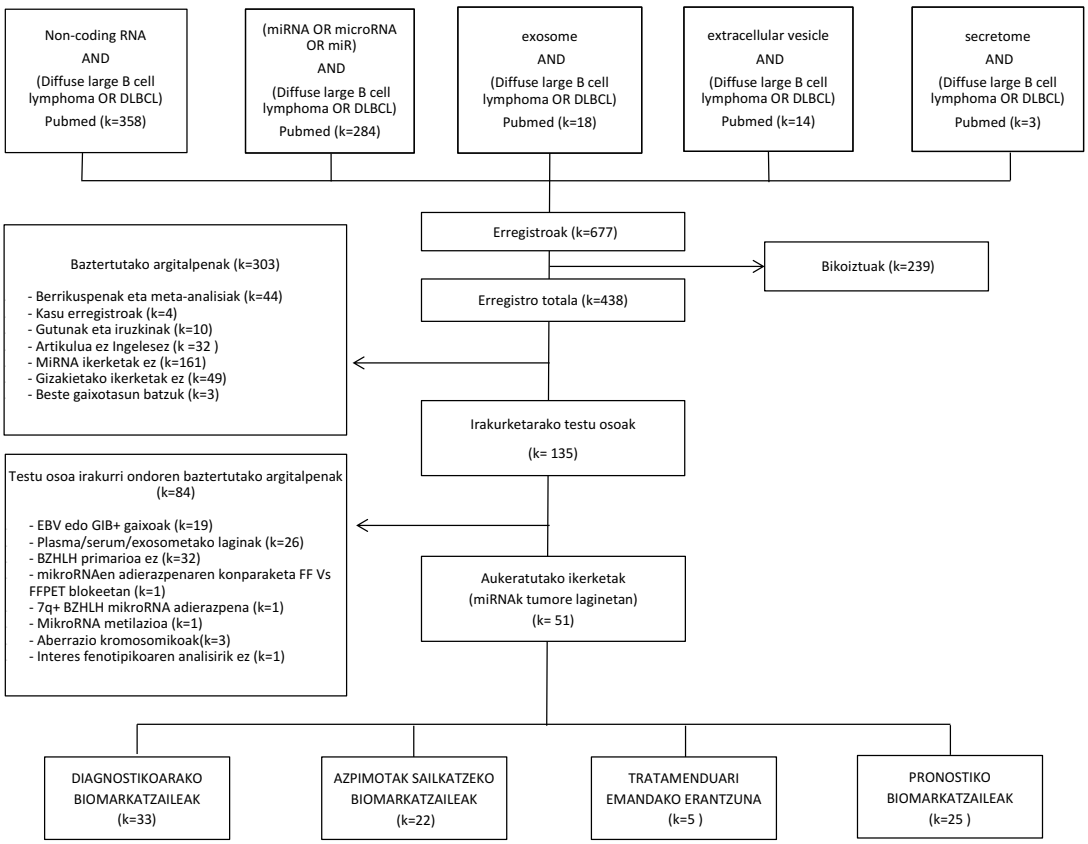


7. irudia: mikroRNA-mRNA sarearen eraikuntzaren lan fluxua.

EMAITZAK

B ZELULA HANDIKO LINFOMA HEDATSUAN MIKRORNA POTENTZIALAK HAUTATZEA LITERATURA SISTEMATIKOKI AZTERTUZ

Bilaketa-estrategiari esker, 677 erregistro aurkitu ziren PubMed datu-basean (8. irudia). Bikoiztutako emaitzak kendu ondoren 438 argitalpen geratu ziren eta horietatik, 303 baztertu egin ziren laburpena irakurri ondoren, ez baitzituzten betetzen inklusio-irizpideak. Ondoren, BZHLHko mikroRNA molekulei buruzko 135 ikerketen testu osoa irakurri zen. Beste 84 artikulu baztertu ziren, besteak beste, arrazoi hauengatik: BZHLHLrekin batera beste patologia batzuk zeudelako, mikroRNAk ez zirelako aztertu tumore-laginean, BZHLH ez-primarioa ere kontuan hartu zutelako, diagnostikoan, azpimota, tratamenduei emandako erantzunean edo pronostikoan mikroRNA molekulek duten funtzioa ez zutelako ebaluatu edo mikroRNA molekulen adierazpen-aldaketak ez zirelako kontuan hartu. Guztira, BZHLHren tumore-laginetan mikroRNA molekulen adierazpen-aldaketak biomarkatzaile gisa duten funtzioa aztertzen zuten 51 ikerketa sartu ziren. Haietatik 33 lanetan, BZHLH diagnostikatzeko ustezko biomarkatzaile gisa hartzen zituzten mikroRNAk, 22tan azpimotak sailkatzeko tresna gisa, bost tratamenduari emandako erantzuna aztertzeko, eta 25 ikerketetan pronostikorako bilatu zituzten markatzaileak.



8. irudia: Ikerketen hautapenaren fluxu-diagrama.

Tumore-ehunetako mikroRNAk BZHLH diagnostikatzeko biomarkatzaile gisa

Hogeita hamahiru ikerketek mikroRNA molekulen adierazpena aztertu zuten, BZHLH kasuak kontrol osasuntsuekin alderatuz (145–150,154,161–186). Hogeita hamairu ikerketa horiei esker, BZHLH pazienteetan kontrol osasuntsuekin alderatuz adierazpen esangarria zuten 152 mikroRNA identifikatu ahal izan ziren; Eranskineko 1. taulan daude.

Bi ikerketa edo gehiagotan era berean erregulazioan aldaketak zituzten mikroRNA molekulei dagokienez, BZHLH pazienteetan gain-adierazita agertzen ziren hiru mikroRNA identifikatu genituen (miR-155-5p (145,147,167,170,174,176,178–181), miR-21-5p (145,161,171,174,178,180,183) eta miR-146a-5p (169,174,179)), nahiz eta ikerketa batzuek ez aurkitu lotura esangarririk (miR-155-5p (149,150), miR-21-5p (147,150), miR-146a-5p (145,150)). Halaber, emaitza kontraesankorrak zituen mikroRNA bat aurkitu genuen: miR-150-5p. Lau ikerketetan, BZHLH pazienteetan azpi-adierazita aurkitu zen (145–148), eta beste ikerketa batean gain-adierazita (149). Beste ikerketa batean, berriz, ez zen lotura esangarririk aurkitu (150) (5. taula).

Tumore-ehunetako mikroRNAk BZHLHren azpimotak sailkatzeko biomarkatzaile gisa

Hogeita bi ikerketetan, BZHLHren ZGB eta ez-ZGB azpimotak bereizteko tumore-ehunetako mikroRNA molekulek duten gaitasuna aztertu zen (145,147,150,151,155,171,173,175–181,187–194). Ikerketa horietan aurkitu zenez, BZHLH ZGB eta ez-ZGB laginen artean, 80 mikroRNA ezberdinek zuten adierazpen esangarria; Eranskinaren 2. taulan ikus daitezke. Adierazpen esangarria zuten 80 mikroRNA molekulen artean, lau aipatu ziren bi ikerketa baino gehiagotan. Aipatzen zenez, ZGB laginetan horietatik hiruk azpi-adierazita (miR-155-5p (145,150,155,178–181,187,192,194), miR-221-3p (150,151,155,180) eta miR-222-3p (150,151,155)) edo adierazpena aldatu gabe zuten (miR-155-5p (147), miR-221-3p (145,191) eta miR-222-3p (145,147,187,191)) eta miR-28-5p molekulak, berriz, gain-adierazita (148,149,185,189) edo aldatu gabe (145,155) aurkitu zen azpimota berean (6. taula).

5. taula: Bi ikerketa baina gehiagotan BZHLHren diagnostikoarekin lotura esangarria duten mikroRNAk.

mikroRNA esangarriak	Emaitza	n BZHLH	n Kontrolak	Lagin iturria	Metodoa	n mikroRNA	Erreferentzia
miR-155-5p	Altu	29	32 (RLH)	Ehuna	qRT-PCR	1	Li 2017
		22	6 (NLN)	Biopsia	qRT-PCR	1	Huskova 2015
		200	11 (NT)	FFPE	qRT-PCR	3	Go 2015
		45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
		90	31 (RLN)	FFPE	qRT-PCR	2	Zhong 2012
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle 2008
		48	6 (NBC)	FF eta FFPE	qRT-PCR	3	Lawrie 2007
		23	2	FF	Semi RT-PCR	1	Eis 2005
		24	14 (NLN)	FFPE	Array	3100 zunda	Tamaddon 2016
		84	4	FFPE	Array	1	Wu 2018
	NS	92	15	FF	sekuentziazioa	miRNAome	Lim 2015
		12	7	FFPE	qRT-PCR	4	Handal 2013
miR-21-5p	Altu	55	20 (NLN)	FF eta FFPE	qRT-PCR	1	Liu 2017
		26	10 (NLN)	FFPE	qRT-PCR	1	Song 2017
		200	11 (NT)	FFPE	qRT-PCR	3	Go 2015
		45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
		48	6 (NBC)	FF eta FFPE	qRT-PCR	3	Lawrie 2007
		24	14 (NLN)	FFPE	Array	3100 zunda	Tamaddon 2016
		45	23(RLNH)	Ehuna	qRT-PCR	1	Chen 2020
	NS	92	15	FF	sekuentziazioa	miRNAome	Lim 2015
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle 2008
miR-146a-5p	Altu	90	31 (RLN)	FFPE	qRT-PCR	2	Zhong 2012
		24	14 (NLN)	FFPE	Array	3100 zunda	Tamaddon 2016
		56	28(RLNH)	Ehuna	qRT-PCR	1	Zhuang 2014
	NS	45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
		92	15	FF	sekuentziazioa	miRNAome	Lim 2015
miR-150-5p	Altu	12	7	FFPE	qRT-PCR	4	Handal 2013
	Baxu	45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
		36	5 (NLN)	Ehuna	qRT-PCR	8	Fassina 2012
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle 2008
		5	4 (RLH)	Ehuna	nanostring	800	Jia 2018
	NS	92	15	FF	sekuentziazioa	miRNAome	Lim 2015

Laburdurak: FF: izoztutako ehuna; FFPE: formalinan finkatutako eta parafinan sartutako ehuna; NA: ez eskuragarri; Altu: BZHLH pazienteetan modu esangarrian gain-adierazita; Baxu: BZHLH pazienteetan modu esangarrian azpi-adierazita; NS: desberditasun ez esangarriak pazienteen eta kontrolen artean; RLH: Linfoma hiperplasia erreaktiboa; NLN: Ehun linfatiko normala; NT: amigdala normala; DC: aurkikuntzaren kohortea; VC: balioztatzearen kohortea; NBC: B zelula normalen lagina.

6. taula: Bi ikerketa baino gehiagotan BZHLHren azpimotekin lotura esangarria duten mikroRNAk.

mikroRNA esangarriak	Emaitza	n ZGB	n ez-ZGB	Lagin iturria	Metodoa	n mikroRNA	Erreferentzia
miR-155-5p	Baxu ZGB	53	95	FFPE	qRT-PCR	8	Go 2015
		32	27	FFPE	qRT-PCR/array	377	Iqbal 2015
		20	34	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
		36	31	FF	qRT-PCR	1	Huang 2012
		21	69	FFPE	qRT-PCR	2	Zhong 2012
		32	28	FFPE	Array	464	Lawrie 2009
		16	18	FF eta FFPE	qRT-PCR	3	Lawrie 2007
		4	19	FF	Semiq. RT-PCR	1	Eis 2005
		41	30	FF	sekuentziazioa	miRNAome	Lim 2015
		32	32	biopsia	qRT-PCR	1	Due 2019
	NS	25	25	FFPE	qRT-PCR	157	Roehle 2008
miR-221-3p	Baxu ZGB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno 2011
		32	28	FFPE	Array	464	Lawrie 2009
		16	18	FF eta FFPE	qRT-PCR	3	Lawrie 2007
		41	30	FF	sekuentziaioa	miRNAome	Lim 2015
	NS	20	20	Ehuna	Array	113	Zhang 2009
		20	34	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
miR-222-3p	Baxu ZGB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno 2011
		32	28	FFPE	Array	464	Lawrie 2009
		41	30	FF	sekuentziaioa	miRNAome	Lim 2015
	NS	25	25	FFPE	qRT-PCR	157	Roehle 2008
		20	20	Ehuna	Array	113	Zhang 2009
		32	27	FFPE	qRT-PCR/array	377	Iqbal 2015
		20	34	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
miR-28-5p	Altu ZGB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno 2011
		32	27	FFPE	qRT-PCR/array	377	Iqbal 2015
		41	30	FF	sekuentziaioa	miRNAome	Lim 2015
		20	20	Ehuna	Array	113	Zhang 2009
	NS	20	34	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
		32	28	FFPE	Array	464	Lawrie 2009

Laburdurak: ZGB: zentro germinaleko B zelula mota; ez-ZGB: aktibatutako B zelula mota; FF: izoztutako ehuna; FFPE: formalinan finkatutako eta parafinan sartutako ehuna; NA: ez eskuragarri; Altu: ZGB pazienteetan modu esangarrian gain-adierazita; Baxu: ZGB pazienteetan modu esangarrian azpi-adierazita; NS: desberdintasun ez esangarriak ZGB eta ez-ZGB pazienteen artean.

Tumore-ehunetako mikroRNAk BZHLHn tratamenduari emandako erantzuna aurresateko biomarkatzaile gisa

Bost ikerketetan, BZHLH ehunetako mikroRNAk R-CHOP tratamenduari emandako erantzunaren biomarkatzaile gisa duten funtzioa aztertu zuten (148,175,176,179,190). Azterketa bakoitzaren ezaugarriak Eranskineko 3. taulan daude jasota. Bost mikroRNA ezberdinek adierazpen esangarria azaltzen zuten tratamenduari emandako erantzun ona eta txarra zuten pazienteen artean. Terapiari emandako erantzun onarekin lotura zuten hiru mikroRNA aurkitu ziren (miR-27b-3p (148), miR-34a-5p (176) eta miR-224-5p (175)). MiR-155-5p (179) eta miR-146-5p (179), berriz, kimioerresistentziarekin erlazioa zutela aurkitu zen. Hala ere, mikroRNA bakoitza azterketa bakarrean aztertu zen, eta emaitza bat ere ez zen erreplikatu.

Tumore-ehunetako mikroRNAk BZHLHren pronostikorako biomarkatzaile gisa

Hogeita bost ikerketetan aztertu zen BZHLH pazienteetan tumore-ehuneko mikroRNA molekulek pronostikorako duten garrantzia (147,148,150–156,167,172,173,175,177–180,184,186,187,190,193–196). Biziraupenarekin erlazio nabarmena zuten 50 mikroRNA aurkitu ziren; Eranskineko 4. taulan daude jasota guztiak.

Bi ikerketa baino gehiagotan bat egiten duten emaitza esangarriak izatearen baldintza betetzen zuten miR-222-3p eta miR-155-5p mikroRNAk identifikatu ziren. Lau ikerketetan, miR-222-3p (151–154) eta miR-155-5p (167,179,187,195) molekulen gain-adierazpena pronostiko txarrarekin erlazionatu zen; horietako batean (154), ez-ZGB taldean soilik pronostiko txarrarekin asoziatu zen. Hala ere, miR-222-3p (147,150,155,156) eta miR-155-5p (147,150,152,153,155,156,178,180) molekulek pronostikoarekin asoziariorik ez zutela ondorioztatu zuten lau eta zortzi ikerketek, hurrenez hurren, eta ikerketa batek emaitza kontraesankorrak aurkitu zituen miR-155 azpi-adierazpena zuten ZGB pazienteen OS eta PFSari dagokionez (194) (7. taula).

7. taula: Bi ikerketa baino gehiagotan BZHLHren pronostikoarekin lotura esangarria duten mikroRNAk.

mikroRNA esangarriak	Emaitza	n BZHLH	Lagin iturria	Metodoa	n mikroRNA	Erreferentzia
miR-222-3p	Altu: OS↓	176	FFPE	qRT-PCR	11	Alencar 2011
	Altu: PFS eta OS↓	36/240	FFPE	qRT-PCR/array	470/9	Montes-Moreno 2011
	Altu: OS eta PFS↓	106	FFPE	qRT-PCR	3	Malumbres 2009
	Altu: OS ez-ZGB-an ↓	74	Biopsia	qRT-PCR	1	Shanshan 2019
	NS	64	FFPE	Array	464	Lawrie 2009
		92	FF	sekuentziazioa	miRNAome	Lim 2015
		58	Biopsia	qRT-PCR	157	Roehle 2008
		83	FFPE	qRT-PCR/array	±900	Shepshelovich 2015
miR-155-5p	Altu: biziraupen baxuagoa	118	FF	qRT-PCR	1	Zhu 2016
	Altu: OS↓	79	FFPE	qRT-PCR	8	Iqbal 2015
	Baxu: PFS↑	90	FFPE	qRT-PCR	1	Zhong 2012
	Altu: PFS↓	82	FFPE	qRT-PCR	1	Wu 2018
	Baxu: OS eta PFS↓	array	Biopsia	qRT-PCR	Array	Due 2019
	NS	176	FFPE	qRT-PCR	11	Alencar 2011
		200	FFPE	qRT-PCR	3	Go 2015
		35	FFeta FFPE	qRT-PCR	3	Lawrie 2007
		64	FFPE	Array	464	Lawrie 2009
		92	FF	sekuentziazioa	miRNAome	Lim 2015
		106	FFPE	qRT-PCR	3	Malumbres 2009
		58	Biopsia	qRT-PCR	157	Roehle 2008
		83	FFPE	qRT-PCR/array	±900	Shepshelovich 2015

Laburdurak: FF: izoztutako ehuna; FFPE: formalinan finkatutako eta parafinan sartutako ehuna; OS: biziraupen globala; PFS: aurrerapenik gabeko biziraupena; EFS: gertaera gabeko biziraupena; RFS: berriz gaixotze gabeko biziraupena; Baxu: Berriz gaixotutako BZHLH pazienteetan azpi-adierazita; Altu: Berriz gaixotutako BZHLH pazienteetan gain-adierazita; NS: desberdintasun ez esangarriak berriz gaixotzen diren pazienteen eta epe luzeko hobekuntza dutenen artean.

MIKRORNA HAUTAGAIAK BALIOZKOTZEA IKERKETAKO POPULAZIOAN

Literaturan deskribatutako diagnostiko-ereduaren analisia ikerketako populazioan

Gure ikerketako populazioan literaturan hautemandako lau mikroRNA molekulak aztertu ziren, erregulazioan aldaketak zituztenak, diagnostikorako biomarkatzaile gisa kasuak baztertzeko duten gaitasuna berresteko.

8. taulan ikus daitekeenez, erregulazioan aldaketak zituzten mikroRNA guztiek antzeko adierazpen-ereduak zituzten ikerketako populazioan, literaturan behatutakoarekin alderatuz (miR-150-5p, padj = $1{,}44x10^{-22}$; miR-146a-5p, padj = $1{,}16x10^{-12}$; miR-155-5p, padj = $1{,}67x10^{-08}$; miR-21-5p, padj = $4{,}03x10^{-05}$).

8. taula: Literaturaren arabera BZHLHn erregulazioa aldatuta duten mikroRNAen adierazpen maila gure ikerketa populazioan.

mikroRNA	"Base Mean"	Log2 "Fold Change"	p-balioa	padj
hsa-miR-150-5p	6310,7	-3,22	$3{,}46x10^{-25}$	$1{,}44x10^{-22}$
hsa-miR-146a-5p	33085,6	2,11	$2{,}05x10^{-14}$	$1{,}16x10^{-12}$
hsa-miR-155-5p	20773,1	1,99	$7{,}26x10^{-10}$	$1{,}67x10^{-08}$
hsa-miR-21-5p	140750,7	1,11	$4{,}37x10^{-06}$	$4{,}03x10^{-05}$

Log2 "Fold change" positiboa edo negatiboa mikroRNAen gain-adierazpena edo azpi-adierazpena adierazten du BZHLH duten pazienteen eta osasuntsu kontrolen artean hurrenez hurren; "Base Mean": zenbaketa normalizatutako balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta.

Literaturan deskribatutako azpimota-ereduaren analisia ikerketako populazioan

Gure ikerketako populazioan aztertu ziren literaturan hautemandako erregulazioan aldaketak zituzten lau mikroRNA molekulak, azpimotak detektatzeko biomarkatzaile gisa kasuak baztertzeko duten gaitasuna berresteko.

Lau mikroRNA molekuletatik batek bakarrik erakutsi du estatistikoki esangarriak diren ezberdintasunak gure populazioan (miR-28-5p, padj = 0,00342) (9. taula).

9. taula: Literaturaren arabera BZHLHren azpimotetan era esangarrian adierazitako mikroRNAen adierazpen maila gure ikerketa populazioan.

mikroRNA	"Base Mean"	Log2 "Fold Change"	p-balioa	padj
hsa-miR-28-5p	475,7	1,24	$3{,}85x10^{-05}$	0,00342
hsa-miR-222-3p	1897,9	-0,79	0,00456	0,09895
hsa-miR-221-3p	4859,2	-0,37	0,22993	0,55006
hsa-miR-155-5p	19078,2	0,12	0,74670	0,91329

Log2 "Fold change" positiboak mikroRNAen gain-adierazpena adierazten du ZGB azpimotan eta negatiboak ez-ZGB azpimotan gain-adierazpena; "Base Mean": zenbaketa normalizatuko balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta.

Literaturan deskribatutako tratamenduari emandako erantzuna aurresateko ereduaren analisia ikerketako populazioan

Literaturan aurkitutako bost mikroRNAk ikerketa bakarrean aztertu zirenez, haiek gure populazioan zuten adierazpena aztertu genuen azaldutako emaitzak baliozkotu ahal zirela berresteko.

10. taulan ikus daitekeenez, batek ere ez du ezberdintasun esangarririk erakutsi adierazpenean, erremisio osoa duten eta errefraktarioak diren pazienteen artean.

10. taula: Literaturaren arabera BZHLHren tratamenduaren erantzunarekin erlazionatutako mikroRNAen adierazpen maila gure ikerketa populazioan.

mikroRNA	"Base Mean"	Log2 "Fold Change"	p-balioa	padj
hsa-miR-224-5p	47,7	0,72	0,11013	0,46446
hsa-miR-155-5p	20773,1	-0,49	0,20472	0,59557
hsa-miR-146a-5p	33085,6	-0,12	0,72557	0,91006
hsa-miR-34a-5p	759,4	-0,02	0,91785	0,97574
hsa-miR-27b-3p	14074,6	0,21	0,50219	0,50218

Log2 "Fold change" positiboak mikroRNAen gain-adierazpena adierazten du erremisio osa duten pazienteetan errefraktarioekin alderatuz eta negatiboak kontrakoa adierazten du; "Base Mean": zenbaketa normalizatutako balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta.

Literaturan deskribatutako pronostiko-ereduaren analisia ikerketako populazioan

Gure ikerketako populazioan aztertu ziren literaturan hautemandako erregulazioan aldaketak zituzten bi mikroRNA molekulak, pronostikorako biomarkatzaile gisa kasuak baztertzeko duten gaitasuna berresteko.

MikroRNA horiek ez zuten ezberdintasun esangarririk erakutsi adierazpenean, gure populazioaren barruan berriro gaixotu diren eta epe luzeko erremisioa duten pazienteen artean (11. taula).

11. taula: Literaturaten arabera pronostikoarekin erlazionatutako mikroRNAen adierazpen maila gure ikerketa populazioan.

mikroRNA	"Base Mean"	Log2 "Fold change"	p-balioa	padj
hsa-miR-222-3p	1984,9	-0,08	0,77088	0,93065
hsa-miR-155-5p	20773,1	-0,03	0,92503	0,97641

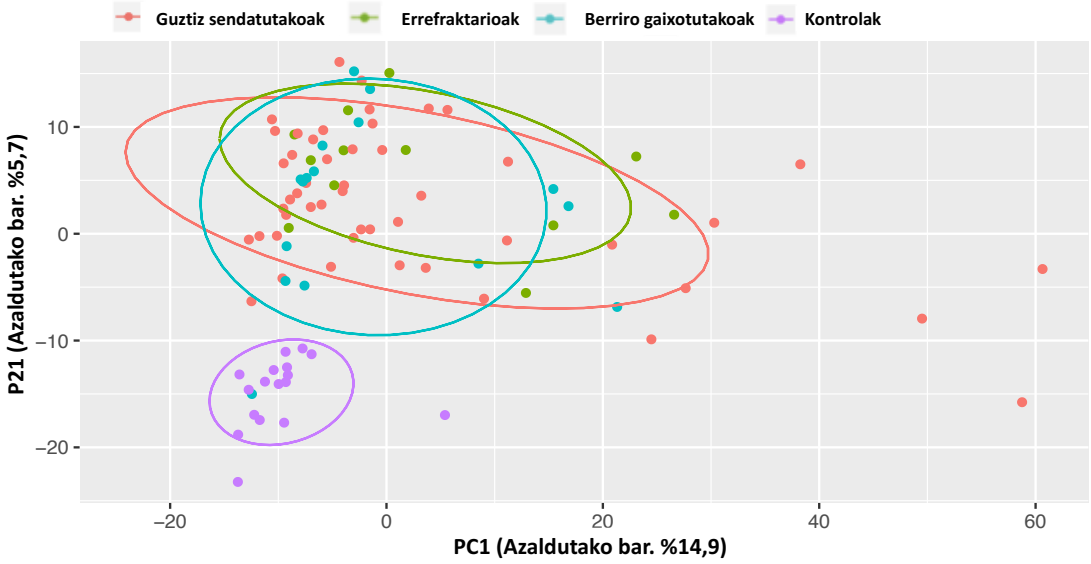
Log2 "Fold change" positiboak mikroRNAen gain-adierazpena adierazten du epe luzeko erantzuna duten pazienteetan diagnonsiaren momentuan berriz gaixotzen direnekin alderatuz eta negatiboak kontrakoa adierazten du; "Base Mean": zenbaketa normalizatuko balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta.

BZHLH-N KARAKTERISTIKOAK DIREN MIKRORNA MOLEKULEN SINADURA BERRIAREN IDENTIFIKAZIOA, RNA TXIKIEN SEKUENTZIAZIO BIDEZ

mikroRNA molekulen analisi osoa

Guztira 1584 mikroRNA identifikatu ziren mikroRNA molekulen sekuentziazio-analisia erabilita. Osagai nagusien analisia (PCA) egin zen mikroRNA guztien adierazpena kontuan hartuz, eta, hala, laginak bariantzaren arabera nola multzokatzen ziren ikusteko (9. irudia). Lehen osagaiak (PC1) sistemaren bariazioaren zatirik handiena erakusten du (azaldutako bariantza osoaren % 14,9); bigarrenari, berriz, bariantza osoaren % 5,7 dagokio. Horren ondorioz, PCA grafikoaren eskualde ezberdinetan kokatu ziren pazienteen laginak: guztiz sendatu direnak, errefraktarioak, berriro gaixotutakoak eta kontrolak. Antza denez, ezberdintasun gutxirekin multzokatzen dira guztiz sendatu diren laginak, errefraktarioak eta berriro gaixotutako pazienteen artean; kontrol taldeko laginak, berriz, guztiz bereizitako taldean multzokatzen dira.

PCA grafikoaren arabera, mikroRNA guztiak bistaratzean erraz bereiz zitezke BZHLH kasu gehienak kontroletatik. Beraz, BZHLH pazienteek mikroRNA molekulen adierazpen bereizgarria dute kontrol taldearekin alderatuz gero. BZHLH pazienteen azpitaldetan ez da ezberdintasun nabarmenik aurkitu tratamenduari emandako erantzunaren eta pronostikoaren arabera.



9. irudia: BZHLH pazienteen eta kontrolen arteko mikroRNAen adierazpenaren osagarri nagusien analisiaren irudikapen grafikoa.

Diagnostikoaren biomarkatzaileak

BZHLH diagnostikatzeko biomarkatzaile berriak izan daitezkeen hautagaien zerrenda lortzeko, mikroRNA bakoitzaren adierazpena konparatu zen BZHLHren diagnostikoa egitean hartutako 78 laginetan eta BZHLH ez zuten pertsonen gongoiletako 17 laginetan. BZHLH diagnostikatu zitzaien

pazienteen laginetan estatistikoki esangarria zen adierazpena 146 mikroRNA molekulek zutela aurkitu genuen kontrol-laginekin alderatuz gero (padj <0,05; log_2 "fold change" > 2/<-2). MikroRNA horietatik 122 gain-adierazita aurkitu ziren BZHLHn, eta 24 mikroRNA, berriz, azpi-adierazita. Eranskineko 5. eta 6. tauletan daude BZHLH pazienteetan gain edo azpi-adierazitako mikroRNA guztiak, kontrolarekin alderatuta (p < 0,05 eta log_2 "fold change"> 0). Gehien gain- edo azpi-adierazitako 20 mikroRNAk 12. eta 13. tauletan azaltzen dira.

12. taula: Gehien gain-adierazitako 20 mikroRNA BZHLH pazienteetan kontrolekin alderatuz.

MikroRNA	"Base Mean"	log2 "Fold Change"	p-balioa	p adj
hsa-miR-210-3p	842,9	3,51	$2,33x10^{-29}$	$1,45\ x10^{-26}$
hsa-miR-944	60,9	4,10	$2,19x10^{-23}$	$6,80\ x10^{-21}$
hsa-miR-12136	76,05	26,94	$3,64x10^{-20}$	$6,47\ x10^{-18}$
hsa-miR-3681-5p	75,3	5,16	$3,33x10^{-20}$	$6,47\ x10^{-18}$
hsa-miR-378i	24,9	3,01	$3,01x10^{-17}$	$4,16\ x10^{-15}$
hsa-miR-4454	183,4	2,35	$1,01x10^{-16}$	$1,04\ x10^{-14}$
hsa-miR-1291	354,7	4,02	$1,84x10^{-16}$	$1,76\ x10^{-14}$
hsa-miR-7974	111,8	3,46	$1,87x10^{-15}$	$1,45\ x10^{-13}$
hsa-miR-183-5p	891,1	3,40	$5,77x10^{-15}$	$3,59\ x10^{-13}$
hsa-miR-146a-5p	33085,5	2,11	$2,05x10^{-14}$	$1,16\ x10^{-12}$
hsa-miR-2467-5p	25,56	2,33	$7,35x10^{-14}$	$3,81\ x10^{-12}$
hsa-miR-4420	8,09	4,63	$2,02x10^{-13}$	$1,01\ x10^{-11}$
hsa-miR-1248	202,5	2,69	$1,03x10^{-12}$	$4,73\ x10^{-11}$
hsa-miR-18a-3p	54,06	2,05	$1,21x10^{-12}$	$5,36\ x10^{-11}$
hsa-miR-129-5p	25,1	4,62	$3,03x10^{-12}$	$1,22\ x10^{-10}$
hsa-miR-147b-3p	92,5	3,41	$3,78x10^{-12}$	$1,47\ x10^{-10}$
hsa-miR-3691-5p	6,6	2,59	$1,09x10^{-11}$	$3,88\ x10^{-10}$
hsa-miR-1246	4,6	4,47	$2,64x10^{-11}$	$8,86x10^{-10}$
hsa-miR-205-5p	67,1	6,11	$3,81x10^{-11}$	$1,22x10^{-09}$
hsa-miR-769-3p	21,5	2,53	$4,00x10^{-11}$	$1,25x10^{-09}$

Log2 "Fold change" positiboak mikroRNAen gain-adierazpena adierazten du BZHLH duten pazienteak eta osasuntsuak alderatuz; "Base Mean": zenbaketa normalizatutako balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta.

13. taula: Gehien azpi-adierazitako 20 mikroRNA BZHLH pazienteetan kontrolekin alderatuz.

MikroRNA	"Base Mean"	log2"FoldChange"	p-balioa	padj
hsa-miR-215-5p	53,8	-4,42	$6{,}74 \times 10^{-35}$	$8{,}39 \times 10^{-32}$
hsa-miR-150-5p	6310,7	-3,22	$3{,}46 \times 10^{-25}$	$1{,}44 \times 10^{-22}$
hsa-miR-224-5p	47,7	-3,30	$5{,}11 \times 10^{-21}$	$1{,}27 \times 10^{-18}$
hsa-miR-194-5p	37,8	-4,33	$6{,}19 \times 10^{-17}$	$7{,}00 \times 10^{-15}$
hsa-miR-452-3p	7,2	-2,33	$2{,}10 \times 10^{-16}$	$1{,}87 \times 10^{-14}$
hsa-miR-335-5p	127,6	-2,74	$6{,}20 \times 10^{-16}$	$5{,}14 \times 10^{-14}$
hsa-miR-145-5p	2060,5	-2,16	$2{,}65 \times 10^{-15}$	$1{,}94 \times 10^{-13}$
hsa-miR-139-5p	48,8	-2,26	$5{,}40 \times 10^{-15}$	$3{,}57 \times 10^{-13}$
hsa-miR-497-5p	332,5	-2,07	$3{,}53 \times 10^{-14}$	$1{,}91 \times 10^{-12}$
hsa-miR-10a-3p	10,2	-2,29	$7{,}45 \times 10^{-12}$	$2{,}73 \times 10^{-10}$
hsa-miR-95-3p	18,1	-2,13	$3{,}79 \times 10^{-11}$	$1{,}22 \times 10^{-09}$
hsa-miR-151b	24,8	-2,21	$6{,}67 \times 10^{-10}$	$1{,}60 \times 10^{-08}$
hsa-miR-551b-3p	7,9	-2,25	$2{,}97 \times 10^{-09}$	$5{,}78 \times 10^{-08}$
hsa-miR-194-5p	234,5	-2,52	$4{,}25 \times 10^{-09}$	$8{,}01 \times 10^{-08}$
hsa-miR-135a-5p	4,3	-3,49	$1{,}92 \times 10^{-08}$	$3{,}07 \times 10^{-07}$
hsa-miR-549a-3p	1,6	-2,39	$2{,}28 \times 10^{-07}$	$3{,}02 \times 10^{-06}$
hsa-miR-549a-5p	3,4	-2,44	$4{,}93 \times 10^{-07}$	$5{,}96 \times 10^{-06}$
hsa-miR-451a	6085,0	-2,07	$4{,}55 \times 10^{-06}$	$4{,}16 \times 10^{-05}$
hsa-miR-670-3p	0,9	-2,72	$8{,}61 \times 10^{-06}$	$7{,}44 \times 10^{-05}$
hsa-miR-135a-3p	0,6	-2,46	$2{,}89 \times 10^{-05}$	0,00021

Log2 "Fold change" negatiboak mikroRNAen azpi-adierazpena adierazten du BZHLH duten pazienteak eta osasuntsuak alderatuz; "Base Mean": zenbaketa normalizatutako balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta.

Azpimotak sailkatzeko biomarkatzaileak

ZGB diren 32 lagin eta ez-ZGB diren 21 laginen adierazpen-profilak aztertzean diagnostikoaren momentuan, adierazpen diferentziala duten zortzi mikroRNA aurkitu ziren (padj <0,05; $\log_2$"fold change" > 2/<-2). Haietatik bost gain-adierazita agertu ziren BZHLH ZGB pazienteetan (miR-129-2-3p, miR-4464, miR-3150b-3p, miR-138-5p eta miR-129-5p), eta beste hiru mikroRNA gain-adierazita zeuden BZHLH ez-ZGB azpimotetan (miR-511-5p, miR-205-5p eta miR-3652). Eranskineko 7. eta 8. tauletan daude BZHLH ZGB eta BZHLH ez-ZGB pazienteetan gain- eta azpi-adierazita dituzten mikroRNA molekulen emaitza guztiak.

14. taula: Modu esangarrian adierazitako mikroRNAk BZHLH ZGB eta ez-ZGB azpimoten artean

MikroRNA	"Base Mean"	log2 "FoldChange"	p-balioa	p adj
hsa-miR-129-2-3p	4,9	4,12	$1,50x10^{-06}$	0,00050
hsa-miR-4464	2,9	3,91	$1,21x10^{-05}$	0,00151
hsa-miR-3150b-3p	49,0	2,00	$1,90x10^{-05}$	0,00211
hsa-miR-138-5p	568,1	2,00	$9,92x10^{-05}$	0,00661
hsa-miR-129-5p	18,2	2,90	0,00014	0,00891
hsa-miR-511-5p	7,6	-2,01	$9,92x10^{-06}$	0,00142
hsa-miR-205-5p	20,3	-3,30	$4,10x10^{-05}$	0,00342
hsa-miR-3652	8,9	-2,44	0,00088	0,03137

Log2 "Fold change" positiboak mikroRNAen gain-adierazpena adierazten du BZHLH ZGB pazienteetan ez-ZGB pazienteekin alderatuz; "Base Mean": zenbaketa normalizatutako balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta.

Tratamenduari emandako erantzunaren biomarkatzaileak

Hamaika mikroRNA (padj <0,05; $\log_2$ aldaketa > 2 or <-2) adierazi ziren modu esangarrian erremisio osoa zuten pazienteen (n = 50) eta paziente errefraktarioen (n = 12) diagnostikoko laginetan. Hamaika horietatik hamar gain-adierazita zeuden erremisio osoa zuten pazienteetan (miR-12136, miR-129-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129-5p eta miR-3928-3p), eta beste bat azpi-adierazita agertu zen (miR-192-5p) (15. Taula). Tratamenduari emandako erantzun on eta txarrekin erlazionatutako mikroRNA guztiak (p < 0,05) Eranskineko 9. eta 10. tauletan daude.

15. taula. Tratamenduari emandako erantzun on edo txarrarekin modu esangarrian erlazionatutako mikroRNAk.

MikroRNA	"Base Mean"	log2"Fold Change"	p-balioa	p adj
hsa-miR-12136	120,0	25,73	$1,29x10^{-13}$	$9,70x10^{-11}$
hsa-miR-129a-5p	42,7	5,09	$2,86x10^{-09}$	$1,08x10^{-06}$
hsa-miR-129-1-3p	7,4	4,08	$1,86x10^{-06}$	0,00035
hsa-miR-3150b-3p	50,7	2,33	$1,60x10^{-05}$	0,00241
hsa-miR-127-3p	3977,2	2,01	$6,14x10^{-05}$	0,00661
hsa-miR-3681-5p	75,3	2,34	0,00016	0,01507
hsa-miR-370-3p	16,1	2,59	0,00041	0,02380
hsa-miR-4464	3,3	3,55	0,00117	0,04641
hsa-miR-129b-5p	27,5	2,88	0,00102	0,04641
hsa-miR-3928-3p	16,5	2,03	0,00113	0,04641
hsa-miR-192-5p	10375,5	-2,41	$1,60x10-^{07}$	$4,01x10^{-05}$

Log2 "Fold change" positiboak mikroRNAen gain-adierazpena adierazten du erremisio osoa duten pazienteetan diagnosiaren momentuan errefraktarioekin alderatuz eta negatiboak kontrakoa adierazten du; "Base Mean": zenbaketa normalizatutako balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta

Pronostikoaren biomarkatzaileak

Epe luzeko erremisioa zuten 50 paziente eta berriro gaixotu ziren 16 pazienteren mikroRNA molekulek diagnostikoa egiteko momentuan zuten adierazpena aztertu zen. Emaitzetan ikusi

zenez, epe luzeko erremisioa zuten pazienteetan zazpi mikroRNA (miR-4444, miR-449c-5p, miR-3681-5p, miR-3928-3p, miR-449b-5p, miR-370-3p eta miR-4424) gain-adierazita agertu ziren modu esangarrian (padj <0,05; log_2 aldaketa > 2). Bestalde, berriro gaixotu ziren pazienteetan lau mikroRNA (miR-133a-2-3p, miR-133a-1-3p, miR-208b-3p eta miR-205-5p) modu esangarrian gain-adierazita zeuden (padj <0,05; log_2 aldaketa< -2) (16. taula). Pronostiko on edo txarrarekin erlazionatutako mikroRNA guztiak (p < 0,05) Eranskineko 11. eta 10. tauletan daude.

16. taula. Pronostikoarekin modu esangarrian erlazionatutako mikroRNAk.

MikroRNA	"Base Mean"	log2"Fold Change"	p-balioa	p adj
hsa-miR-4444	10,3	2,25	$6,20x10^{-05}$	0,0089
hsa-miR-449c-5p	9,8	2,22	$4,75x10^{-05}$	0,0089
hsa-miR-3681-5p	75,3	2,03	0,00021	0,014
hsa-miR-3928-3p	16,5	2,02	0,00018	0,014
hsa-miR-449b-5p	7,23	2,22	0,00036	0,021
hsa-miR-370-3p	16,1	2,12	0,00071	0,029
hsa-miR-4424	110,8	2,32	0,0010	0,041
hsa-miR-133a-2-3p	470,9	-3,8	$1,53x10^{-06}$	0,001
hsa-miR-133a-1-3p	69,29	-2,52	$6,78x10^{-05}$	0,0089
hsa-miR-208b-3p	12,94	-3,08	0,00014	0,013
hsa-miR-205-5p	62,4	-3,36	0,0002	0,014

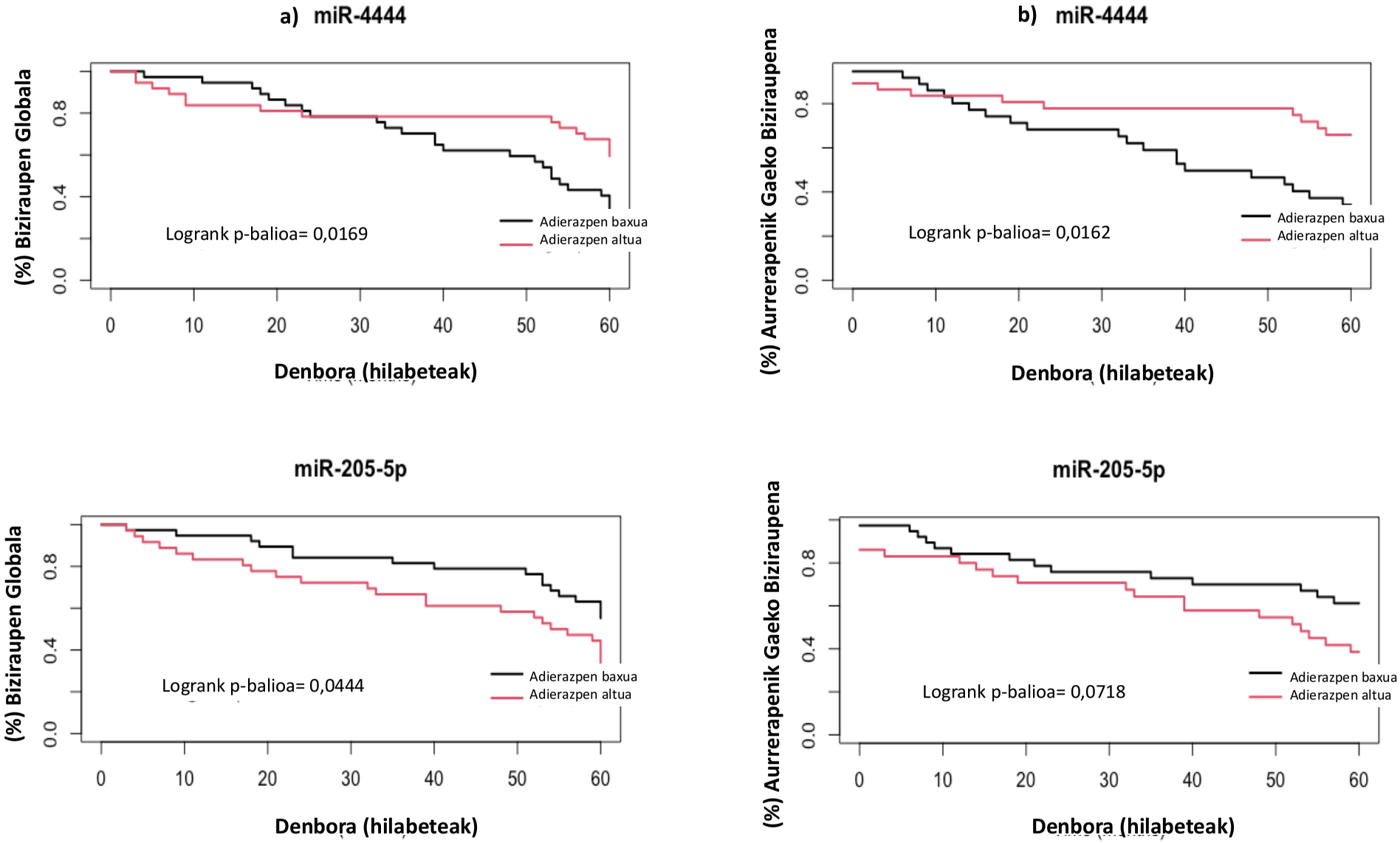
Log2 "Fold change" positiboak mikroRNAen gain-adierazpena adierazten du epe luzeko erantzuna duten pazienteetan diagnosiaren momentuan berriz gaixotzen direnekin alderatuz eta negatiboak kontrakoa adierazten du; "Base Mean": zenbaketa normalizatutako balioen batezbestekoa adierazten du, tamaina faktorearen arabera banatuta, lagin guztiak hartuta.

mikroRNA molekulen adierazpen-mailak biziraupenean duen inpaktua

Pazienteen biziraupen-kurba sortzeko diagnostikotik bost urteko PFS eta OS hartu ziren oinarritzat. Pazienteak adierazpen handiko eta txikiko taldetan banatu ziren, pronostiko onarekin erlazionatutako zazpi mikroRNAen batezbesteko adierazpenaren arabera: miR-4444 (2,83), miR-449c-5p (2,75), miR-3681-5p (4,98), miR-3928-3p (3,02), miR-449b-5p (2,75), miR-370-3p (2,73), miR-4424 (3,92); eta pronostiko txarrarekin erlazionatutako lau mikroRNAen arabera: miR-133a-1-3p (3,73), miR-133a-2-3p (3,83), miR-208b-3p (1,97), miR-205-5p (2,32).

Pronostiko onarekin erlazionatutako mikroRNA molekulen artean, ezberdintasun esangarriak ikusi ziren OS eta PFSetan, miR-4444 molekularen adierazpen altuaren eta baxuaren taldeen artean OSren p-balioa = 0,0169 eta PFSren p-balioa = 0,0162 (10. irudia). Horrez gain, pronostiko txarrarekin erlazionatu izan diren mikroRNAk kontuan hartuta, ezberdintasun esangarriak identifikatu genituen miR-205-5p molekularen adierazpen altua eta baxua zuten taldeen artean (p = 0,0444) (10. irudia).

Cox-en arrisku proportzionalen (Cox PH) aldagai anitzeko analisia egin genuen, mikroRNA horiek OS eta PFSrekin erlazionatuta zeuden jakiteko, BZHLH pazienteen bilakaera azaltzen duten bi adierazle finkatuekin (azpimota eta IPI) loturarik izan gabe. Analisi horien emaitzek erakutsi zutenez, miR-205-5p mikroRNA OS (p=0,02) eta PFS (p=0,02) txarragoekin zegoen asoziaturik, IPI eta azpimota edozein izanda ere.



10. irudia: Pronostikoarekin lotutako mikroRNAen biziraupen analisia

MikroRNA-mRNA INTERAKZIO-SAREAREN ANALISIA

Esperimentu bidez baliozkotutako ituak identifikatzea

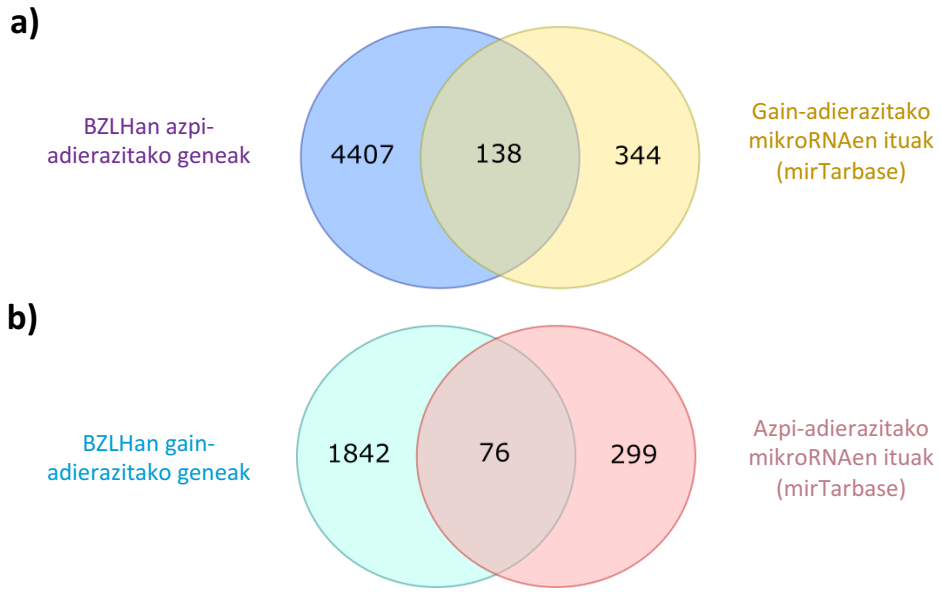
Diagnostikoko analisian (BZHLH *vs* kontrolak) adierazpen aldakorra zuten eta esperimentu bidez baliozkotutako mikroRNA molekulen ituak, mirTarbase datu-basea erabilita identifikatu ziren. Esperimentu bidez baliozkotutako 482 itu identifikatu genituen, guztira, gure ikerketan gain-adierazita aurkitutako 122 mikroRNA molekuletarako. Azpi-adierazitako 25 mikroRNA molekulei dagokienez, berriz, 375 gene aurkitu ziren (11. irudia).

Adierazpen aldakorra zuten geneak identifikatzea BZHLHn

GEOn aurkitu eta DLCBL pazienteen datuak zituzten 15 ikerketetatik batek bakarrik erakusten zituen kontrol osasuntsuen datuak, eta hori dela eta, gure analisian sartzeko egokia suertatu zen (6. irudia). GSE56315 adierazpen-profilaren datu multzoak BZHLH zuten pertsonen 55 lagin eta pertsona osasuntsuen 33 lagin zituen. Gain-adierazitako 1918 gene eta azpi-adierazitako 4545 gene aurkitu ziren BZHLH zuten pazienteetan, kontrolerako banako osasuntsuekin alderatuz gero, GEOren datu multzoan.

Integrazioa, ohar funtzionalak eta seinale-bideen aberaste-analisiak

BZHLHn azpi-adierazitako geneen artean 138 gene izan ziren gain-adierazitako mikroRNAen ituak (Eranskineko 13. taula), eta BZHLHn gain-adierazitako geneetatik 76 izan ziren azpi-adierazitako mikroRNA molekulen itu (Eranskineko 14. taula) (11. irudia). Zenbait genek mikroRNA bat baino gehiagorekin interakzioak zituztela ikusi zen (17. taula eta 18. taula). Adibidez, *FOXO1*, *BCL2*, *GS3KB* eta *PTEN* geneak, BZHLHn azpi-adierazita zeudenak, hurrenez hurren, 7, 5, 5 eta 4 mikroRNAren itu dira.



11. irudia: a) BZHLHan azpi-adierazitako geneak eta gain-adierazitako mikroRNAen gainjartzea adierazten duen Venn diagrama. b) BZHLHan gain-adierazitako geneak eta azpi-adierazitako mikroRNAen gainjartzea adierazten duen Venn diagrama.

17. taula: Azpi-adierazitako itu geneekin elkarrekintzak erakusten dituzten gain-adierazitako mikroRNAk.

Gene ituak	mikroRNA
AKT2	hsa-miR-612; hsa-miR-2861
ALDH5A1	hsa-miR-210-3p; hsa-miR-147b
APC	hsa-miR-135a-5p; hsa-miR-663a; hsa-miR-129-5p
BCL2	hsa-miR-7-5p; hsa-miR-182-5p; hsa-miR-205-5p; hsa-miR-135a-5p; hsa-miR-9-5p
CCND2	hsa-miR-182-5p; hsa-miR-146a-5p
CDK6	hsa-miR-129-5p; hsa-miR-320a
CREB1	hsa-miR-182-5p; hsa-miR-9-5p
CXCR4	hsa-miR-146a-5p; hsa-miR-9-5p; hsa-miR-663a; hsa-miR-146a-3p
ELAVL1	hsa-miR-9-5p; hsa-miR-146a-5p
EZR	hsa-miR-183-5p; hsa-miR-205-5p
FBXW7	hsa-miR-182-5p; hsa-miR-182-3p; hsa-miR-155-3p
FOXO1	hsa-miR-182-5p; hsa-miR-183-5p; hsa-miR-183-5p; hsa-miR-9-5p; hsa-miR-9-3p; hsa-miR-135a-5p; hsa-miR-135a-5p
FOXO3	hsa-miR-182-5p; hsa-miR-9-5p
GSK3B	hsa-miR-183-5p; hsa-miR-182-5p; hsa-miR-9-5p; hsa-miR-129-1-3p; hsa-miR-1246
HES1	hsa-miR-9-3p; hsa-miR-9-5p
HIF3A	hsa-miR-210-3p; hsa-miR-147a
IGF2BP3	hsa-miR-9-5p; hsa-miR-129-5p
ITGB1	hsa-miR-183-5p; hsa-miR-9-3p
MAPK1	hsa-miR-320a; hsa-miR-9-3p; hsa-miR-129-5p
MTSS1	hsa-miR-182-5p; hsa-miR-135a-5p
NOTCH1	hsa-miR-129-5p; hsa-miR-9-5p; hsa-miR-146a-5p
PTEN	hsa-miR-182-5p; hsa-miR-155-3p; hsa-miR-205-5p; hsa-miR-320a
RAC1	hsa-miR-320a; hsa-miR-146a-5p
RECK	hsa-miR-182-5p; hsa-miR-183-5p
ROCK1	hsa-miR-146a-5p; hsa-miR-135a-5p
RUNX2	hsa-miR-205-5p; hsa-miR-320a; hsa-miR-135a-5p
SIAH1	hsa-miR-135a-5p; hsa-miR-944
SMAD4	hsa-miR-146a-5p; hsa-miR-182-5p; hsa-miR-205-5p; hsa-miR-183-5p
SOX2	hsa-miR-1181; hsa-miR-146a-5p
SP1	hsa-miR-129-5p; hsa-miR-612
TGFB1	hsa-miR-663a; hsa-miR-146a-5p
TP53	hsa-miR-612; hsa-miR-663a; hsa-miR-155-3p
YY1	hsa-miR-7-5p; hsa-miR-205-5p

18. taula: Gain-adierazitako itu geneekin elkarrekintzak erakusten dituzten azpi-adierazitako mikroRNAk.

Gene ituak	mikroRNA
BCL2	hsa-miR-451a; hsa-miR-497-5p; hsa-miR-135a-5p; hsa-miR-224-5p; hsa-miR-139-5p
CD44	hsa-miR-216a-5p; hsa-miR-145-5p
CDH2	hsa-miR-194-5p; hsa-miR-145-5p
CEBPD	hsa-miR-95-3p; hsa-miR-135a-5p
EGFR	hsa-miR-145-5p; hsa-miR-135a-5p
KLF4	hsa-miR-145-5p; hsa-miR-135a-5p
KRAS	hsa-miR-217; hsa-miR-224-5p
MTDH	hsa-miR-145-5p; hsa-miR-217
PTEN	hsa-miR-217; hsa-miR-216a-5p
SMAD4	hsa-miR-483-3p; hsa-miR-224-5p
STAT1	hsa-miR-145-5p; hsa-miR-150-5p
VEGFA	hsa-miR-145-5p; hsa-miR-150-5p
ZEB2	hsa-miR-215-5p; hsa-miR-335-5p

Bestalde, mikroRNA batzuek hainbat iturekin interakzioak dituztela ikusi zen (19. taula eta 20. taula). Adibidez, litekeena da miR-9-5p molekula ia 25 itu-generen azpi-adierazpenean parte

hartzea, eta miR-146a-5p eta miR-182-5p, berriz, BZHLHn azpi-adierazitako 24 eta 20 gene artean erregulatuko lituzkete hurrenez hurren.

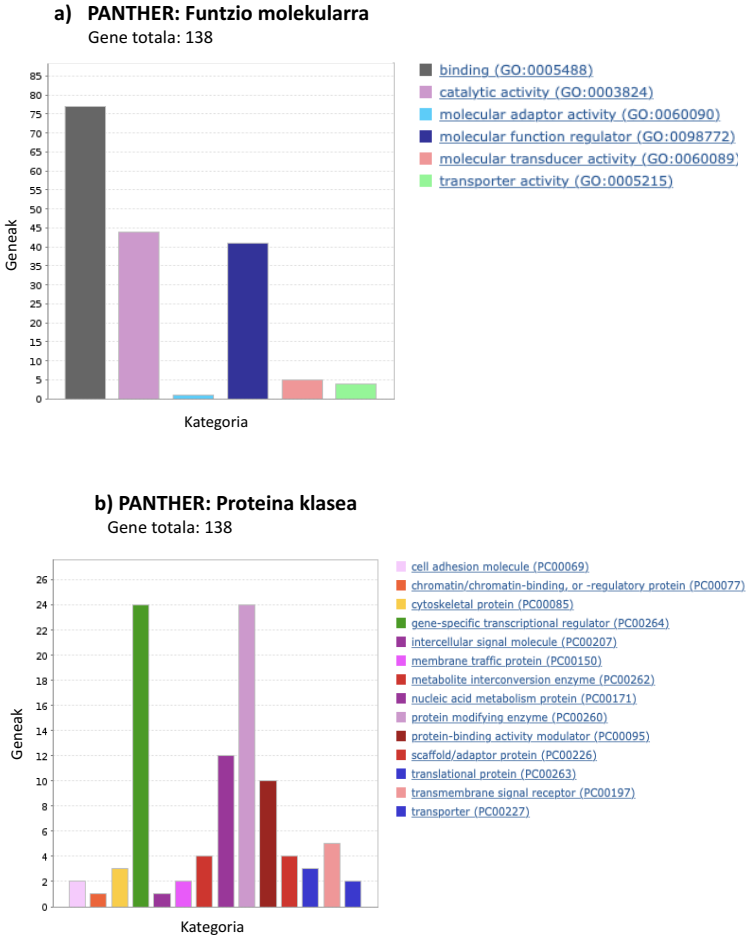
19. taula: Gain-adierazitako mikroRNAekin elkarrekintzak erakusten dituzten azpi-adierazitako itu geneak.

mikroRNA	Gene ituak
hsa-miR-1181	*SOX2; STAT3*
hsa-miR-1246	*GSK3B; DYRK1A; PRKAR1A*
hsa-miR-129-1-3p	*GSK3B; PDCD2*
hsa-miR-129-5p	*APC; CDK6; IGF2BP3; MAPK1; NOTCH1; SP1; FNDC3A; PDPK1; SOX4*
hsa-miR-135a-5p	*APC; BCL2; FOXO1; MTSS1; ROCK1; RUNX2; SIAH1; BMPR2; KLF8; RBAK*
hsa-miR-141-5p	*SPAG9; WNK1*
hsa-miR-146a-5p	*CCND2; CXCR4; ELAVL1; NOTCH1; RAC1; ROCK1; SMAD4; SOX2; TGFB1; BCLAF1; BRCA2; CNOT6L; CPM; FAF1; FANCM; HOXD10; IRAK2; NFAT5; PRKCE; RHOA; SIKE1; SOS1; STAT1; WASF2*
hsa-miR-147a	*HIF3A; PSMA3*
hsa-miR-155-3p	*FBXW7; PTEN; TP53*
hsa-miR-182-3p	*FBXW7; STAT5B*
hsa-miR-182-5p	*BCL2; CCND2; CREB1; FBXW7; FOXO1; FOXO3; GSK3B; MTSS1; PTEN; RECK; SMAD4; ATF1; CYLD; FLOT1; RARG; TP53BP1; TP53INP1; TRIM8; TSC22D3; ZFAND4*
hsa-miR-183-5p	*EZR; FOXO1; GSK3B; ITGB1; RECK; SMAD4; BTRC; EGR1; PPP2CB*
hsa-miR-18a-3p	*ATM; KRAS*
hsa-miR-19b-1-5p	*CASP8; FGFR2*
hsa-miR-205-5p	*BCL2; EZR; PTEN; RUNX2; SMAD4; YY1; ACSL4; CCNJ; E2F5; EGLN2; IL24; LMNA; SRC*
hsa-miR-208b-3p	*CACNB2; QKI*
hsa-miR-210-3p	*ALDH5A1; HIF3A; FOXN3; FOXP3; INPP5A; MNT; PTBP3; PTPN1; PTPN2; RAD52; XIST*
hsa-miR-944	*SIAH1; PTP4A1*
hsa-miR-320a	*CDK6; MAPK1; PTEN; RAC1; RUNX2; ARPP19; CTNNB1; ITGB3; MCL1; MTDH; NFATC3; SUZ12; YWHAZ*
hsa-miR-612	*AKT2; SP1; TP53*
hsa-miR-663a	*APC; CXCR4; TGFB1; TP53; JUND*
hsa-miR-7-5p	*BCL2; YY1; BAX; CUL5; ILF3; KMT5A; MSH3; PIK3CG; REL; SKP2; SNCA; XIAP*
hsa-miR-9-3p	*FOXO1; HES1; ITGB1; MAPK1; DLG1; DMD; TUG1*
hsa-miR-9-5p	*BCL2; CREB1; CXCR4; ELAVL1; FOXO1; FOXO3; GSK3B; HES1; IGF2BP3; NOTCH1; TGFBR2; AP3B1; BCL2L11; CAMKK2; CUL4A; DICER1; DRD2; ETS1; FOXP1; MALAT1; MTHFD1; POU2F2; PRKAA1; RAP2A; REST*

20. taula: Azpi-adierazitako mikroRNAekin elkarrekintzak erakusten dituzten gain-adierazitako itu geneak.

mikroRNA	Gene ituak
hsa-miR-135a-5p	*BCL2; CEBPD; EGFR; KLF4; APC; BMPR2; HOXA10; MTSS1*
hsa-miR-139-5p	*BCL2; ACTC1; ADGRL4; FAM162A; RHOT1*
hsa-miR-145-5p	*CD44; CDH2; EGFR; KLF4; MTDH; STAT1; VEGFA; ABHD17C; APH1A; CD28; EPAS1; FZD7; ITGB8; JAG1; MEST; MMP1; MMP12; MYO5A; MYO6; MYOCD; PARP8; RREB1; SERINC5; SERPINE1; SPTLC1; SRGAP1; YES1*
hsa-miR-150-5p	*STAT1; VEGFA; PRKCA; SRCIN1*
hsa-miR-194-5p	*CDH2; CHD1; HBEGF; IL10; ITGA9; ITSN1; PTPN12; SOCS2*
hsa-miR-215-5p	*ZEB2; NID1; WNK1*
hsa-miR-216a-5p	*CD44; PTEN; CEMIP*
hsa-miR-95-3p	*CEBPD; CELF2*
hsa-miR-217	*KRAS; MTDH; PTEN; NR4A2; PTPN14; ROBO1*
hsa-miR-224-5p	*BCL2; KRAS; SMAD4; AP2M1; DPYSL2; EDNRA; TRIB1*
hsa-miR-335-5p	*ZEB2; BRCA1; ID4; MERTK; PLAUR; SOX4; TCEAL9; TNC*
hsa-miR-451a	*BCL2; MIF; MMP2; MMP9*
hsa-miR-483-3p	*SMAD4; DLC1; IGF1*
hsa-miR-497-5p	*BCL2; CHEK1; Reck*

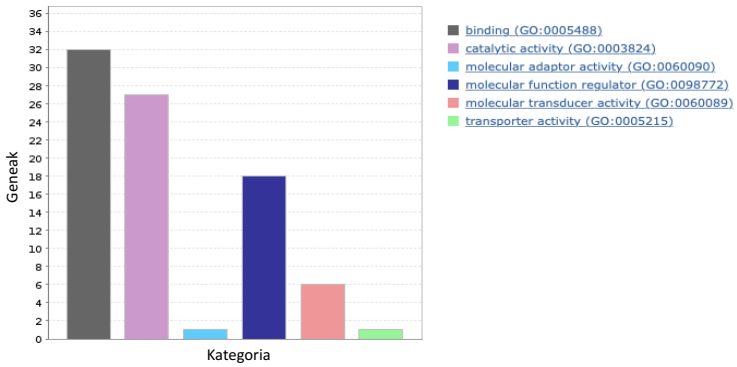
Xehetasun biologiko gehiago bilatu nahian, gene-ontologia (GO) aberaste-analisia egin zen PANTHER programa erabilita. Bi talde funtzionaletan sailkatu ziren adierazpen diferentziala zuten geneak (DEG): funtzio molekularraren eta proteina-klasearen arabera. Gain-adierazita zeuden mikroRNAekin interakzioak zituzten gain-adierazitako 138 geneetan, gehien aberastutako funtzio molekularrak proteinekiko lotura (77 gene), jarduera katalitikoa (44 gene) eta funtzio molekularraren (41 gene) erregulatzaileak izan ziren (12.a irudia). Proteina-klaseen analisiari dagokionez, geneekiko espezifikoa den transkripzio-erregulatzaileak (24 gene), proteinak eraldatzeko entzimak (24 gene) eta azido nukleikoaren metabolismoko proteinak (12 genes) izan ziren aberastuenak (12.b irudia).



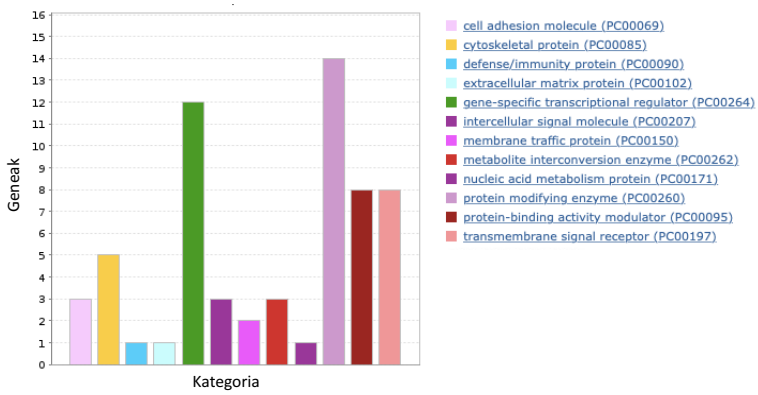
12. irudia: Gain-adierazitako mikroRNAekin elkarrekintzak erakusten dituzten eta azpi-adierazitako itu geneen aberaste-analisia.

Azpi-adierazitako mikroRNA molekulekin interakzioak zituzten gain-adierazitako 76 geneetan, gehien aberastutako funtzio molekularrak proteinekiko loturak (32 gene), jarduera katalitikoa (27 gene) eta funtzio molekularraren erregulatzaileak (18 gene) izan ziren baita (13.a irudia). Proteina-klaseen analisiari dagokionez, proteinak eraldatzeko entzima (14 gene), geneekiko espezifikoa den transkripzio-erregulatzaileak (12 gene), proteinak eraldatzeko entzima eta mintzean zeharreko seinale-hartzailea (8 gene) izan ziren aberastuenak (13.b irudia).

a) PANTHER: Funtzio molekularra
Gene totala: 78

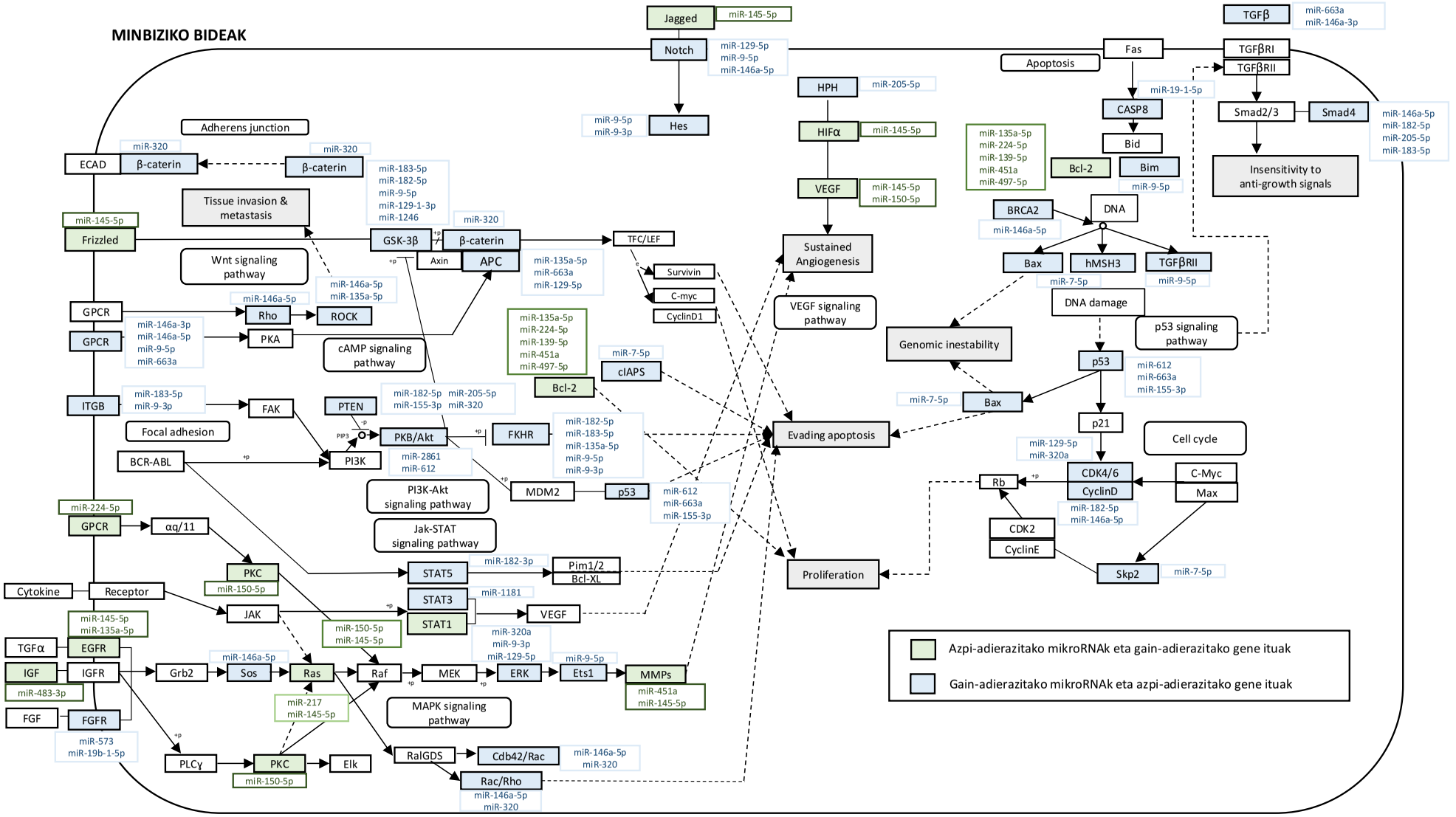


b) PANTHER: Proteina klasea
Gene totala: 78



13. irudia: Azpi-adierazitako mikroRNAekin elkarrekintzak erakusten dituzten eta gain-adierazitako itu geneen aberaste-analisia.

Itu-gene esangarriak dituzten mikroRNAek eragin dezaketen bidezidorrak ebaluatzeko, bidezidorrak aberasteko analisia egin zen *ConsensusPathDB* web-tresna erabilita. Azpi-adierazitako eta gain-adierazitako itu-geneen hamar bide esangarrietatik, minbiziko bideak maiz agertu ziren bi kasuetan (p-balioa $3{,}98 \times 10^{-23}$ eta $1{,}75 \times 10^{-09}$, hurrenez hurren) (21. taula eta 22. taula) (ikus 14. irudia). Bide horietan, gain-adierazitako mikroRNAek 38 gene izan zituzten itu (Eranskineko 15. taula), eta azpi-adierazitako mikroRNAek, berriz, 17 gene izan zituzten itu (Eranskineko 16. taula). Minbiziko bidean gain- eta azpi-adierazitako geneen ituak Eranskineko 17 eta 18. tauletan azaltzen dira. Are gehiago, hamar biderik esangarrienetan, FoxO seinale-bidea, tirosina kinasaren hartzaileen bidezko seinaleztapena eta PI3K-Akt seinale-bideak asko agertu ziren azpi-adierazitako itu-geneetan (21. taula). Gain-adierazitako itu-geneen kasuan, hamar biderik nabarmenetan asko adierazi zen tirosina kinasen hartzaileen bidearen bidezko seinaleztapena, 14 gene itu dituelarik (22. taula).



14. irudia: Minbizi bidezidorreko geneak eta haien itu diren gain-adierazitako eta azpi-adierazitako mikroRNAk (KEGG datu basetik egokitua)

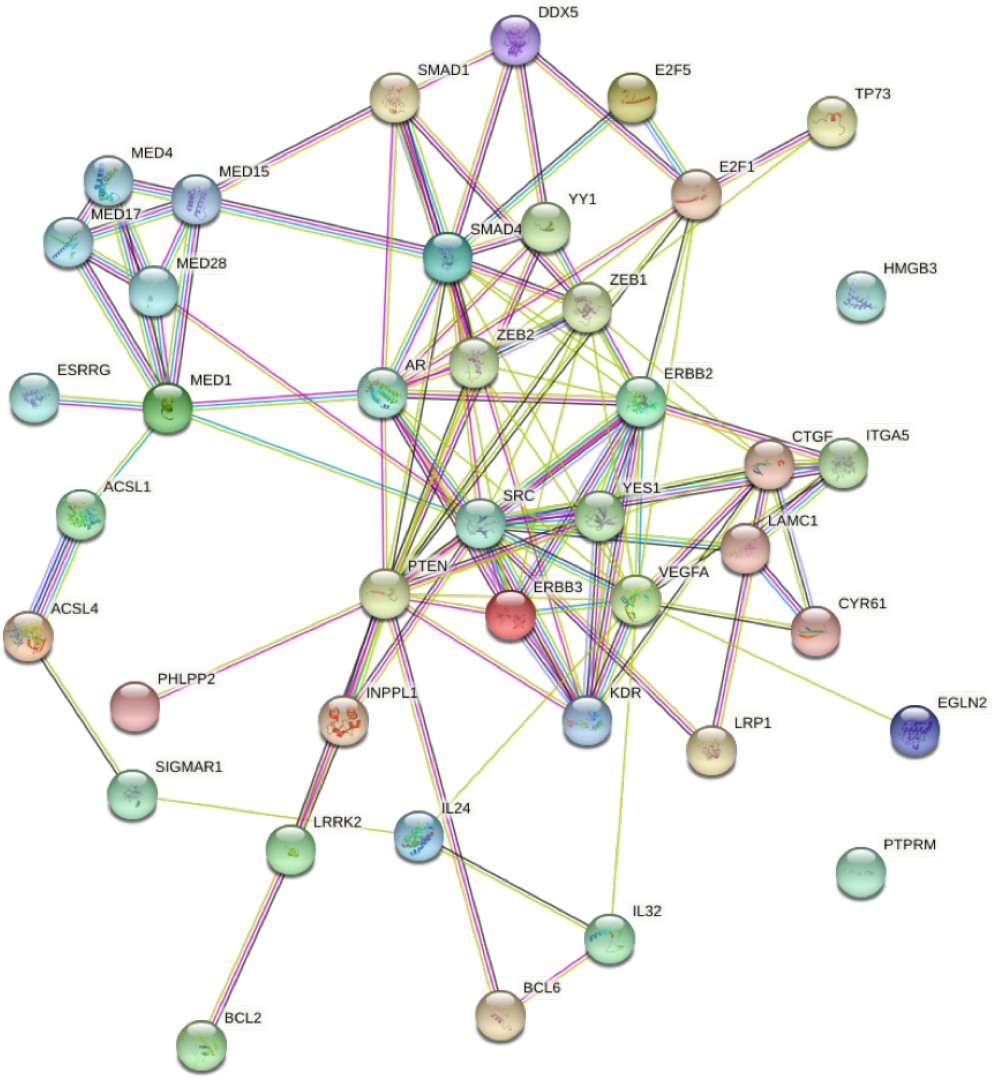
21. taula: Azpi-adierazitako itu geneentzako gehien aberastutako 10 bidezidorrak.

Bidezidorraren izena	Tamaina	Kandidatuak	p-balioa	q-balioa	Bidezidor iturria
Minbiziaren bidezidorrak - Homo sapiens	526	38 (7,2%)	$3,98x10^{-23}$	$2,93x10^{-20}$	KEGG
Minbizi kolorrektala - Homo sapiens	86	17 (19,8%)	$5,11x10^{-18}$	$1,88x10^{-15}$	KEGG
Gaixotasunaren seinale transdukzioa	248	24 (9,7%)	$1,54x10^{-17}$	$3,8x10^{-15}$	Reactome
Minbizian eragile diren miRNAk - Homo sapiens	299	25 (8,4%)	$1,01x10^{-16}$	$1,87x10^{-14}$	KEGG
B Hepatitisa - Homo sapiens	144	18 (12,5%)	$2,52x10^{-15}$	$3,72x10^{-13}$	KEGG
Gaixotasuna	510	29 (5,7%)	$6,92x10^{-15}$	$8,51x10^{-13}$	Reactome
FoxO bidezidorraren seinaleztapena - Homo sapiens	132	17 (13,0%)	$8,35x10^{-15}$	$8,81x10^{-13}$	KEGG
AGE-RAGE bidezidorraren seinaleztapena konplikazio diabetikoetan - Homo sapiens	99	15 (15,2%)	$3,29x10^{-14}$	$3,04x10^{-12}$	KEGG
Tirosina kinasa hartzaile bidezko seinaleztapena	423	26 (6,1%)	$3,83x10^{-14}$	$3,14x10^{-12}$	Reactome
PI3K-Akt seinaleztapen bidezidorra - Homo sapiens	354	24 (6,8%)	$4,63x10^{-14}$	$3,33x10^{-12}$	**KEGG**

22. taula: Gain-adierazitako itu geneentzako gehien aberastutako 10 bidezidorrak.

Bidezidorraren izena	Tamaina	Kandidatuak	p-balioa	q-balioa	Bidezidor iturria
Minbizian eragile diren miRNAk - Homo sapiens	299	15 (5,0%)	$4,38x10^{-11}$	1,42x10-08	KEGG
EGFR Transaktibazioa gastrina bidez	10	5 (50,0%)	$9,69x10^{-10}$	1,53x10-07	Reactome
Minbiziaren bidezidorrak - Homo sapiens	526	17 (3,2%)	$1,75x10^{-09}$	1,53x10-07	KEGG
Maskuriko minbizia - Homo sapiens	41	7 (17,1%)	$1,88x10^{-09}$	1,53x10-07	KEGG
Proteoglikanoak minbizian - Homo sapiens	201	11 (5,5%)	$9,01x10^{-09}$	5,84x10-07	KEGG
Gastrin-CREB seinaleztapen bidezidorra PKC eta MAPK bidez	19	5 (26,3%)	$4,3x10^{-08}$	2,05x10-06	Reactome
Tirosina kinasa hartzaile bidezko seinaleztapena	423	14 (3,3%)	$4,44x10^{-08}$	2,05x10-06	Reactome
AGE-RAGE seinaleztapen bidezidorra konplikazio diabetikoetan - Homo sapiens	99	8 (8,1%)	$5,46x10^{-08}$	2,21x10-06	KEGG
SHC1 gertaerak ERBB2 seinaleztapenean	22	5 (22,7%)	$9,63x10^{-08}$	3,32x10-06	Reactome
Seinaleztapena SCF-KIT bidez	43	6 (14,0%)	$1,02x10^{-07}$	3,32x10-06	Reactome

mikroRNA-mRNA interakzio-sarearen analisiaren estrategiaren proteina-proteina interakzioen sarea (PPI) eraikitzea izan zen. Analisi honetarako, alde batetik, miR-205 erregulatzen eta adierazpen aldakorra zuten geneak miR-205en mikroRNA–itu mRNA interakzio-sarea sortzeko erabili ziren. Bestalde, baliozkotutako 36 ituak erabili ziren elkarrekintzak identifikatzeko mirTarbase datu basea erabiliz (15. irudia). Interakzio sarea, miR-205rekin eraiki zen soilik, izan ere, miR-205-5p erlazioa erakutsi zuen OS eta PFS txarragoekin, IPI eta azpimota edozein dela ere. Mailarik altuena zuten bi nodoak *ERBB3* eta *PTEN* izan ziren.



15. irudia: miR-205ren 36 itu geneen proteina-proteina interakzio sarea.

EZTABAIDA

Ikerketa honen lehenengo helburua, BZHLH diagnostikatzeko, sailkatzeko, haren pronostikoa egiteko eta tratamenduari emandako erantzuna neurtzeko balio duten mikroRNA molekulen sorta zehaztea izan zen. Horretarako, BZHLHrako esangarriak ziren mikroRNA molekulen sinadura berria identifikatzea erabaki zen, diagnostikoa, azpimotak karakterizatzeko eta tratamenduari emandako erantzuna hobetzeko. Helburu hori betetzeko, berrikuspen sistematikoa egin genuen BZHLHrako biomarkatzaile gisa proposatutako mikroRNAk identifikatzeko eta espainiar populazioan mikroRNA bibliografiko horiek balioztatu genituen. Ondoren, mikroRNA sinadura berri bat proposatu genuen RNA txikien sekuentziazioaren teknikaren emaitzak erabiliz. BZHLHren patogenesian mikroRNA molekulen ekintza-mekanismoa ikertzea zen bigarren helburua; horretarako, mikroRNA-mRNA interakzio-sarea eraiki genuen. Horren bidez, BZHLHren mekanismo patologikoak hobeto ulertu ahal izango dira, eta biziraupen-tasak hobetu.

LITERATURAN DESKRIBATUTAKO MIKRORNA POTENTZIALAK HAUTATZEA B ZELULA HANDIETAKO LINFOMA HEDATSUAN

Egungo literaturaren analisi sakona egin genuen BZHLH duten pazienteen diagnostikorako, azpimotak karakterizatzeko, tratamenduari emandako erantzuna eta pronostikoa aurreikusteko garaian tumore-biopsietan mikroRNA molekulen adierazpenak biomarkatzaile gisa izan dezakeen funtzioari buruz.

BZHLHn mikroRNAk diagnostikorako biomarkatzaile izateko egokitasunari dagokionez, 33 artikulu aurkitu ziren. Bi ikerketa baino gehiagotan bat egiten zuten emaitzekin, BZHLH pazientetan azpi-adierazita zeuden hiru mikroRNA identifikatu ziren (miR-155-5p (145,147,167,170,174,176,178–181), miR-21-5p (145,161,171,174,178,180,183) eta miR-146a-5p (140,146,154). Horietatik miR-155-5p eta miR-21-5p molekulek dituzte emaitzarik sendoenak, ikerketa gehienetan ikusi denez, gain-adierazita daude BZHLH pazienteetan.

MiR-155-5p gehien aztertutako mikroRNA izan zen. Aipatu diren ikerketetatik hamarretan, BZHLH pazienteetan gain-adierazita zegoela detektatu zen (145,147,167,170,174,176,178–181), eta beste bi ikerketetan ez zen erlazio nabarmenik aurkitu (149,150). MiR-155-5p eta BZHLHren arteko lotura nabarmenik aurkitu ez dituzten bi ikerketetatik batek zuen guztietan lagin-tamainarik txikiena (19 paziente) (149), eta beste azterketak irizpide zorrotzagoak jarraitu zituen estatistikoki esangarriak ziren loturak detektatzeko (150). Gure baliozkotze-ikerketak ere BZHLH laginetan mikroRNA hau gain-adierazita dagoela berresten du, eta horrek argi erakusten du mikroRNA horren adierazpena gaitzaren biomarkatzaile egokitzat jo daitekeela. Emaitza horrekin bat eginez, aurretik egindako ikerketek proposatu dute miR-155 onko-miR bat izan daitekeela, tumore askotan aktibatzen baita horren adierazpena (esaterako, prostata-minbizian, bularreko minbizian eta beste tumore batzuetan, batez ere ehun linfatikoari eragiten dietenetan) (197–199). BZHLHren bereizgarriak diren geneak (hala nola *SOSC* eta *SHIP1*) daude mikroRNA horren itu baliozkotuen artean, eta datu hori proposatu da molekulak BZHLHn duen inplikazioa azaltzeko (200).

Bestalde, bederatzi ikerketa independentetan aztertu zen miR-21-5p molekularen erregulazioa; BZHLH pazienteetan nabarmenki emendatuta zegoela ikusi zuten zazpi ikerketetan (157–

159,161,164,165,170,173,176,179), baina beste bi ikerketetan ez zen asoziazio estatistiko esangarririk aurkitu (147,150). Gure baliozkotze-ikerketan ere mikroRNA horren gehiegizko adierazpena berretsi zen. BZHLH pazienteetan mikroRNA horren gain-erregulazioak gaitzean funtzioren bat bete dezakela adierazten du. Behaketa horrekin bat eginez, miR-21 molekula minbizi gehienetan erregulatu gabe dagoela aurkitu da (kolon eta ondesteko minbizian, adibidez), eta onkogene gisa funtzionatzen duela (201). Oro har, miR-21 molekula onko-miR gisa hartzen da, hainbat fosfatasaren adierazpena inhibitzen baitu —*PDCD4* (*Programmed Cell Death 4*) eta PTEN (*Phosphatase and Tensin Homolog*)—, eta AKT eta MAPK seinaleztapen bidezidorren jarduera kontrolatzen baitu (202); horrekin azal liteke BZHLHn duen funtzioa.

Bestalde, miR-150-5p molekulak emaitza kontraesankorrak ditu. Lau ikerketetan, BZHLH pazienteetan azpi-adierazita zegoen (145–148), eta beste ikerketa batean gain-adierazita (149). Gainerako ikerketan, berriz, ez zen lotura esangarririk aipatu (150). Hala ere, mikroRNA gain-adierazita aurkitu zuen *Hans et al.*-en ikerketaren lagin-tamaina txikiak azal lezake kontraesan hori (BZHLH pazienteak n = 12 eta kontrolak n = 7), lagin-tamaina horrek fidagarriak ez diren emaitzak eman baititzake. Are gehiago, mikroRNA hori izan da azpi-adierazitako mikroRNAetatik bigarren emaitzarik deigarriena gure baliozkotze-populazioan. Gainera, mikroRNA horren funtzio garrantzitsuaren alde egiten dute egindako *in vitro* ikerketek. Frogatu da miR-150 gain-adierazpenak zelulen proliferazioa murrizten duela eta apoptosia induzitzen duela NK/T-zelulen linfoma-lerroetan (203). Halaber, miR-150 *MYB* itu duen hematopoiesi arruntaren erregulatzaile garrantzitsua dela jakinarazi da (204). Horrenbestez, hematopoiesian duen oinarrizko funtzioa dela eta, erraz ondoriozta daiteke miR-150 molekulak tumore-ezabatzaile orokor gisa funtziona dezakela BZHLHn.

Hogeita bi ikerketetan aztertu da mikroRNA molekulek BZHLH azpimotetan sailkatzeko duten erabilgarritasuna. Erregulatu gabeko lau mikroRNA aurkitu ziren, guztira, bi ikerketa baino gehiagotan (miR-155-5p (145,150,155,178–181,187,192,194), miR-221-3p (150,151,155,180), miR-222-3p (150,151,155) eta miR-28-5p (150,151,187,191)). Hala ere, miR-222-3p eta miR-28-5p emaitza kontraesankorrak eman zituzten, ez baitaude BZHLHren sailkapenarekin era esangarrian erlazionatuta lau ikerketetan lehena (145,147,187,191) eta bi ikerketetan bigarrena (145,155)). Gure populazioan azpimarratzekoa da baliozkotu ahal izan dugun mikroRNA bakarra miR-28-5p izan dela, eta, horregatik, ezin dugu kanpoan utzi molekula horrek biomarkatzaile izateko duen aukera. Litekeena da BZHLH paziente batzuen azpimotaren sailkapena GAP edo IHK bidez egin izanagatik agertzea desadostasunak; izan ere, zaila da ikerketak konparatzea, aldakortasun handia baitago IHK tindagaiak (errepikakortasun zaila) eta interpretazioak egiterakoan. Kasu honetan, miR-155-5p eta miR-221-3p mikroRNAek erakutsi dituzte emaitzarik sendoenak. Hamaika ikerketetatik hamarretan, miR-155-5p gain-adierazita aurkitu zen ez-ZGB azpitaldean. Loturarik aurkitu ez zuen ikerketan, IHK erabili zen azpimotak sailkatzeko, eta betebehar zorrotzagoak zituen adierazpen diferentziala zuten mikroRNA molekulentzat (199). Emaitza horrekin bat eginez, gure baliozkotze-ikerketan (zeinean IHK erabilita sailkatu baitziren laginak) ez genuen ezberdintasunik aurkitu miR-155-5p molekularen adierazpenean ZGB eta ez-ZGB azpimotak alderatzean. Horrek adieraz lezake sailkapenen desberdintasunak mikroRNA horren adierazpenari eragiten diotela. Bestalde, miR-221-3p gain-adierazita agertu zen ez-ZGB azpitaldean, molekula hori aztertu zen sei ikerketetatik lautan. Gure baliozkotze-ikerketan, ordea, ez genuen ezberdintasun esangarririk aurkitu haren adierazpenean. Lehen aipatu dugun

eztabaida kontuan hartuz gero, biomarkatzaile gisa duen erabilgarritasuna zalantzan jartzen da azpimotak sailkatzeko.

R-CHOP tratamenduari emandako erantzuna aurresateko biomarkatzaile gisa balio izan dezaketen mikroRNAei dagokienez, bost ikerketa aurkitu ziren eta haietan ez da adostasunik lortu kontuan hartu ditugun mikroRNA molekulen inguruan (148,175,176,179,190). Gain-adierazitako mikroRNAen artean, miR-27-3p (148), miR-34a-5p (176) eta miR-224-5p (175) kimiosentikortasunarekin erlazionatu ziren, eta miR-155-5p eta miR-146-5p (179), berriz, kimioerresistentziarekin (Eranskineko 3. taula). Behin behineko-emaitza hauek berresteko ikerketa gehiago egin behar badira ere, gure baliozkotze-ikerketak ez du mikroRNA horiek R-CHOP tratamenduari emandako erantzuna aurresateko biomarkatzaile gisa duten balioaren alde egiten.

Azkenik, mikroRNAk BZHLHren pronostikoan duten inplikazioa 20 ikerketetan aztertu zen, eta 50 mikroRNA esangarri aurkitu ziren. (147,148,150–156,167,172,173,175,177–180,184,186,187,190,193–196). Horien artean, miR-222-3p (151–154) eta miR-155-5p (167,179,187,195) molekulen adierazpena pronostikoarekin erlazionatuta zegoela ikusi zen, bi ikerketa baino gehiagotan bat zetozen emaitzak aurkezten zituzten. Gainera, ez-ZGB azpitaldean bilakaerarik txarrenarekin erlazionatu zen miR-155-5p molekula (154). Hala ere, mikroRNA horiek ikerketa askotan aztertu izan dira, gure baliozkotze-ikerketa barne, eta haietan ez zen inolako loturarik aurkitu pronostikoarekin. Horrek esan nahi du aztertutako mikroRNA bat ere ezin dela finkatu pronostikoaren markatzaile fidagarri gisa. Azpimarragaria da ikerketa gehienek ez zutela tratamendu-erregimen espezifikoa adierazi. Parametro hori garrantzi handikoa da pronostikoaren biomarkatzaileak bilatzeko orduan, tratamendu-erregimen espezifikoarekiko menpe baitago pronostikoa.

Hainbat muga aurkitu ziren berrikuspen sistematiko hau egiteko garaian. Alde batetik, lanean sartutako ikerketek heterogeneotasun handia zuten laginen iturriari, erabilitako kontrol motei eta adierazpen-analisirako metodologiari dagokionez. Metodologien aldakortasun hori izan daiteke azterketen artean dauden ezberdintasunen jatorria. Berrikuspen batean ezberdintasun horien eragina zehaztea zaila denez, garrantzitsua litzateke etorkizunean egiten diren ikerketetarako ikerketa-metodologia bateratu eta estandar bat izatea, erreplikatzeko gaitasuna eta ikerketen arteko alderaketak errazte aldera.

Horrez gain, ikerketa batetik bestera esangarritasun estatistikorako mugako balioa ezberdina izatea ere heterogeneotasun-iturri izan daitekeela uste dugu. Are gehiago, estatistikoki esangarriak diren emaitzak soilik argitaratzeko joera dago, eta horrek alborapena eragiten du emaitzetan. Argitaratutako literaturan dauden muga horiek guztiek trinkotasun eza areagotzen dute emaitza askotan, eta, hala, zaildu egiten du BZHLHn biomarkatzaile gisa aztertutako mikroRNA batzuen funtzioari buruz ondorio nagusi batzuk ateratzea.

Azkenik, ikerketetan mikroRNA hautapena hartu dira kontuan, eta horrek konpara daitekeen emaitza kopurua mugatzen du; gainera, hobe ezagutzen diren mikroRNA molekuletan kokatzen du eztabaida, beste mikroRNA batzuk alde batera utzita. Horrenbestez, beharrezkotzat jotzen dugu mikroRNA multzo zabalago batekin egindako eskala handiko ikerketak egitea, hurrengo belaunaldiko sekuentziazioa eta antzeko teknikak erabilita, BZHLHn biomarkatzaile gisa erabiltzeko aukera duten mikroRNAk sakonago eta alborapen gabe identifikatzeko.

MIKRORNA MOLEKULEN ADIERAZPENAREN SINADURA BZHLH-N RNA TXIKIEN SEKUENTZIAZIOAN OINARRITUTA

Literaturaren berrikuspen sistematikoari esker identifikatutako mugak kontuan hartuta, BZHLHn mikroRNA molekulen sinadura berria definitzeko ikerketa egitea erabaki genuen. Horretarako, RNA txikien sekuentziazio osoa erabili genuen. Sekuentziazio osoaren bidez, mikroRNA molekulen adierazpen-profilak lortzen dira, bai lehendik deskribatutakoenak bai hautagai berrienak. Hautagai horiek ez dira sartu beste ikerketetan, zeinetan "*microarray*" eta alderantzizko transkripzioaren PCR kuantitatiboa (RT-qPCR) teknologiak erabili baitira gehien.

MikroRNAen adierazpenaren sinadura BZHLH diagnostikatzeko

BZHLH laginetan eta kontrol-laginetan mikroRNA molekulek duten adierazpena konparatzerakoan behatu ziren ezberdintasunik handienak talde batetik bestera. Identifikatutako mikroRNA guztien adierazpenak kontuan hartuta bereizketa garbia dagoela kontrol-taldearen laginen eta BZHLH laginen artean erakusten du PCA grafikak (9. irudia). Horrek esan nahi du BZHLH laginen eta kontrol-laginen mikroRNA profilak oso ezberdinak direla. PCA egitean taldeetako mikroRNA profiletan ezberdintasun handiak agertzeaz gain, erregulazioan aldaketak dituzten hainbat mikroRNA detektatu genituen konparaketak banaka egiterakoan.

Diagnostikoaren momentuko BZHLH laginetako eta kontrol-laginetako mikroRNAen adierazpenak konparatzean, 146 mikroRNAek adierazpen diferentziala zutela ikusi zen. MikroRNA horietatik 112 gain-adierazita zeuden BZHLH pazienteetan. Are gehiago, mikroRNA gehienen erregulazioaren aldakortasuna ez da aurretiaz aipatu BZHLH edo bestelako gaitzetan. BZHLH laginetan gehien gain-adierazitako mikroRNAk, miR-210-3p eta miR-994 izan ziren; azpi-adierazitako mikroRNAk, berriz, miR-215-5p eta miR-150. Azken horiek, pertsona osasuntsuetan gehiago adierazten zirela aurkitu zen.

Ikerketek erakutsi dutenez, miR-210 gain-adierazita agertzen da linfometan, eta zelulen zikloaren erregulatzaile nagusietako bat izan daitekeela proposatu da (205). Gure aurkikuntzekin bat datozen emaitzen artean, bi ikerketetan aipatu zen BZHLH pazienteetan miR-210-3p gain-adierazita zegoela (146,147), B-zelula arruntetako jarduerarekin alderatuz gero; hala ere, beste bi ikerketetan ez zen aurkitu estatistikoki esangarririk zen loturarik (150,165). Datu interesgarri bat ere aurkitu zen: mikroRNA molekulen adierazpen-profilak ikertzean, minbizi mota askotan detektatu da miR-210 molekularen adierazpenaren igoera (próstata (206), urdail-hesteak (207), bularreko (208) edo birikako minbizian (209), adibidez). Horrenbestez, beste egileei esker gaur arte bildutako datuek eta ikerketa honetan azaltzen ditugunak erakusten dutenez, miR-210-3p molekulak onkogene funtzioa izan dezake BZHLHn. Aurkikuntza berriek adierazten dutenez, miR-210 molekularen gain-adierazpenak tumoreen aurkako erantzun immunitarioaren eraginkortasuna jaitsi dezake, eta tumoreek immunozaintza saihesteko gaitasuna hobetu, zelulen bidezko zitotoxikotasunaren eraginkortasuna apalduta —T linfozito zitotoxikoen (CTL) eta zelula hiltzaile naturalen (*NK, natural killer cells*) jarduna, hain zuzen ere—(210). Gure interakzio-sarearen analisiak erakusten duenez, 3' muturreranzko ituek (*PTPN1,* esaterako) parte hartzen dute aipatu mekanismo horretan.

MiR-944 molekulari dagokionez, haren gain-adierazpenik ez da aurretik deskribatu BZHLHren kasuan, ezta bestelako gaitz hematopoietikoetan ere. Hala ere, tumore mota askotan ikusi da miR-944 molekularen adierazpena aldatuta dagoela, eta, gaitzaren arabera, tumoreak ezabatzeko funtzioa edo funtzio onkogenikoa duela. Alde batetik, bularreko minbizian (211), utero-lepoko minbizian (212) eta endometrioko minbizian (213), esaterako, miR-944 gain-adierazita aurkitu da, eta horrek esan dezake miR-944ak tumorearen garapena sustatzen duela, zelulen ugalketa, migrazioa eta inbasioa sustatzen baititu. Bestalde, beste ikerketa batzuetan ikusi denez, tumore-ezabatzaile funtzioa ere har dezake miR-944 molekulak, kolon eta ondesteko minbizi-ehunean haren erregulazioa nabarmen txikitzen baita (214,215). Gure ikerketan lortutako emaitzen arabera, bularreko, utero-lepoko eta endometrioko minbizian duen joera berdina hartzen du BZHLHn, hau da, onkogene funtzioa du. Hala ere, oraindik ez dira ezagutzen miR-944 molekulak BZHLHn izan ditzakeen funtzio biologikoak eta ekintza-mekanismoak. Egindako interakzio-sarearen analisiaren bidez BZHLHn garrantzitsuak diren miR-944 molekularen itu hauek identifikatu genituen: *HECW2, S100PBP, PTP4A1* eta *SIAH1*. *HECW2* geneak ongi karakterizatu gabe dagoen HECT domeinuko ubikitina ligasa kodetzen du; aurkitu denez, entzima horrek p73 tumore-ezabatzailea egonkortzen du eta haren transkripzio-jarduera areagotuz (216). Tentagarria da proposatzea miR-944 molekulak *HECW2* genearen adierazpena ezaba lezakeela, horrekin p73ren erregulazioa txikituz eta, beraz, tumoreen sorrera sustatuz. Hala ere, ikerketa gehiago egin behar dira *HECW2* geneak BZHLHn eta p73-miR-944 erregulazio-sarean duen funtzioa zehazteko. MiR-944 molekularen beste itu batek, *S100PBP* geneak, pankreako minbiziko zelula-lerroetan zelulen inbasioa eta atxikimendua ezabatzen duela ikusi da (217). BZHLHn *S100PBP* genearen erregulazioa txikituta dagoenez, interesgarria litzateke BZHLHren zelula-inbasioan ere parte hartzen duen edo ez zehaztea. Azkenik, beste azterketa batzuetan deskribatu denez, giza bularreko minbizian *SIAH1* genearen erregulazioa txikitzeak zelulen ugalketa, kolonien sorrera, migrazioa eta inbasioa, zelulek S fasea abiaraztea eta apoptosia inhibitzea sustatzen ditu (218). Hortaz, miR-944ek BZHLHn eskema bera jarraituko balu gaixotasun honen garapenerako mikroRNA honen ekintza mekanismoa azal dezake.

Erregulazioa txikituta duten mikroRNA molekulei dagokienez, gure populazioan gehien azpi-adierazitako molekula miR-125 izan zen. Horrekin bat eginez, miR-125 molekulak BZHLHn duen adierazpena dagoeneko aztertu zuten Wu *et al.*-ek (219), eta haiek ere azpi-adierazita aurkitu zuten BZHLH pazienteetan. Beste gaitz hematologiko batzuei dagokienez, Wang *et al.*-ek (2010) (220) aipatu zuten leuzemia mieloide akutuan (AML) ohikoa zela miR-125 azpi-adierazita aurkitzea. Are gehiago, minbizi mota askotan gehien ikertu den mikroRNA da miR-125, eta ikerketetan ikusi denez, tumore-ezabatzaile funtzioa edo onkogene-funtzioa izan dezake tumore motaren arabera. Kolon eta ondesteko minbizian (221,222), mikrozitikoa ez den birika-minbizian (223) eta bularreko minbizian (224) tumore-ezabatzaile gisa identifikatu zen azpi-adierazita lehendabizikoz. Urdaileko minbizian, aldiz, onkogene-funtzioa hartzen du gain-adierazita baitago (225) eta korrelazioa baitu tumorearen inbasioaren eta minbiziaren fasearen garapenarekin (226,227). Datu horiek argi erakusten dute zein konplexua den minbiziaren erregulazioa, eta, tumorearen jatorrizko ehunaren arabera, miR-215 tumoreen garapenean eta ezabatzean duen funtzio bikoitzean islatzen da hori. Gure kasuan, miR-125 BZHLHn izan dezakeen ezabatzaile-funtzioari buruzko xehetasun berriak ematen dituzte gure emaitzek.

Interes handiko beste aurkikuntza FRA1H (1q41–q42.1) gune ahularen *1q41.1* zatiko *miR-215-194-1* multzoan dago miR-125, miR-194-5p molekularekin batera (gure ikerketan gehien azpi-

adierazita zeuden mikroRNA guztien artean laugarrena), eta gune ahul hori ezabatu egiten da minbizi mota askotan (228). Bi mikroRNA horien batera azpi-adieraztearen fenomenoaren berri eman da beste minbizi batzuetan, esaterako, giltzurrun-zeluletako kartzinoman (229) eta mieloma anizkoitzean (230). Molekula horien ekintza-mekanismoei dagokienez, Pichiorri *et al.*-ek (230) ikusi zuten mikroRNA horien gain-adierazpena aurkezten zuten mieloma anizkoitzeko zeluletan *MDM2* genearen erregulazioa azpi-adierazita zegoela proteinen eta mRNA molekulen mailan. Azpi-adierazpen horrek alderantzizko erlazioa du p53-ren adierazpen handiagoarekin eta p21-en aktibazioarekin. Horrek azal lezake molekula horiek duten tumore-ezabatzaile funtzioa. BZHLHn miR-215 eta miR-194 molekulek prozesu zelularrei eragiteko dituzten mekanismoak are gehiago aztertzeko, interakzio-sarearen analisia egin genuen, eta tumorearen garapenean parte hartzeagatik ezagunak diren itu batzuk identifikatu ziren. Horien artean dago 2B motako aktibina-hartzailea *(ACVR2B)*. Molekula hori bi mikroRNA horien itu gisa deskribatu da haurtzaroko giltzurrun-neoplasian (231). Hartzaile horrek funtsezko funtzioa du aktibina aktibatzeko garaian, eta, aldi berean, beta hazkuntza-faktore transformatzailearen (TGF)-β seinale-bidearen osagai garrantzitsua izan daiteke, eta kartzinogenesian eragin dezake. Aurkikuntza horiek *miR-215-194-1* multzoak tumoreak ezabatzeko duen funtzioaren eta egon daitezkeen ekintza-mekanismoen alde egingo lukete, eta, beraz, are gehiago ikertzeko gai interesgarri bihurtzen ditu.

BZHLHn azpi-adierazita dauen mikroRNAen artean, miR-150-5p ere aurkitu genuen; molekula hori dagoeneko aztertu da BZHLHko hainbat ikerketetan. Leuzemietan eta linfomatan gertatzen den miR-150 molekularen erregulazioaren txikitzea deskribatu da jada. Litekeena da mikroRNA horrek oso funtzio espezifikoa izatea neoplasia linfatiko ezberdinen garapenean (BZHLH barne), aurreko atalean azaldu dugunez.

Bost mikroRNA horien (miR-210-3p, miR-944/miR-215-5p, miR-150-5p eta miR-194-5p) erabilera konbinatuta asko areagotuko litzateke mikroRNA markatzaile bakarrak diagnostikatzeko duen zehaztasuna. Edonola ere, ikerketa gehiago egin behar dira bost mikroRNA horien adierazpen-profilarekin BZHLH beste tumore motatatik bereizteko aukera dagoen edo ez argitzeko.

Laburbilduz, aurretik deskribatu diren erregulazioa galdutako hainbat mikroRNA berresteaz gain, BZHLHn erregulazioa galdu duten beste mikroRNA batzuen aurkikuntzaren berri ematen dugu, zeinak ez baitira ikertu minbizi mota honetan. Are gehiago, gehien gain-adierazitako 20 mikroRNA molekulen multzotik (12. taula) miR-146a-5p, miR-129-5p eta miR-205 dira aurretiaz BZHLHrako ikertu diren bakarrak. Gehien azpi-erregulatutako 20 mikroRNA multzoari dagokionez (13. taula), miR-224-5p, miR-145-5p, miR-497-5p, miR-151b eta miR-451 soilik ikertu dira aurretiaz BZHLHn.

Emaitza horiek argi erakusten dute mikroRNA molekulen profilak zehaztea tresna diagnostiko berria, fidagarria eta estandarizatu daitekeena izan daitekeela BZHLH karakterizatzeko. BZHLHren diagnostikoaren ezaugarri analitikoak are gehiago hobetzeko, lagungarria izan daiteke mikroRNA sinadura berri horiek ohiko biomarkatzaileekin batera erabiltzea.

MikroRNA molekulen adierazpenaren sinadura BZHLHren azpimotak sailkatzeko

BZHLH ZGB eta BZHLH ez-ZGB azpimotek aktibazio onkogenikorako mekanismo, anormaltasun genomiko eta emaitza kliniko ezberdinak dituzte (10,69). R-CHOPekin edo R-CHOPen antzekoak diren erregimenekin tratatzean, BZHLH ZGB duten pazienteek biziraupen hobeagoa dute IPIaren balioa edozein dela ere (31,232). Hala ere, ez dago argi azpimotetan sailkatzeak pronostikorako duen balioa, batez ere kontuan hartzen badugu azpimotetan sailkatzeko metodo ezberdinak erabiltzen direla eta emaitzak irregularrak lortzen direla. Sekzio honetan, bi azpimotetan mikroRNA molekulen adierazpena aldatzen den edo ez aztertu genuen, baita adierazpen horrek esparru klinikoan sailkapena errazten lagunduko dezakeen edo ez.

Diagnostikoa egitean hartutako ZGB eta ez-ZGB laginetako mikroRNAen adierazpen-profilak konparatzean, zortzi mikroRNAtan adierazpen esangarria aztertu zen. Haietatik bost (miR-129-2-3p, miR-4464, miR-3150b-3p, miR-138-5p eta miR-129-5p) gain-adierazita zeuden BZHLH ZGB pazienteetan, eta beste hiru (miR-511-5p, miR-205-5p, eta miR-3652) BZHLH ez-ZGB azpimotan gain-adierazita zeuden.

BZHLH ZGB pazienteetan gain-adierazitako mikroRNAei dagokienez, miR-129-5p eta miR-138-5p gain-adierazpena ZGB azpimotan aurretik azaldu dira (147,150,155,187). Are gehiago, ZGB azpimotan gain-adierazita dauden mikroRNAk, zentro germinaletako linfomen sorrerarekin erlazionatutako transkritoak itu izaten dituzte (233), batez ere, miR-129-3p/miR-129-5p eta miR-138-5p molekulen kasuan. MiRTarBase sareko tresna erabiliz aurresan ziren mikroRNA horien itu-geneak. Horien artean zeuden, besteak beste, funtzio hauekin erlazionatutako geneak: zelula-zikloa (*CDKN1A*), MAPK eta NFkB seinale-bidea *(MAPK1).* MikroRNA horien adierazpena handia izanez gero, ziklinaren mendekoa den kinasa-inhibitzailearen —*CDKN1A* (p21 izenez ere ezaguna)— adierazpena era negatiboan erregulatuko luke, eta, hala, zelulek zelula-zikloaren G1 puntua gaindituko lukete. Horregatik proposatzen dugu mikroRNA horiek ezinbestekoak direla zentroblastoentzat zelula-zikloaren G1 fasetik S fasera igarotzeko, *CDKN1A* genearen adierazpena murriztuz (234). Beste datu interesgarri bat ere aurkitu genuen: *TP53* genea miR-129-3p/miR-129-5p eta miR-138-5p molekulen itu-genea da. Zelula-zikloaren etena, DNAren konponketa, apoptosia, seneszentzia eta autofagia zuzentzen dituen funtsezko tumore-ezabatzailea da *TP53* genea (235,236). Linfomaren sorreran eta gaitzaren garapenean eragiten du *TP53* geneak gaizki funtzionatzeak, eta p53-ren funtzionamendu normala ezinbestekoa da tumoreak ezabatzeko. *TP53* genearen mutazioek pronostikoari dagokionez duen esanahiak ez du emaitza bateraturik eman hainbat minbizitan. Hala ere, azkenaldian egindako ikerketek erakutsi dutenez, *TP53* geneko mutazioek BZHLH ZGB mailatan banatzen dute eta bakoitzak OS ezberdina du (237). BZHLH ez-ZGBren kasuan, ordea, ez du halakorik egiten. Horrek adieraz lezake BZHLH ZGB *TP53* genearen mendekoa dela, eta BZHLH ez-ZGB, berriz, *TP53* genearekiko independentea. Horrek aipatu ditugun mikroRNA molekulen adierazpenak azpimota bakoitzean dituen ezberdintasunak azalduko lituzke. Bestalde, ikerketa honetan azpi-adierazita behatu diren miR-4464 eta miR-3150b-3p molekulak ez dira inoiz deskribatu orain arte BZHLHren patogenesian. Azkenik, ez-ZGB azpimotan gehien gain-adierazitako hiru mikroRNAk (miR-511-5p, miR-205-5p eta miR-3652) ere ez dira inoiz deskribatu orain arte BZHLHren patogenesian.

Gerta daiteke mikroRNA horien adierazpen diferentzialak erregulazio-bide ezberdinak azaleratzea, eta erabilgarriak izan daitezke BZHLH pazienteak sailkatzeko. Lehenago ikusi

dugunez, zentro germinaleko B zelula da BZHLH ZGBren ohiko baliokidea, eta BZHLHren ez-ZGB azpimotak zentro germinalaren osteko plasmablastoen antza du (10,74). NF-κB bidearen aktibazio osagarria da BZHLH ez-ZGBren ezaugarri bereizgarrietako bat. Zentro germinaleko zelula antzekoa (ZGB) duten BZHLHek PI3K/AKT bidearen aktibazio onkogenikoaren mende daudela erakutsi dute (233). Hala ere, ez dago argi PI3K/AKT bidea aktibatzearen atzean dagoen mekanismoa eta BZHLHn izan dezakeen funtzio onkogenikoa.

BZHLH azpimotetan adierazpen diferentziala duten zortzi mikroRNA aurkitu ditugun arren, goian aipatu bezala, muga bat dugu ikerketa honetan: Han-en IHK algoritmoa erabili zen pazienteak sailkatzeko. Ikerketek erakutsi dutenez, IHKn oinarritutako sailkapenak GAPn oinarritutako sailkapenarekin aurresandakoaren antzeko emaitza eman arren (eta GAP da urrezko estandarra) (25), IHK tindagaiak erreproduzitzean dagoen aldakortasuna eta immunotindatzeari eragiten dioten faktore subjektibo eta teknikoen ondoriozko interpretazioa izan daitezke emaitza bateraturik ez lortzearen arrazoiak (238). Horren harira, berrikuspen sistematikoa egiterakoan, GAP eta IHK erabili zuten ikerketen artean ezberdintasunak behatu genituen ikerketa mota bakoitzean identifikatutako mikroRNAei dagokienez. Hori dela eta, ikerketa gehiago egin behar dira ezberdintasun horiek argitzeko, eta, bien bitartean, are interesgarriagoa litzateke tratamenduari emandako erantzunarekin edo pronostikoarekin erlazionatutako mikroRNAk identifikatzea, azpimotetan adierazpen diferentziala dutenak aztertu beharrean.

Terapiari emandako erantzuna aurresateko mikroRNA sinadura

BZHLH duten pazienteen % 10–20 errefraktarioak izango dira lehen aukerako kimioterapia ematean, eta % 30–40 berriro gaixotuko dira erremisio totala eta gero. Paziente horiek hasieratik identifikatzea garrantzitsua izan daiteke, lehen aukerako kimioterapia hobeto esleitzeko; izan ere, lehen aukerako indukzio-tratamenduak huts egin ondoren BZHLH duten pazienteen emaitzak ezin baino txarragoak izaten dira (239). Hori dela eta, lehentasuna du eraginkortasun terapeutikoa era eraginkorrean baloratzeko adierazle berriak identifikatzea. Hala ere, agente kimioterapeutikoekiko erresistentzia garatzearen atzean dauden mekanismoak ez dira ongi ulertzen oraindik. MikroRNA molekulek, funtzio biologikoen erregulatzaileak direnez, farmakoekiko erresistentzian ere eragiten dute. Hori dela eta, BZHLH pazienteek tratamenduari ematen dioten erantzunarekin lotura duten mikroRNA berriak identifikatzeko helburua jarri genuen; mikroRNA horiek biomarkatzaile gisa erabili ahal izango lirateke pazienteak multzokatzeko eta terapia are gehiago pertsonalizatzeko. Hori bereziki garrantzitsua da kontuan hartuta BZHLHren kasuan mikroRNA molekulen adierazpenaren eta tratamenduari ematen zaion erantzunaren arteko erlazioa gutxi aztertu dela.

Ikerketa honetan, RNA txikien sekuentziazioa erabiliz, tratamenduari emandako erantzun onarekin erlazionatu zen hamar mikroRNAen gain-adierazpena (miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p eta miR-3928-3p). Horrez gain, miR-192-5p gain-adierazpena R-CHOP terapiarekiko kimioerresistentziarekin erlazionatu zen. Nabarmentzekoa da mikroRNA horien erregulazioaren profilak ez direla inoiz aztertu BZHLHn, bai ordea beste minbizi mota batzuen testuinguruan.

Aurkitu dugunez, miR-12136 tratamenduari guztiz erantzuten dioten pazienteetan gehien gain-adierazitako mikroRNA da; adierazpena 25 bider edo gehiago handitu dela behatu da. Are gehiago, duela gutxi aurkitu da miR-12136, eta, beraz, oso gutxi dakigu mikroRNA horri buruz.

Molekula honi buruz lortutako emaitzek argi erakusten dute ikerketa gehiago egiteko hautagai interesgarria dela. Azken molekula horren antzera, ikusi genuen orain arte ikertu gabeko miR-3681-5p eta miR-3928-3p mikroRNAk ere tratamenduari emandako erantzun onarekin eta pronostiko onarekin erlazionatuta zeudela. MiR-129-5p ere tratamenduari emandako erantzun onarekin erlazionatuta dago (15. taula). Are gehiago, gure emaitzekin bat eginez, miR-129-5p azpi-adierazpenak kimioerresistentziarekin korrelazioa duela behatu da aurretiaz bularreko minbizian. MikroRNA horren gain-adierazpenak erregimen terapeutiko batzuei emandako erantzuna zein den jakiteko datu garrantzitsua izatearen alde egiten du informazio horrek (240). Horrez gain, gure populazioan tratamenduari emandako erantzun onarekin erlazionatutako miR-127 molekulak bularreko minbizian p53 bidezko apoptosiaren aktibazioan funtzioren bat izan dezakeela deskribatu da dagoeneko; BZHLHn tratamenduari emandako erantzun onarekin duen lotura azaltzen duen ekintza-mekanismo bat izan daiteke hori (241). Kontuan hartu behar da aurretik egindako ikerketa batzuetan miR-192 eta kimioerresistentzia erlazionatzen zituztela biriketako (242), laringeko eta hipofaringeko minbizietan (243). Emaitza horiek guztiek argi erakusten dute mikroRNA molekula horiek zer funtzio duten BZHLHn gertatzen den kimioerresistentzian.

R-CHOPekin tratatutako BZHLH pazienteen tratamenduaren erantzunarekin erlazionatutako sinadura-mikroRNA hauek proposatzen ditugu: miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p, miR-3928-3p eta miR-192-5p. Etorkizunean, mikroRNA molekuletan oinarritutako tratamenduak ohiko kimioterapia-tratamenduarekin konbinatuta eskaintzea izan liteke BZHLH errefraktarioa duten pazienteak klinikoki kudeatzeko bidea. Lerro honetan, miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p eta miR-3928-3p gain-adierazpena aukera hona izan liteke mikroRNA hauen adierazpen baxua aurkezten duten pazienteetan, erantzun ona duten korrelazioagaitik. Aipatutako hau, pre-mikroRNAk, mikroRNA geneak edo mikroRNA "*mimic*"-ak erabiliz lortu daiteke (244–246). Beste alde batetik, miR-129-5p inaktibazioa kontrako zentzuko mikroRNA oligonukleotidoak, mikroRNA belaki edo mikroRNA inhibitzaileak (245,246) estrategia terapeutiko berri bat izan daiteke mikroRNA hau gain-adierazita duten gaixoentzat. Testu inguru honetan, mikroRNAtan oinarritutako terapia minbizian etorkitzun handiko estrategia den arren, zelulen ituetara heltzea da erronka nagusia. Zorionez, zentzu honetan aurrerapen handiak egiten ari dira.

Horien bidez, errefraktario izateko aukera handia duten pazienteak identifikatu ahal izango lirateke, eta horientzat lehen lerroko tratamendua optimizatzeko aukera egongo litzateke. Tratamenduen eraginkortasun terapeutikoa hobetzen eta tratamenduak pertsonalizatzen lagunduko luke informazio horrek; izan ere, mikroRNA molekulen adierazpena aztertuta, paziente bakoitzari hobekien egokitzen zaion tratamendua aukeratu ahal izango litzateke.

BZHLHn emaitza klinikoarekin erlazionatuta dauden mikroRNA sinadurak

Tratamenduari emandako erantzunaz gain, BZHLH pazienteen hasierako tratamendu-plangintza egiterakoan ezinbestekoa izan daiteke bizirik irauteko aukera zehatza aurresateko gaitasuna. Gaur egun, IPI da BZHLHn pronostikoa aurresateko urrezko estandarra. Hala ere, sistema horrek ez ditu islatzen BZHLH pazienteetan behatzen den zelulen morfologiaren heterogeneotasuna, immunitate-fenotipoa eta biologia molekularra. Are gehiago, erituximab-en garaian, huts egin

du B zeluletako linfoma zuten pazienteen pronostikoa aurresatean, proportzio handi batean (247). Aurretik egindako ikerketek hainbat ondorio aurkeztu dituzte BZHLH pazienteen emaitza klinikoari benetan eragiten dioten faktore klinikoei buruz, eta kontraesanak agertu dira ikerketa horien artean. Hori dela eta, biomarkatzaile berriak beharko lirateke ezaugarri klinikoekin batera konbinatzeko. Ikerketetan bildutako datuek erakutsi dutenez, mikroRNAen adierazpena izan daiteke paziente baten emaitza aurresateko biomarkatzaile gakoa. Pronostikoarekin erlazionatutako mikroRNAk azpimotekin erlazionatutakoak baino tresna hobea dira pazienteak arriskuaren arabera sailkatzeko lanetan, eta, gainera, erabilgarriak izan litezke agente terapeutiko espezifikoak garatzeko.

Epe luzeko erremisioa zuten pazienteetan gain-adierazitako zazpi mikroRNA (miR-4444, miR-449c-5p, miR-3681-5p, miR-3928-3p, miR-449b-5p, miR-370-3p eta miR-4424) eta berriz gaixotu ziren pazienteetan lau mikroRNA (miR-133a-3p, miR-133a-3p, miR-208b-3p eta miR-205-5p identifikatu genituen.

Horietatik bi (miR-3681-5p eta miR-3928-3p) ez dira inoiz aztertu BZHLH kasuetan. Izan ere, duela gutxi karakterizatu dira miR-3681-5p eta miR-3928-3p, eta horregatik ez dira beste ikerketetan sartu. Biziraupenaren analisiak erakusten duenez, miR-4444 lotura nabarmena du OS eta PFS hobearekin. Orain arte, miR-4444 molekulak BZHLHn duen adierazpen-ereduak eta pronostikoan horrek duen esanahia ez dira era sistematikoan ikertu, baina aipatu izan da aurretik ondesteko adenokartzinomaren pronostiko txarrarekin erlazionatutako sei mikroRNA molekulen eredu baten barruan (248). MiR-4444 molekulak itu ezberdinak izatea eta bide ezberdinetan parte hartzeko aukera edukitzea izan daiteke emaitza kontraesankor horiek argituko lituzkeen azalpen bat. Hala izango balitz, funtzio ezberdinak beteko lituzke zelula motaren, gene-adierazpenaren tumore-ereduaren eta terapia motaren arabera. Horren eraginez, orain arteko emaitzak ezingo lirateke konparatu. Ikerketa gehiago behar dira BZHLHn duen funtzioa ulertzeko.

MiR-205 ere OS okerragoarekin era esangarrian erlazionatu da immunokimioterapiarekin era uniformean tratatu ziren BZHLH pazienteen taldean. Are gehiago, aldagai anitzeko analisiak erakutsi zuenez, miR-205-5p OS eta PFS okerragoarekin erlazionatuta dago, IPI eta azpimota edozein izanda ere. Azpimota ez da biziraupenarekin erlazionatu, ziurrenez, pazienteak erituximab-ekin tratatu direlako; tratamendu horrek BZHLH ez-ZGBrekin erlazionatuta dagoen pronostiko txarra gainditzen duela aurkitu baita (249). Edonola ere, mikroRNA adierazpen-ereduak IPI egoerarekin eta BZHLH azpimotarekin erlazionatuz gero, pronostiko ezberdinetan hobeto sailkatu ahal izango dira pazienteak, eta horrek tratamendua are hobeto pertsonalizatzea ekar dezake. Ikerketa honetan aurkitu dugunez, miR-205-5p molekularen adierazpena oso erabilgarria izan daiteke BZHLHren kasuan, pronostiko txarrarekin eta biziraupenarekin erlazionatuta egoteaz gain, BZHLH pazienteetan eta ez-ZGB pazienteetan erregulazioa handituta duelako.

MikroRNA honek BZHLHn dituen ekintza-mekanismoei buruzko ikerketa sakontzeko, proteina-proteina interakzioen (PPI) sare bat eraiki zen, mikroRNA-mRNA interakzio-sarearen zati gisa aztertu ziren miR-205 eta haren itu-geneak (15. irudia). PPI sare horretan, *ERBB3* proteinak zituen interakzio-nodorik gehien. *ERBB3* proteinak epidermiseko hazkuntza-faktoreen hartzaile-familiako kide bat kodetzen du. Haren mutazio somatiko batzuk ohikoak dira kolon eta

ondesteko eta urdaileko minbizietan, baina arraroak minbizi hematologikoetan. Oraindik ez dakigu proteina horrek zer funtzio duen. Hogeita hamasei itu-proteinen artean, *PTEN* ere funtsezko nodo-proteina da. PI3K/AKT seinale-bidearen erregulatzaile negatibo nagusia da *PTEN*, eta azkenaldian argitaratutako ikerketen arabera, BZHLH kasuen %25–30ean aktibatuta dago sorreran (250). *PTEN* proteinaren galera tumore solido askoren pronostiko okerragoekin erlazionatu da, BZHLH barne, eta era esangarrian erlazionatu da gaitz garatuarekin, kimioterapiarekiko erresistentziarekin eta bizirik irauteko aukera txikiarekin (251–254). Hori dela eta, horrek azal lezake BZHLHn duen funtzioa.

Guztia batera hartuta, gure datuek adierazten dute miR-4444 eta miR-205 molekulak BZHLHren patogenesiarekin erlazionatuta daudela eta pazientearen pronostikoan zerikusia dutela. Guk dakigula, ikerketa honetan lehen aldiz frogatu da miR-444 eta miR-205 molekulek BZHLH duten pazienteen pronostikoa aurresateko balioa dutela eta tratamendurako norabide berria eskaintzen dutela. Horregatik ondorioztatu dugu miR-4444 azpi-adierazpena eta miR-205 gain-adierazpena duten pazienteek onurak izan ditzaketela tratamenduaren hasieran programa terapeutiko trinkoago bat jasoz gero. Gure emaitzek tratamendu pertsonalizatuago bat garatzera bideratzen gaituzte, zeinean mikroRNAen adierazpenak lagundu egingo baitu paziente bakoitzarentzat egokiena den tratamendua aukeratzen. Hala ere, bakoitzaren mekanismo espezifikoa eta pronostikorako balioa are gehiago aztertu eta egiaztatu behar dira.

MikroRNAak BZHLHren pronostikoarekin erlazionatu dituzten ikerketa gehienak errituximab aplikatzen hasi aurretik tratatutako BZHLH pazienteekin egin ziren. Beraz, ikerketa honek abiapuntu interesgarria eskaintzen du testuinguru horretan. Gure emaitzak kohorte-tamaina egokia duten ikerketa independenteen bidez baliozkotu beharko liratekeela uste dugu, pronostikorako biomarkatzaile sendoak baliozkotzeko.

B ZELULA HANDIETAKO LINFOMA HEDATSUAN MIKRORNA MOLEKULEK DUTEN EKINTZA-MEKANISMOA ARGITZEA MIKRORNA-mRNA INTERAKZIO-SAREAREN ANALISIA ERABILIZ

Erregulazioan aldaketak dituzten mikroRNA molekulen mekanismo osoari buruzko xehetasunak argitzeko asmoz, mikroRNA-mRNA interakzio-sarea eratu genuen. Izan ere, mikroRNA molekulen erregulazioan aldaketek mikroRNA molekulen itu-geneen adierazpen aberrantea eragiten dute, eta horrek, aldi berean, linfomagenesia azkartzen du. Integrazio-analisiaren bidez ikusi zen BZHLHn 214 genek paira zitzaketela mikroRNA molekulen erregulazio aldakorraren ondorioak, mikroRNA molekulen mekanismoarekin bat zetorren adierazpen-eredua baitzeukaten. Ildo horretatik jarraituz, ikerketa honen bidez behatutako erregulazioaren emendioa zuten mikroRNA molekulak izan daitezke BZHLHn 138 generen adierazpena txikitzearen eragileak. Halaber, mikroRNAen azpi-adierazpenak azal lezake 76 geneen gain-adierazpena. Funtzioen aberaste-analisia eginez, proteina lotzaileen eta katalitikoen (transkripzio-faktoreak eta kinasak, adibidez) gain-adierazpena ikusi zen gene horietan. *In silico* analisien bidez zehaztu ahal izan zen gene horiek asko agertzen direla minbiziaren bidezidorretan, batez ere, PI3K-Akt eta MAPK seinale-bideetan. Interesgarria da jakitea bide horietako hainbat gene ikerketa honetan aurkitu diren eta erregulazio aldakortasuna duten mikroRNA bat baino gehiagoren itu direla, eta mikroRNA ugarik gene horietako bat baino gehiago dituztela itu bide horietan; hori dela eta, BZHLHren patogenesia areagotzearen

erantzule izateko hautagai sendoak dira. Erregulazioan aldaketak jaso dituzten mikroRNAk BZHLHn eragiten duten moduari dagozkien mekanismoak aztertu genituen, gehien inplikatuta dauden itu-geneetan eta mikroRNA molekulatan arreta jarrita.

MikroRNA bat baino gehiagoren itu diren eta azpi-adierazita dauden generik interesgarrienen artean *FOXO1* eta *PTEN* daude. PI3K-Akt bidean parte hartzen dute (255), eta hurrenez hurren, bost eta lau mikroRNA molekulen itu direla aurresan da. p27, p21, FasL eta Bim proteinen transkripzio-faktorea da *FOXO1*. Proteina horiek ezabatzaileak dira, G_1/S trantsizioa blokeatzen baitute eta apoptosia eta DNAren konponketa induzitu. *FOXO1* geneak oinarrizko funtzioa betetzen du B zelulekiko espezifikoa den diferentziazio-programa ezartzen eta mantentzen. Baina, horrez gain, zelulen heriotza eragiten du BCR seinaleztapen desegokiaren eraginez (256,257). *FOXO1* genearen funtzioa galtzeak tumoregenesia sustatzen du, estresarekiko erresistentzia, proliferazioaa eta zelulen biziraupena areagotzen baititu (258). Apoptosia pairatu beharko luketen zelula anormalek bizirik iraungo lukete *FOXO1* genearen funtziorik ez izateagatik, eta, horrek, tumorea hedatzea dakar. Horrenbestez, *FOXO1* genearen funtzioa galtzeak zelulen zikloaren etetea induzitzeko gaitasuna txikitzen du, eta horrek tumorea garatzea eragiten du (259). Tumore linfoideei dagokienez, *FOXO1* genea tumore-ezabatzailea bezala deskribatu da Hodgkin-en linfoman (260), eta, BZHLHn, gene horren erregulazioa asaldatuta dagoela aipatu izan da (261). Deskribatu denez, *FOXO1* geneak *MYC* protoonkogenea erreprimitu egiten du eta *BCL2L1* apoptosiaren inhibitzailea mediastinoko B zelulen linfoma primarioan (262). Gainera, *FOXO1* genearen fosforilazioak eta inaktibazioak alderantzizko korrelazioa du leuzemia mieloide akutua (AML) duten pazienteen biziraupen-denborarekin (263). Ikerketa honetan ikusi denez, 5 mikroRNAk, miR-182-5p, miR-183-5p, miR-135a-5p, miR-9-5p eta miR-9-3p, dute *FOXO1* zuzeneko itu gisa eta haren adierazpena ezabatu egiten dute. MikroRNAek gene hori isiltzen dutenez, litekeena da BZHLHren patogenesian parte hartzea. Ildo beretik jarraituz, lehenago aipatu dugun *PTEN* tumore-ezabatzaile bat da, *FOXO1* genearen 5' muturrean eragiten du eta *AKT* negatiboki erregulatzen du. *PTEN* bidezko erregulaziorik gabe, *AKT* aktibatuak *FOXO1* fosforilatu eta nukleotik zitoplasmara bidaltzen du, eta proteasomek degradatu egiten dute han (264). Gure ikerketaren arabera, miR-155, miR-320a, miR-205 eta miR-182-5p molekulen itu da *PTEN*. Beraz, gene horren adierazpena murriztu eta *AKT*-ren erregulazio negatiboa eten dezake. MiR-182-5p molekulak bi geneak ditu itu gisa, eta PI3K/AKT bidea modulatzen duen funtsezko mikroRNA bat izan daiteke, *PTEN* genearen 5' muturra eta *FOXO1* genearen 3' muturra baitira haren ituak. Gure aurkikuntzekin bat eginez, aurretiaz deskribatu da Hodgkin-en linfoma klasikoan miR-182 eta miR-183 mikroRNA molekulen eta *FOXO1* genea ezabatzearen arteko harremana (260).

GSK3β genearen adierazpenaren murriztea emaitza oso interesgarria da ere. Izan ere, miR-183-5p, miR-182-5p, miR-9-5p, miR-129-1-3p eta miR-1246 molekulen itu izan daitekeela aurresan da. *GSK3β* oinarrizko proteina kinasa bat da, zeluletan hainbat proteina fosforilatzen dituena, eta, beraz, hainbat bideri eragiten die, besteak beste Wnt seinale-bideari. Bide horrek ezinbesteko funtzioa du hainbat minbiziren tumoregenesian, BZHLH barne (265,266). Wnt bidea ez badago, β-katenina suntsitzeko konplexuak fosforilatzen du β-katenina, eta proteolisira bideratzen du molekula —ardatza inhibitzen duen proteina (*AXIN*), poliposis coli adenomatoso proteinak (*APC*) eta glukogeno sintasa kinasa 3β proteinak (*GSK3β*) osatzen dute konplexua— (267). Wnt seinale-bideak *GSK3β* inaktibatzen du eta ez du uzten β-katenina fosforilatzen. Hala, β-katenina egonkortu egiten da zitoplasman. β-katenina pilatzen joaten da, eta nukleora

translokatzen da. Han TCF/LEF konplexuari lotzen zaio eta haien transkripzio-jarduera izugarri handitzen du. Protoonkogeneak daude TCF/LEF konplexuak erregulazioa emendatzen dieten geneen artean; esaterako, *c-myc* eta *cyclin-D1*. Azken horrek zelulen proliferazioa sustatzen du eta apoptosia saihesten du (268). Horrenbestez, miR-183-5p, miR-182-5p, miR-9-5p, miR-129-1-3p eta miR-1246 molekulek *GSK3β* genearen erregulazioa murrizten badute, β-kateninaren pilaketa eta protoonkogeneen aktibazioa areagotuko lukete. *GSK3β* genea ezabatzeak, gainera, farmakoekiko erresistentzia areagotzen du minbizi-zeluletan, Wnt/β-katenina seinaleztapena erregulatzeko gaitasunari esker (269). Are gehiago, mikroRNA horiek gehiegi adieraztearen eraginez, BZHLH zelulen ugalketa, inbasioa eta apoptosiaren ezabatzea areagotuko litzateke, *GSK3β* genea erregulatzen baitute.

Datuen arabera, miR-182-5p, miR-183-3p eta miR-9-5p molekulen arteko elkarlanak laguntzen dio BZHLHren patogenesiari funtzio onkogenikoan, PI3K/AKT/FOXO1/Wnt bidea guztiz erregulatzen baitute.

Horrez gain, ikusi genuen miR-612, miR-663a eta miR-155-3p molekulak *TP53*-ren erregulatzaile negatibo zuzenak direla. p53 tumore-ezabatzaileak oinarrizko funtzioa betetzen du, egonkortasun genomikoa eta tumoreen ezabatzea mantentzen baitu. Halaber, zelularen zikloaren hasiera eta zelulen banaketa kontrolatzen du p53k, transkripzio-faktore bat baita (270). Mutazioen bilaketa zabal bat egitean ikusi zen *TP53* genean funtzioaren galera zekarten mutazioak agertzen zirela tumore gaiztoetatik %50 baino gehiagotan (271). Are gehiago, p53 hainbat tumore motarekin erlazionatuta dagoela eta mikroRNA jakin batzuek erregulatzen dutela frogatu da (272,273). Hiru mikroRNA molekuletatik miR-612 lehendik erlazionatu da esofagoko zelula ezkatatsuen kartzinomaren garapenarekin eta metastasiarekin, eta *TP53* genearen mRNA eta proteina-adierazpenaren inhibizioan eragiten du (274). Litekeena da mikroRNA horiek *TP53*-ren mRNA molekularen 3´-UTR muturrera lotzea, eta, hala, *p53*-ren mailaren eta funtzioaren erregulazioa txikitzea. Horrek era negatiboan erregulatzen du p53 bidezko apoptosia eta zelula-zikloaren etena, eta, beraz, mikroRNA horiek p53-ren sareko osagai garrantzitsuak dira.

Azkenik, analisi honetan aipatzeko modukoa da miR-146a-5p molekulak duen funtzioa: diagnostikorako biomarkatzaile izateko hautagaia den gehien gain-adierazitako mikroRNA. mikroRNA horrek BZHLHn erregulazioa txikituta duten 11 geneekin interakzioa duela ikusi da; gene horien artean daude, besteak beste, *BRCA2, RHOA, SOS1, STAT1, CCND2, CXCR4, NOTCH1, RAC1, ROCK1, SMAD4 eta TGFB1. BRCA1* genearekin erlazionatutako bularreko minbiziaren patogenesian parte hartzen duen oinarrizko mikroRNA gisa deskribatu izan da miR-146a-5p (275). Horri dagokionez, *BRCA1* geneak bularreko minbiziaren tumoreak ezabatzeko funtzioa du, DNAren kalteak konpontzen baititu (276). Horrez gain, nahiz eta orain arte miR-146a-5p eta *BRCA1* genearen arteko atzeraelikadura-zikloa linfomarako deskribatu ez den, BZHLHn miR-146a-5p molekulak *BRCA1* genearen itzulpena inhibitzen duela proposatu dugu. Are gehiago, Luo *et al.*-ek aurkitu zutenez, *SMAD4* genearen adierazpen-galera BZHLH zelulen proliferazioarekin erlazionatuta egon daiteke, β hazkuntza-faktorearen (*TGF-β*) seinale-bide goretsiz (277). *NOTCH1* gene tumore-ezabatzailea da, eta haren jarduera nabarmen txikitzen da azaleko minbizian (278). Aukera erakargarria litzateke p53-ren funtzioaren murrizketak *NOTCH1* genearen adierazpenaren erregulazioa txikitzea (279).

BZHLHn mikroRNAen azpi-adierazpenaren ondorioz gain-adierazita dauden geneei dagokienez, *BCL2* geneak du emaitzarik esangarriena. Berez, 135a-5p, miR-234-5p, miR-139-5p, miR-451a eta miR-497-5p molekulek erregulatzen dute, baina horien mikroRNA molekulen azpi-adierazpenaren ondorioz genearen gain-adierazpena eragin dezake. Datuek erakutsi dutenez, apoptosiaren kontrakoak diren osagaiak (*Bcl-2*, esaterako) asko adieraztea da B zeluletan linfomagenesiari ekarpen handiena egiten dion faktore nagusietako bat (280). Hori aurkitu zenetik, ikerketa askok zehaztu dute minbizian parte hartzen duen onkogene garrantzitsuenetakoa dela *BCL2*, apoptosia inhibitzen baitu eta linfomak garatzea eragiten baitu, batez ere *c-MYC* genearen gehiegizko adierazpenarekin batera agertzen denean (281). *BCL2* proteina mitokondrietan kokatzen da gehien batean; biziraupena sustatzen du eta apoptosia inhibitu, *C* zitokromoa mitokondrietatik zitoplasmara askatzea saihestuz. BZHLH kasuen 50ean Bcl-2a gain-adierazita dago translokazioaren eta beste mekanismo batzuen bidez (Bcl-2 irabazia/anplifikazioaren bidez, adibidez) (282). B zeluletako minbizi askok, BZHLHk barne, t(14;18)(q32;q21) translokazio kromosomikoa dute. Translokazio horren ondorioz, 14q32 gunean dauden kate astuneko immunoglobulina sustatzaileak *BCL2* genearen promotoretik gertu kokatzen dira, eta, hala, handitu egiten da *BCL2* genearen adierazpenaren erregulazioa. mikroRNA bidez *BCL2* genea isiltzeko prozesua galtzeak ere adierazpena handitzen lagunduko luke. Izan ere, aurretik egindako ikerketek berretsi egin dute mikroRNAak (miR-15 eta miR-16, adibidez) ezabatzea edo haien erregulazioa txikitzea *BCL2* genea gehiegi adieraztearekin erlazionatuta dagoela (283). Beraz, miR-135a-5p, miR-234-5p, miR-139-5p, miR-451a eta miR-497-5p azì-adierazpenak *BCL2* genearen gain-adierazpena susta lezake BZHLHn eta, hala, linforma-zelulen apoptosia gutxitu. miR-234-5p ez beste mikroRNA guztia hauek daude gure populazioko BZHLHn gehien azpi-adierazita dauden 20 mikroRNA nagusien multzoan. Beraz, interesgarria litzateke lotura horiek beste populazio batzuetan aztertzea.

Diagnostikorako biomarkatzaile izateko hautagai den miR-145-5p mikroRNAren azpi-adierazpenak gene batzuen gain-adierazpenean lagun dezake, besteak beste: *STAT1, MMP1, JAG1, VEGFA, EPAS1* eta *FZD7.* Gure emaitzen arabera, *STAT1* genearen erregulazioa txikitu egiten da beste linfometan (284) eta minbizietan (285). *STAT1* geneak apoptosia sustatu ohi du. Horretarako, zelularen gainazaleko heriotza-hartzaileen familiako kideen eta haien lotugaien adierazpena sustatzen du (286). Hala ere, *STAT1* geneak BZHLHn duen funtzioari buruzko iritzi ezberdinak daude, *STAT1* geneak BZHLHn onkogene gisa jarduten duela baitiote beste ikerketa batzuek (287). Hori dela eta, datu esperimental gehiago behar dira *STAT1* geneak BZHLHn duen funtzioa egiaztatzeko. Bestalde, *MMP1* genearen gain-adierazpena bereziki interesgarria da, beste minbizi batzuetan (esofagoko minbizian, adibidez) gain-adierazita dagoela aipatu baita (288). Tumorearen aurrerapenean sortzen den mikroingurunean gertatzen diren aldaketa askoren bitartekari dira matrizeko metaloproteinasak (MMPak). Horien artean dago *MMP1*, zeinak zelulaz kanpoko matrizearen hainbat osagai degradatzen baititu, eta horrek minbizi-zelulei inbaditzen eta metastasia egiten laguntzen die (289). *JAG1* geneari dagokionez, hainbat ikerketa independentetan aipatu da *NOTCH1* genearen eta *Jagged1* lotugaiaren gain-adierazpena gertatzen dela mieloma anizkoitzean gaitzak aurrera egiten duenean (290). *EGFR* ere maila altuan aurkitu ohi da minbizi-zelula mota batzuetan, eta zelula horiek haztea eta zatitzea eragiten du (291). Urdaileko minbizien %30 kasuetan, *EGFR* gain-adierazita dagoela ikusi da, eta biomarkatzaile eraginkorra izan daiteke EGFRren kontrako terapiaren onura klinikoa

aurresateko (292). Hala ere, ez da ezagutzen BZHLHn *EGFR*ren adierazpenaren erregulazioak zer mekanismo duen.

MikroRNAei buruzko jakintza geroz eta handiagoa da eta tumoregenesian eta tumoreen aurrerapenean duten parte-hartze estuari buruz. mRNA-mikroRNA interakzioen analisi honen bidez, erregulazio aldakorra duten mikroRNA molekulek BZHLHren patogenesiari eragiteko duten mekanismo bat proposatu dugu, eta PI3K/AKT/FOXO1/Wnt bideak eta *BCL2*-ren parte-hartzeak duten garrantzia nabarmendu dugu. Litekeena da mikroRNA batzuek elkarlanean jardutea (miR-182-5p, miR-183-3p eta miR-9-5p), eta beste batzuk (miR-146a-5p, adibidez) BZHLHn parte hartzen duten geneen erregulatzaile nagusiak izatea. Metodo honen bidez, gene sorta handietatik biomarkatzaile izan daitezkeen hautagaiak identifikatu ditugu, adierazpen diferentziala duten gene sorta txikietatik egin beharrean. Ikerketa honetan identifikatutako mikroRNA-mRNA adierazpen bateratua itu gisa erabil daiteke BZHLH detektatzeko garaian.

MUGAK ETA INDARGUNEAK

Ikerketa honen garapenean zehar zenbait muga eta indargune aurkitu genituen.

BZHLH pazienteen gongoil linfatikoen FFPE laginetan mikroRNA molekulen adierazpena aztertu dugu ikerketa honetan. Gongoil linfatikoen FFPE biopsiak eskuragarri izateari esker, ikerketa-populazioaren ia 20 urteko tartea aztertzeko aukera izan dugu, hots, 1999 eta 2018. urte bitartean BZHLH diagnostikatu zaien pazienteen laginak. Bost urteko biziraupen orokorra eta aurrerapenik gabeko biziraupena gaixotasunari dagokion amaiera-puntuko emaitza sendo gisa hartu badugu ere, epe luze horretan jarraipena egiteko aukera izatea abantaila dela deritzogu. Gainera, Euskadiko hiru ospitale nagusietako pazienteak sartu dira ikerketan, eta, horri esker, paziente-populazio garrantzitsua lortu dugu. Hala ere, FFPE blokeetatik erauzitako RNAren kalitatea ez da oso ona; 300 base baino laburragoak diren zatitan degradatzen da normalean, (293,294) eta formaldehidoarekin finkatzerakoan kimikoki eraldatzen da metiliol taldeen erruz (295). FFPE blokeetatik erauzitako RNAk aldaketak jasan ohi baditu ere, mRNA molekulekin alderatuz gero, mikroRNAk erresistenteagoak dira RNasaren bidezko degradazioarekiko, tamaina txikiagaitik eta bigarren mailako egituragaitik ziurrenez (296). Are gehiago, aurretik egindako ikerketetan ondorioztatu da gordetako FFPE ehun-laginetatik erauzitako mikroRNA molekulen adierazpenak korrelazio ona zuela izoztutako lagin freskoekin, eta formaldehidoaren bidezko finkapenak ez duela gehiegi aldatzen mikroRNA molekulen egonkortasuna (297). Inolako zalantzarik gabe, RNA molekulak hobeto gordeko lirateke izoztutako lagin freskoetan, baina material mota hori ez dago hain eskuragarri. MikroRNA molekulen analisi hauetarako, FFPE laginak erabilgarriak dira, eta atzera begira egindako ikerketa handiak egiteko aukera ematen du, zeinetan emaitzak pazienteen parametro klinikoekin korrelazioan kokatu baitaitezke.

Hurrengo belaunaldiko sekuentziazio-teknologia erabiliz egin zen mikroRNA profilen analisi osoa. Gaur egun, RT-PCRa edo microarray teknologia erabilita egiten da mikroRNA adierazpenaren neurketa kuantitatiboa. Guk dakigula, minbizian mikroRNA sinadurak identifikatzeko mikroRNA sekuentziazioa erabili duten ikerketa gutxi daude, orain arte batek erabili du hurrengo belaunaldiko sekuentziazioa (NGS) BZHLH ikertzeko asmoz (150). NGS

metodoak gero eta gehiago erabiltzen dira deskribatutako mikroRNA guztiak identifikatzeko aukera ematen dutelako. Horrelakorik ezin daiteke egin beste teknikekin, eta, gure ikerketan ikusi da, gaitzaren prozesuekin erlazionatutako mikroRNA asko ez direla aztertu orain arte, hoiek aztertzea oso garrantzitsua denean.

Bestalde, pazienteak hiru ospitaletan diagnostikatu zituzten, eta jarraipen-ohitura ezberdinak izan zituzten. Hori heterogeneotasun-iturri bada ere, kontuan hartu behar da tratamendu-protokoloak antzekoak izan direla. Azkenik, BZHLHn biomarkatzaile izateko aukera duten mikroRNA asko ez dira aztertu orain arte, eta, beraz, haien funtzioa baliozkotu egin beharko litzateke beste populazio batzuetan. BZHLH laginen kohorte handiagoetan prospekzio-ikerketak egitea beharezkoa da gure aurkikuntzak are gehiago baliozkotzeko eta paziente bakoitzaren mikroRNA profilean oinarritutako aukera terapeutiko berriak aztertzeko.

MikroRNA-mRNA sareak era esperimentalean baliozkotutako datu bilduma handietan oinarrituta daude. Hala ere, mikroRNA sareen analisiak badu muga bat: paziente ezberdinen erregulazioan aldaketak zituzten mikroRNA eta geneetan oinarrituta eraiki zela sarea. Metodo horren beste desabantaila mikroRNA sareek inferentzia kausal okerretara jotzea eragin dezaketela, eta horrek emaitza kontraesankorrak ematen ditu gene-adierazpenean. Egoera ideal batean, paziente beraren mikroRNA eta geneen adierazpenak erabiliko lirateke mikroRNA molekulen erregulazio-sareak aurresateko. Hala ere, muga hori onartu beharra dugu ikerketaren beraren diseinua dela eta. Interakzio horiek laborategian baliozkotzea da falta den erronkarik handiena.

ONDORIOAK

B-zelula handiko linfoma hedatsuan:

1. Lau mikroRNAk (miR-150-5p, miR-146a-5p, miR-155-5p eta miR-21-5p) azaldu zuten aldakortasuna erregulazioan BZHLHn literaturan oinarrituz eta emaitza hauek gure pazienteen kohortean balidatu ziren. Hala ere, beste ikerketa lanetan aztertutako mikroRNA kopuru murriztua eta haien emaitza kontraesangarriak direla eta, ez dute ondorio sendorik ekartzen eta beraz, ezin daiteke gaixotasunaren sailkapenerako, tratamenduaren erantzunerako eta pronostikorako sinadura identifikatu.
2. MikroRNA adierazpenean aldakortasuna argia identifikatu zen gure kohorteko BZHLH laginetan eta kontroletan RNAseq teknika erabiliz. MiR-210-3p eta miR-944 gain-adierazpen esangarriena izan zuten eta miR-215-5p eta miR-150-5p aldera, azpi-adierazpen esangarriena izan zuten.
3. Azpimoten ezberdintasunei dagokiela, miR-129-2-3p gain-adierazpenik handiena erakutsi zuen mikroRNA izan zen ZGB BZHLH azpimotan eta miR-511-5p ez-ZGB BZHLHn. MiR-129-2-3pen gain-adierazpenaren papera ZGB azpimotan izan daiteke linfomen zentro germinalaren eraketan erregulazio aldaketak dituzten transkritoak bere itu izatea.
4. miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p, miR-3928-3p eta miR-192-5p osatzen duten mikroRNA sinadura arrisku handiko paziente errefraktarioak identifikatu ditzake. Gainera, etorkizunean mikroRNAn oinarritutako terapiaren itutzat har daitezke.
5. MiR-4444 eta miR-205-5p adierazpena BZHLHren pronostiko ona eta txarraren markatzaileak izan daitezke hurrenez hurren.
6. MikroRNA espezifikoen gain-adierazpena BZHLHren patogenesian parte hartu dezakete PI3K/AKT/FOXO1/Wnt bidezidorren erregulazioaren bidez. Izan ere, bidezidor hauetako geneak identifikatu ditugun desregulatutako MikroRNAen ituak dira.
7. MiR-9-5p, miR-146a-5p eta miR-182-5p master erregulatzaileak izan daitezke BZHLHn adierazpen aldakorra duten gene anitz erregulatzen dituztelako.
8. NGS balio handiko teknika da gutxi ikertutako mikroRNAk aztertzea ahalbidetzen duelako.

Laburbilduz, gure ikerketa lanak mikoRNAk BZHLHren diagnosian, sailkapenean, tratamenduaren erantzunean, pronostikoan eta patogenesian paper garrantzitsua jokatzen dutela azpimarratzen du.

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ERANSKINA

1. eranskin taula: Literaturan modu esangarrian adierazitako mikroRNAk BZHLH pazienteetan kontrolekin alderatuz.

MikroRNA esangarriak	Emaitza	n BZHLH	n Kontrola	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-21	Altu	45	23	Ehuna	qRT-PCR	1	Chen 2020
miR-214	Baxu	15	15	FF	qRT-PCR	1	Sun J-R 2019
miR-222-3p	Altu	74	26	FF	qRT-PCR	1	Sun S 2019
miR-101	Baxu	30	30 (NLN)	FF	qRT-PCR	1	Huang Y 2019
miR-195	Baxu	36	NA	FF	qRT-PCR	1	Wang 2019
miR-101	Baxu	72	30	FF	qRT-PCR	1	Huang 2019
miR-27a	Altu	409	477	Ehuna	qRT-PCR	1	Tang 2019
miR-let-7a	Baxu	13	11	FF	qRT-PCR	1	Malpeli 2018
miR-23a	Altu	70	30	FFPE	qRT-PCR	1	Xu 2018
miR-155	Altu	82	-	FFPE	qRT-PCR	2550 array	Wu 2018
miR-195	Baxu	20	-	Ehuna	qRT-PCR	1	He 2018
miR-155	Altu	29	32 (RLH)	Ehuna	qRT-PCR	1	Li 2017
miR-4532 **miR-1915-3p** **miR-187-3p** **miR-4485** **miR-4284** **miR-4508** **miR-1973** **miR-663a** **miR-877-5p** **miR-3195** **miR-596** **miR-4516** **miR-4488** **miR-636** **miR-27b-3p** **miR-150-5p** **miR-200b-3p** **miR-205-5p** **miR-342-3p** **miR-203**	Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Baxu Baxu Baxu Baxu Baxu Baxu	5	4 (RLH)	Ehuna	nanostring	800	Jia 2017
miR-21	Altu	55	20 (NLN)	FF eta FFPE	qRT-PCR	1	Liu 2017
miR-21	Altu	26	10 (NLN)	FFPE	qRT-PCR	1	Song 2017
miR-10a	Baxu	9	9 (RLH)	FF	qRT-PCR	1	Fan 2016
miR-4284 **miR-21-5p** **miR-142-3p** **miR-155-5p** **miR-3182** **miR-16-5p** **miR-142-5p** **miR-451a** **miR-29a-3p** **miR-342-3p** **miR-17-5p** **miR-20a-5p** **miR-let-7g-5p** **miR-23b-3p** **miR-19b-3p** **miR-15a-5p** **miR-491-3p** **miR-4484** **miR-143**	Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Altu Baxu Baxu	24	14 (NLN)	FFPE	array	3100 probes	Tamaddon 2016

1. eranskin taula: Literaturan modu esangarrian adierazitako mikroRNAk BZHLH pazienteetan kontrolekin alderatuz. (Jarraipena).

MikroRNA esangarriak	Emaitza	N BZHLH	n Kontrola	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-125b-5p	Baxu						
miR-30c-1-3p	Baxu						
miR-4534	Baxu						
miR-7491	Baxu						
miR-711	Baxu						
miR-5571	Baxu						
miR-630-5p	Baxu	24	14 (NLN)	FFPE	array	3100 probes	Tamaddon 2016
miR-483-5p	Baxu						
miR-4741	Baxu						
miR-4778-5p	Baxu						
miR-3158-5p	Baxu						
miR-320a	Baxu						
miR-101	Baxu						
miR-224	Baxu	258	40 (NLN)	FFPE	qRT-PCR	1	Ni 2015
miR-155	Altu	22	6 (NLN)	Biopsia	qRT-PCR	1	Huskova 2015
miR-16-1	Altu						
miR-16-2	Altu						
miR-27a	Altu						
miR-103	Altu	63	5 ZGB zelulak	FF	qRT-PCR	11	Troppan 2015
miR-185	Altu						
miR-199	Altu						
miR-497	Altu						
miR-21	Altu						
miR-17-92	Altu	200	11 (NT)	FFPE	qRT-PCR	3	Go 2015
miR-155	Altu						
miR-126	Altu						
miR-10b	Altu						
miR-145	Altu						
miR-126	Altu						
miR-424	Altu						
miR-134	Altu						
miR-199a-2	Altu						
miR-127	Altu						
miR-379	Altu						
miR-127	Altu						
miR-199b	Altu						
miR-143	Altu						
miR-144	Altu						
miR-199b	Altu						
miR-139	Altu						
miR-199a-2	Altu	92	15	FF	sekuentziazioa	miRNAome	Lim 2015
miR-130a	Altu						
miR-542	Altu						
miR-125a	Altu						
miR-218-2	Altu						
miR-99b	Altu						
miR-125b-2	Altu						
miR-10a	Altu						
miR-145	Altu						
miR-455	Altu						
miR-214	Altu						
miR-628	Altu						
miR-146b	Altu						
miR-100	Altu						
miR-497	Altu						
miR-1301	Altu						

1. eranskin taula: Literaturan modu esangarrian adierazitako mikroRNAk BZHLH pazienteetan kontrolekin alderatuz (Jarraipena).

MikroRNA esangarriak	Emaitza	n BZHLH	n Kontrola	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-451a	Altu						
miR-1247	Altu						
miR-574	Altu						
miR-195	Altu						
miR-340	Altu						
miR-326	Altu						
miR-196a-2	Altu						
miR-146b	Altu						
miR-338	Altu						
miR-675	Altu						
miR-337	Altu						
miR-511-2	Altu						
miR-10393-3p	Altu						
miR-99a	Altu						
miR-22	Altu						
miR-let-7c	Altu						
miR-217	Altu						
miR-654	Altu						
miR-452	Altu						
miR-503	Altu						
miR-455	Altu						
miR-let-7b	Altu						
miR-let-7e	Altu						
miR-450a-2	Altu						
miR-136	Altu						
miR-let-7a-2	Altu						
miR-196b	Altu						
miR-362	Altu						
miR-224	Altu	92	15	FF	sekuentziazioa	miRNAome	Lim 2015
miR-203a	Baxu						
miR-205	Baxu						
miR-20b	Baxu						
NOVELM00290	Baxu						
miR-4491	Baxu						
miR-3150b	Baxu						
miR-28	Baxu						
miR-20b	Baxu						
miR-129-2	Baxu						
miR-3917	Baxu						
miR-10392-5p	Baxu						
miR-363	Baxu						
miR-486	Baxu						
miR-3934	Baxu						
miR-138-1	Baxu						
miR-3681	Baxu						
NOVELM00288	Baxu						
miR-27a	Baxu						
miR-23a	Baxu						
miR-589	Baxu						
miR-10397-5p	Baxu						
miR-4746	Baxu						
miR-151a	Baxu						
NOVELM00113	Baxu						
miR-331	Baxu						
miR-17	Baxu						
miR-942	Baxu						
miR-629	Baxu						

1. eranskin taula: Literaturan modu esangarrian adierazitako mikroRNAk BZHLH pazienteetan kontrolekin alderatuz (Jarraipena).

MikroRNA esangarriak	Emaitza	n BZHLH	n Kontrola	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-128-2	Baxu						
miR-15b	Baxu						
miR-3615	Baxu						
miR-200b	Baxu						
miR-181a-1	Baxu	92	15	FF	sekuentziazioa	miRNAome	Lim 2015
miR-130b	Baxu						
miR-582	Baxu						
miR-616	Baxu						
miR-185	Baxu						
miR-146-a	Altu	56	28 (RLH)	Ehuna	qRT-PCR	1	Zhuang 2014
miR-23a	Altu	104	28	FFPE	qRT-PCR	1	Wang 2014
miR-146b	Baxu	106	30 (RLH)	FFPE	qRT-PCR	939	Wu 2014
miR-320d	Baxu						
miR-200c	NS	61	13 (NLN)	Ehuna	qRT-PCR	1	Berglund 2013
miR-150	Baxu						
miR-29b	Baxu						
miR-29a	Baxu						
miR-142-3p	Baxu						
miR-142-5p	Baxu						
miR-145	Baxu	45 (DC); 75 (VC)	10 (DC); 6 (VC)(NLN)	FF eta FFPE	qRT-PCR/array	177	Caramuta 2013
miR-143	Baxu						
miR-195	Baxu						
miR-497	Baxu						
miR-494	Altu						
miR-638	Altu						
miR-21	Altu						
miR-155	Altu						
miR-16	Baxu	12	7	FFPE	qRT-PCR	4	Handal 2013
miR-150	Altu	90	31 (RLN)	FFPE	qRT-PCR	2	Zhong 2012
miR-155	Altu						
miR-146a	Altu						
miR-18b	Altu						
miR-19b	Altu	36	5 (NLN)	Ehuna	qRT-PCR	8	Fassina 2012
miR-20a	Altu						
miR-92	Altu						
miR-93	Altu						
miR-106a	Altu						
miR-150	Baxu						
miR-210	Altu	58	7 (NLN)	FFPE	qRT-PCR	157	Roehle 2008
miR-155	Altu						
miR-106a	Altu						
miR-17-5p	Altu						
miR-150	Baxu						
miR-145	Baxu						
miR-328	Baxu						
miR-139	Baxu						
miR-99a	Baxu						
miR-10a	Baxu						
miR-95	Baxu						
miR-149	Baxu						
miR-let-7e	Baxu						
miR-320	Baxu						
miR-151	Baxu						
miR-21	Altu	48	6 (NBC)	FF eta FFPE	qRT-PCR	3	Lawrie 2007
miR-155	Altu						

1. eranskin taula: Modu esangarrian adierazitako mikroRNAk BZHLH pazienteetan kontrolekin alderatuz. (Jarraipena).

MikroRNA esangarriak	Emaitza	n BZHLH	n Kontrola	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-221	Altu	48	6 (NBC)	FF eta FFPE	qRT-PCR	3	Lawrie 2007
miR-155	Altu	23	2	FF	Semi RT-PCR	1	Eis 2005

Laburdurak: FF: izoztutako ehuna; FFPE: formalinan finkatutako eta parafinan sartutako ehuna; NA: ez eskuragarri; Altu: BZHLH pazienteetan modu esangarrian gain-adierazita; Baxu: BZHLH pazienteetan modu esangarrian azpi-adierazita; NS: desberditasun ez esangarriak pazienteen eta kontrolen artean; RLH: Linfoma hiperplasia erreaktiboa; NLN: Ehun linfatiko normala; NT: amigdala normala; DC: aurkikuntzaren kohortea; VC: balioztatzearen kohortea; NBC: B zelula normalen lagina.

2. eranskin taula: Literaturan BZHLH azpimotetan modu esangarrian adierazitako mikroRNAk.

MikroRNA esangarriak	Emaitza	n ZGB	n non-ZGB	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-155	Baxu ZGB	248	242	FFPE	qRT-PCR	1	Due 2019
miR-197	Altu ZGB	14	37	FFPE	qRT-PCR	array	Yang 2018
miR-21	Baxu ZGB	19	36	FF and FFPE	qRT-PCR	1	Liu 2017
0	-	29	29	FF	qRT-PCR	1	Marques 2016
0	-	6	15	Biopsia	qRT-PCR	1	Huskova 2015
miR-155	Baxu ZGB	53	95	FFPE	qRT-PCR	8	Go 2015
miR-28-3p	Altu ZGB	32	27	FFPE	qRT-PCR/array	377	Iqbal 2015
miR-28-5p	Altu ZGB						
miR-331-5p	Altu ZGB						
miR-589	Altu ZGB						
miR-129-3p	Altu ZGB						
miR-597	Altu ZGB						
miR-542-3p	Baxu ZGB						
miR-155	Baxu ZGB						
miR-1270	Altu ZGB	41	30	FF	sekuentziazioa	miRNAome	Lim 2015
miR-129-1-3p	Altu ZGB						
miR-129-2-3p	Altu ZGB						
miR-129-5p	Altu ZGB						
miR-138-1-3p	Altu ZGB						
miR-138-5p	Altu ZGB						
miR-151a-3p	Altu ZGB						
miR-151b	Altu ZGB						
miR-181a-5p	Altu ZGB						
miR-196b-5p	Altu ZGB						
miR-210-3p	Altu ZGB						
miR-28-3p	Altu ZGB						
miR-28-5p	Altu ZGB						
miR-301a-5p	Altu ZGB						
miR-3074-5p	Altu ZGB						
miR-30e-3p	Altu ZGB						
miR-3150b-3p	Altu ZGB						
miR-331-3p	Altu ZGB						
miR-339-3p	Altu ZGB						
miR-3681-5p	Altu ZGB						
miR-3934-3p	Altu ZGB						
miR-423-3p	Altu ZGB						
miR-4746-5p	Altu ZGB						
miR-582-3p	Altu ZGB						
miR-582-5p	Altu ZGB						
miR-5989-3p	Altu ZGB						

2. eranskin taula: Literaturan BZHLH azpimotetan modu esangarrian adierazitako mikroRNAk (Jarraipena).

MikroRNA esangarriak	Emaitza	n ZGB	n non-ZGB	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-664a-3p	Altu ZGB						
miR-744-5p	Altu ZGB						
miR-10397-5p	Baxu ZGB						
miR-106a-5p	Baxu ZGB						
miR-10b-5p	Baxu ZGB						
miR-148a-5p	Baxu ZGB						
miR-155-5p	Baxu ZGB						
miR-17-5p	Baxu ZGB						
miR-20a-5p	Baxu ZGB						
miR-21-3p	Baxu ZGB						
miR-221-3p	Baxu ZGB						
miR-222-3p	Baxu ZGB						
miR-222-5p	Baxu ZGB	41	30	FF	sekuentziazioa	miRNAome	Lim 2015
miR-29b-1-5p	Baxu ZGB						
miR-30b-3p	Baxu ZGB						
miR-30d-3p	Baxu ZGB						
miR-320a	Baxu ZGB						
miR-363-3p	Baxu ZGB						
miR-424-5p	Baxu ZGB						
miR-503-5p	Baxu ZGB						
miR-625-3p	Baxu ZGB						
miR-625-5p	Baxu ZGB						
miR-92a-a-5p	Baxu ZGB						
NOVELM00288	Baxu ZGB						
0	-	140	118	FFPE	qRT-PCR	1	Ni 2015
miR-199a **miR-497**	Altu ZGB Altu ZGB	36	17	FF	qRT-PCR	11	Troppan 2015
miR-320d	Baxu ZGB	47	59	FFPE	qRT-PCR	2	Wu 2014
miR-155 **miR-146a**	Baxu ZGB Baxu ZGB	20	34	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
miR-155	Baxu ZGB	36	31	FF	qRT-PCR	1	Huang 2012
0	-	10	8	FF	qRT-PCR	2	Kim 2012

2. eranskin taula: Literaturan BZHLH azpimotetan modu esangarrian adierazitako mikroRNAk (Jarraipena).

MikroRNA esangarriak	Emaitza	n ZGB	n non-ZGB	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-155	Baxu ZGB	21	69	FFPE	qRT-PCR	2	Zhong 2012
miR-146a	Baxu ZGB						
miR-331	Altu ZGB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno 2011
miR-151	Altu ZGB						
miR-28	Altu ZGB						
miR-454-3p	Altu ZGB						
miR-222	Baxu ZGB						
miR-144	Baxu ZGB						
miR-451	Baxu ZGB						
miR-221	Baxu ZGB						
0	-	8	17	FFPE	qRT-PCR	3	Nie 2010
miR-129	Altu ZGB	32	28	FFPE	Array	464	Lawrie 2009
miR-138	Altu ZGB						
miR-199b	Altu ZGB						
miR-421	Altu ZGB						
miR-520h	Altu ZGB						
miR-569	Altu ZGB						
miR-616	Altu ZGB						
miR-620	Altu ZGB						
miR-653	Altu ZGB						
miR-132	Baxu ZGB						
miR-146b	Baxu ZGB						
miR-155	Baxu ZGB						
miR-186	Baxu ZGB						
miR-190	Baxu ZGB						
miR-194	Baxu ZGB						
miR-21	Baxu ZGB						
miR-213	Baxu ZGB						
miR-221	Baxu ZGB						
miR-222	Baxu ZGB						
miR-301a-5p	Baxu ZGB						
miR-30d	Baxu ZGB						
miR-340	Baxu ZGB						
miR-363	Baxu ZGB						
miR-422b	Baxu ZGB						
miR-518a	Baxu ZGB						

2. eranskin taula: Literaturan BZHLH azpimotetan modu esangarrian adierazitako mikroRNAk (Jarraipena).

MikroRNA esangarriak	Emaitza	n ZGB	n non-ZGB	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-660	Baxu ZGB	32	28	FFPE	Array	464	Lawrie 2009
miR-106b	NA	20	20	Ehuna	Array	113	Zhang 2009
miR-140-3p	NA						
miR-142-3p	NA						
miR-142-5p	NA						
miR-151-5p	NA						
miR-16	NA						
miR-184	NA						
miR-191	NA						
miR-19a	NA						
miR-19b	NA						
miR-20a	NA						
miR-28-5p	NA						
miR-299-5p	NA						
miR-30c	NA						
miR-30e	NA						
miR-32	NA						
miR-526b	NA						
miR-583	NA						
miR-129	NA	25	25	FFPE	qRT-PCR	157	Roehle 2008
miR-133a	NA						
miR-133b	NA						
miR-138	NA						
miR-151	NA						
miR-155	NA						
miR-199b	NA						
miR-27b	NA						
miR-155	Baxu ZGB	16	18	FF and FFPE	qRT-PCR	3	Lawrie 2007
miR-21	Baxu ZGB						
miR-221	Baxu ZGB						
miR-155	Baxu ZGB	4	19	FF	Semi. RT-PCR	1	Eis 2005

Laburdurak: ZGB: zentro germinaleko B zelula mota; ez-ZGB: aktibatutako B zelula mota; FF: izoztutako ehuna; FFPE: formalinan finkatutako eta parafinan sartutako ehuna

3. eranskin taula: Literaturan R-CHOP tratamenduari emandako erantzun on edo txarrarekin modu esangarrian erlazionatutako mikroRNAk.

MikroRNA esangarriak	Emaitza	n BZHLH	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-27	FR	201	Ehuna	nanostring	800	Jia 2017
miR-34a	FR	62	FF	qRT-PCR	1	Marques 2016
0	-	22	Biopsia	qRT-PCR	1	Huskova 2015
miR-224	FR	258	FFPE	qRT-PCR	1	Ni 2015
miR-146	UFR					

Laburdurak: UFR: erantzun ez onuragarria; FR: erantzun onuragarria; FF: izoztutako ehuna; FFPE: formalinan finkatutako eta parafinan sartutako ehuna

4. eranskin taula: Literaturan pronostikoarekin modu esangarrian erlazionatutako mikroRNAk.

MikroRNA esangarriak	Emaitza	n BZHLH	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-222-3p	Altu: ↓ OS (ez-ZGB)	74 Non ZGB	Ehuna	qRT-PCR	1	Shanshan 2019
miR-155	Baxu: ↓ OS eta PFS (ZGB)	73	Ehuna	qRT-PCR	1	Due 2019
miR-101	Baxu: biziraupen baxuagoa	72	FF	qRT-PCR	1	Huang 2019
miR-197	Baxu: ↓ PFS	51	FFPE	qRT-PCR	array	Yang 2018
miR-155	Altu: ↓ PFS	82	FFPE	qRT-PCR	array	Wu 2018
miR-101-3p	NS	100	FFPE	qRT-PCR	1	Cui 2017
miR-27b	Baxu: ↓ OS	202	Ehuna	qRT-PCR	1	Jia 2017
miR-34a	Altu: ↑ OS	62	FF	qRT-PCR	1	Marques 2016
miR-155	Altu: biziraupen baxuagoa	118	FF	qRT-PCR	1	Zhu 2016
miR-23a	Altu: ↓ OS	104	FFPE	qRT-PCR	1	Wang 2014
miR-155 **miR-16** **miR-363** **miR-24**	Altu: ↓ OS Altu: ↓ OS Altu: ↓ OS Altu: ↑ OS	79	FFPE	qRT-PCR	8	Iqbal 2015
miR-214-5p **miR-28-5p** **miR-324-5p** **miR-339-3p** **miR-5586-5p** **NOVELM00203M**	Altu: ↑ OS eta EFS Altu: ↑ OS eta EFS Altu: ↓ OS eta PFS Altu: ↑ OS eta EFS Altu: ↑ OS eta EFS Altu: ↓ OS eta PFS	92	FF	sekuentziazioa	miRNAome	Lim 2015
miR-224	Altu: ↑ OS eta PFS	258	FFPE	qRT-PCR	1	Ni 2015
miR-17-5p **miR-19-3p** **miR-20a-5p** **miR-106a-5p** **miR-150-5p** **miR-342-3p** **miR-181a-5p** **miR-140-3p**	Altu: pronostiko txarra Altu: pronostiko txarra Altu: pronostiko txarra Altu: pronostiko txarra Baxu: pronostiko txarra Baxu: pronostiko txarra Baxu: pronostiko txarra Baxu: pronostiko txarra	83	FFPE	qRT-PCR/array	±900	Shepshelovich 2015
miR-199a **miR-497**	Altu: ↑ OS eta DFS Altu: ↑ OS eta DFS	58	FF	qRT-PCR	11	Troppan 2015
miR-17-92 **miR-21**	Altu: ↓ OS eta PFS Altu: ↓ OS eta PFS	200	FFPE	qRT-PCR	3	Go 2015
miR-146b-5p **miR-320d**	Baxu: ↓ PFS Baxu: ↓ PFS eta OS	12 (DC); 106 (VC)	FFPE	qRT-PCR	939	Wu 2014

4. eranskin taula: Literaturan pronostikoarekin modu esangarrian erlazionatutako mikroRNAk (Jarraipena).

MikroRNA esangarriak	Emaitza	n BZHLH	Lagin iturria	Metodoa	MikroRNA znbk	Erreferentzia
miR-200c	Altu: ↓ OS	61	Ehuna	qRT-PCR	1	Berglund 2013
miR-146a **miR-155**	Baxu: ↑ PFS Altu: ↑ PFS	90	FFPE	qRT-PCR	2	Zhong 2012
miR-181 **miR-18a** **miR-222**	Baxu: ↑ PFS Altu: ↓ OS Altu: ↓ OS	176	FFPE	qRT-PCR	11	Alencar 2011
miR-221 **miR-331** **miR-222** **miR-93** **miR-148a** **miR-151** **miR-28-5p** **miR-451** **miR-491**	Altu: ↑ OS eta PFS Altu: ↑ OS eta PFS Altu: ↓ OS eta PFS Altu: ↓ OS eta PFS NA NA NA NA NA	36/240	FFPE	qRT-PCR/array	470/9	Montes-Moreno 2011
miR-100 **miR-199a** **miR-199b** **miR-23a** **miR-24** **miR-27a** **miR-30e** **miR-330** **miR-425** **miR-302** **miR-608** **miR-637** **miR-222** **miR-let-7g** **miR-195** **miR-19a** **miR-21** **miR-23a** **miR-27a** **miR-34a** **miR-127**	Altu: ↑ EFS Altu: ↑ EFS Altu: ↑ EFS Altu: ↑ EFS Altu: ↑ EFS Altu: ↑ EFS Altu: ↑ EFS Altu: ↑ EFS Altu: ↑ EFS Altu: ↓ OS eta PFS Altu: ↓ EFS Altu: ↓ EFS Altu: ↓ OS eta PFS Baxu: ↑ EFS Baxu: ↑ EFS Baxu: ↑ OS Baxu: ↑ OS Baxu: ↑ OS Baxu: ↑ OS Baxu: ↑ OS Altu: ↓ OS eta PFS	64 106 58	FFPE FFPE Biopsia	Array qRT-PCR qRT-PCR	464 3 157	Lawrie 2009 Malumbres 2009 Roehle 2008
miR-21	Altu: RFS	35	FF eta FFPE	qRT-PCR	3	Lawrie 2007

Laburdurak: FF: izoztutako ehuna; FFPE: formalinan finkatutako eta parafinan sartutako ehuna; OS: biziraupen globala; PFS: aurrerapenik gabeko biziraupena; EFS: gertaera gabeko biziraupena; RFS: berriz gaixotze gabeko biziraupena.

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 "fold change" >0).

MikroRNA	Ids	"BaseMean"	Log2 "Fold Change"	p-balioa	padj
hsa-miR-210-3p	MIMAT0000267	843,0	3,51	$2{,}33\times10^{-29}$	$1{,}45\times10^{-26}$
hsa-miR-944	MIMAT0004987	60,9	4,10	$2{,}19\times10^{-23}$	$6{,}80\times10^{-21}$
hsa-miR-12136	MIMAT0049032	76,1	26,94	$3{,}64\times10^{-20}$	$6{,}47\times10^{-18}$
hsa-miR-3681-5p	MIMAT0018108	75,3	5,16	$3{,}33\times10^{-20}$	$6{,}47\times10^{-18}$
hsa-miR-425-3p	MIMAT0001343	152,2	1,60	$1{,}04\times10^{-17}$	$1{,}62\times10^{-15}$
hsa-miR-378i	MIMAT0019074	25,0	3,01	$3{,}01\times10^{-17}$	$4{,}16\times10^{-15}$
hsa-miR-106b-3p	MIMAT0004672	1394,7	1,32	$3{,}80\times10^{-17}$	$4{,}73\times10^{-15}$
hsa-miR-4454	MIMAT0018976	183,5	2,35	$1{,}01\times10^{-16}$	$1{,}04\times10^{-14}$
hsa-miR-1291	MIMAT0005881	354,8	4,02	$1{,}84\times10^{-16}$	$1{,}76\times10^{-14}$
hsa-miR-7974	MIMAT0031177	111,8	3,46	$1{,}87\times10^{-15}$	$1{,}45\times10^{-13}$
hsa-miR-183-5p	MIMAT0000261	891,2	3,40	$5{,}77\times10^{-15}$	$3{,}59\times10^{-13}$
hsa-miR-146a-5p	MIMAT0000449	33085,6	2,11	$2{,}05\times10^{-14}$	$1{,}16\times10^{-12}$
hsa-miR-2467-5p	MIMAT0019952	25,6	2,33	$7{,}35\times10^{-14}$	$3{,}81\times10^{-12}$
hsa-miR-4420	MIMAT0018933	8,1	4,63	$2{,}02\times10^{-13}$	$1{,}01\times10^{-11}$
hsa-miR-19a-3p	MIMAT0000073	1660,1	1,77	$7{,}91\times10^{-13}$	$3{,}79\times10^{-11}$
hsa-miR-1248	MIMAT0005900	202,5	2,69	$1{,}03\times10^{-12}$	$4{,}73\times10^{-11}$
hsa-miR-18a-3p	MIMAT0002891	54,1	2,05	$1{,}21\times10^{-12}$	$5{,}36\times10^{-11}$
hsa-miR-21-3p	MIMAT0004494	6544,0	1,86	$1{,}90\times10^{-12}$	$7{,}88\times10^{-11}$
hsa-miR-129a-5p	MIMAT0000242_1	25,1	4,62	$3{,}03\times10^{-12}$	$1{,}22\times10^{-10}$
hsa-miR-147b-3p	MIMAT0004928	92,6	3,41	$3{,}78\times10^{-12}$	$1{,}47\times10^{-10}$
hsa-miR-3691-5p	MIMAT0018120	6,6	2,59	$1{,}09\times10^{-11}$	$3{,}88\times10^{-10}$
hsa-miR-128-1-5p	MIMAT0026477	16,5	1,38	$1{,}90\times10^{-11}$	$6{,}55\times10^{-10}$
hsa-miR-1246	MIMAT0005898	4,6	4,47	$2{,}64\times10^{-11}$	$8{,}86\times10^{-10}$
hsa-miR-205-5p	MIMAT0000266	67,2	6,11	$3{,}81\times10^{-11}$	$1{,}22\times10^{-09}$
hsa-miR-769-3p	MIMAT0003887	21,6	2,53	$4{,}00\times10^{-11}$	$1{,}25\times10^{-09}$
hsa-miR-10395-5p	MIMAT0041621	120,1	2,24	$4{,}74\times10^{-11}$	$1{,}44\times10^{-09}$
hsa-miR-3917	MIMAT0018191	7,5	2,67	$1{,}05\times10^{-10}$	$3{,}10\times10^{-09}$
hsa-miR-4424	MIMAT0018939	110,8	4,39	$1{,}29\times10^{-10}$	$3{,}72\times10^{-09}$
hsa-miR-7706	MIMAT0030021	100,7	2,22	$2{,}11\times10^{-10}$	$5{,}97\times10^{-09}$
hsa-miR-4449	MIMAT0018968	77,2	1,99	$2{,}52\times10^{-10}$	$6{,}96\times10^{-09}$
hsa-miR-210-5p	MIMAT0026475	9,4	2,51	$5{,}01\times10^{-10}$	$1{,}30\times10^{-08}$
hsa-miR-19a-5p	MIMAT0004490	20,6	2,30	$6{,}23\times10^{-10}$	$1{,}55\times10^{-08}$
hsa-miR-7-1-5p	MIMAT0000252_2	87,2	1,92	$6{,}17\times10^{-10}$	$1{,}55\times10^{-08}$
hsa-miR-182-5p	MIMAT0000259	4036,2	2,63	$6{,}65\times10^{-10}$	$1{,}60\times10^{-08}$
hsa-miR-320a-5p	MIMAT0037311	16,4	3,77	$6{,}97\times10^{-10}$	$1{,}64\times10^{-08}$
hsa-miR-155-5p	MIMAT0000646	20773,1	1,99	$7{,}26\times10^{-10}$	$1{,}67\times10^{-08}$
hsa-miR-1307-3p	MIMAT0005951	443,6	1,61	$1{,}56\times10^{-09}$	$3{,}34\times10^{-08}$

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 “fold change” >0) (Jarraipena).

MikroRNA	Ids	“Base Mean”	“Log2 Fold Change”	p-balioa	padj
hsa-miR-1843	MIMAT0039764	78,3	1,49	$2,03x10^{-09}$	$4,22x10^{-08}$
hsa-miR-34a-5p	MIMAT0000255	759,4	1,11	$2,32x10^{-09}$	$4,72x10^{-08}$
hsa-miR-191-3p	MIMAT0001618	22,5	2,26	$2,57x10^{-09}$	$5,16x10^{-08}$
hsa-miR-185-3p	MIMAT0004611	41,1	2,21	$2,94x10^{-09}$	$5,78x10^{-08}$
hsa-miR-4518	MIMAT0019055	11,7	2,25	$3,33x10^{-09}$	$6,37x10^{-08}$
hsa-miR-3609	MIMAT0017986	56,4	2,31	$4,58x10^{-09}$	$8,32x10^{-08}$
hsa-miR-629-5p	MIMAT0004810	91,4	1,58	$4,62x10^{-09}$	$8,32x10^{-08}$
hsa-miR-3922-3p	MIMAT0018197	17,2	2,73	$7,13x10^{-09}$	$1,27x10^{-07}$
hsa-miR-9-1-5p	MIMAT0000441	128,0	3,32	$9,08x10^{-09}$	$1,59x10^{-07}$
hsa-miR-141-5p	MIMAT0004598	20,2	3,65	$1,60x10^{-08}$	$2,65x10^{-07}$
hsa-miR-19b-1-5p	MIMAT0004491	12,6	2,08	$1,60x10^{-08}$	$2,65x10^{-07}$
hsa-miR-3651	MIMAT0018071	63,2	1,88	$1,81x10^{-08}$	$2,96x10^{-07}$
hsa-miR-3176	MIMAT0015053	14,2	1,85	$1,90x10^{-08}$	$3,07x10^{-07}$
hsa-miR-130b-3p	MIMAT0000691	381,5	1,37	$4,01x10^{-08}$	$6,23x10^{-07}$
hsa-miR-3620-5p	MIMAT0022967	4,8	3,65	$5,74x10^{-08}$	$8,81x10^{-07}$
hsa-miR-92a-1-5p	MIMAT0004507	43,9	1,75	$6,64x10^{-08}$	$1,01x10^{-06}$
hsa-miR-1307-5p	MIMAT0022727	4232,8	2,31	$7,57x10^{-08}$	$1,13x10^{-06}$
hsa-miR-330-5p	MIMAT0004693	126,3	1,52	$7,71x10^{-08}$	$1,14x10^{-06}$
hsa-miR-5100	MIMAT0022259	153,9	2,32	$1,20x10^{-07}$	$1,74x10^{-06}$
hsa-miR-16-1-3p	MIMAT0004489	13,0	1,29	$1,22x10^{-07}$	$1,74x10^{-06}$
hsa-miR-10392-5p	MIMAT0041615	28,4	2,00	$1,51x10^{-07}$	$2,14x10^{-06}$
hsa-miR-3614-5p	MIMAT0017992	14,7	2,28	$1,73x10^{-07}$	$2,42x10^{-06}$
hsa-miR-3150b-3p	MIMAT0018194	50,7	2,38	$1,84x10^{-07}$	$2,52x10^{-06}$
hsa-miR-3677-3p	MIMAT0018101	13,6	2,27	$1,83x10^{-07}$	$2,52x10^{-06}$
hsa-miR-296-3p	MIMAT0004679	26,7	2,39	$2,02x10^{-07}$	$2,73x10^{-06}$
hsa-miR-1304-3p	MIMAT0022720	36,2	1,41	$2,05x10^{-07}$	$2,74x10^{-06}$
hsa-miR-208b-3p	MIMAT0004960	8,0	4,29	$2,66x10^{-07}$	$3,45x10^{-06}$
hsa-miR-769-5p	MIMAT0003886	2112,0	1,64	$2,80x10^{-07}$	$3,59x10^{-06}$
hsa-miR-9-1-3p	MIMAT0000442	8,1	3,14	$3,05x10^{-07}$	$3,87x10^{-06}$
hsa-miR-146a-3p	MIMAT0004608	27,7	2,13	$3,12x10^{-07}$	$3,92x10^{-06}$
hsa-miR-130b-5p	MIMAT0004680	91,9	1,28	$4,43x10^{-07}$	$5,46x10^{-06}$
hsa-miR-103a-2-5p	MIMAT0009196	8,7	1,08	$4,80x10^{-07}$	$5,86x10^{-06}$
hsa-miR-548k	MIMAT0005882	79,1	1,24	$5,14x10^{-07}$	$6,15x10^{-06}$
hsa-miR-3610	MIMAT0017987	26,0	2,95	$5,37x10^{-07}$	$6,19x10^{-06}$
hsa-miR-10394-3p	MIMAT0041620	13,2	2,92	$5,37x10^{-07}$	$6,19x10^{-06}$
hsa-miR-4775	MIMAT0019931	7,2	1,41	$5,24x10^{-07}$	$6,19x10^{-06}$
hsa-miR-17-3p	MIMAT0000071	334,1	1,14	$5,34x10^{-07}$	$6,19x10^{-06}$

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 "fold change" >0) (Jarraipena).

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-181a-3p	MIMAT0000270	1153.4	1.35	$6.56x10^{-07}$	$7.42x10^{-06}$
hsa-miR-4521	MIMAT0019058	106.9	1.85	$7.20x10^{-07}$	$8.07x10^{-06}$
hsa-miR-3913-2-5p	MIMAT0018187_1	13.4	1.97	$7.97x10^{-07}$	$8.77x10^{-06}$
hsa-miR-6753-3p	MIMAT0027407	1.3	3.50	$8.61x10^{-07}$	$9.39x10^{-06}$
hsa-miR-3689f	MIMAT0019010	5.6	5.38	$1.10x10^{-06}$	$1.19x10^{-05}$
hsa-miR-378a-3p	MIMAT0000732	7208.5	1.42	$1.13x10^{-06}$	$1.21x10^{-05}$
hsa-miR-15a-3p	MIMAT0004488	4.9	1.52	$1.39x10^{-06}$	$1.48x10^{-05}$
hsa-miR-4487	MIMAT0019021	2.3	2.26	$1.60x10^{-06}$	$1.64x10^{-05}$
hsa-miR-4741	MIMAT0019871	3.9	3.00	$1.62x10^{-06}$	$1.65x10^{-05}$
hsa-miR-766-5p	MIMAT0022714	9.0	2.78	$1.67x10^{-06}$	$1.68x10^{-05}$
hsa-miR-4517	MIMAT0019054	3.5	2.28	$2.00x10^{-06}$	$1.99x10^{-05}$
hsa-miR-25-5p	MIMAT0004498	25.2	1.37	$2.08x10^{-06}$	$2.05x10^{-05}$
hsa-miR-4746-5p	MIMAT0019880	3.9	1.88	$2.14x10^{-06}$	$2.09x10^{-05}$
hsa-miR-10527-5p	MIMAT0041997	13.1	1.73	$2.32x10^{-06}$	$2.25x10^{-05}$
hsa-miR-30b-3p	MIMAT0004589	6.6	1.50	$2.38x10^{-06}$	$2.30x10^{-05}$
hsa-miR-3944-3p	MIMAT0018360	3.2	3.56	$2.61x10^{-06}$	$2.49x10^{-05}$
hsa-miR-4485-3p	MIMAT0019019	2.3	2.78	$3.14x10^{-06}$	$2.98x10^{-05}$
hsa-miR-576-3p	MIMAT0004796	18.8	1.98	$3.18x10^{-06}$	$3.00x10^{-05}$
hsa-miR-1303	MIMAT0005891	10.2	1.93	$4.24x10^{-06}$	$3.94x10^{-05}$
hsa-miR-21-5p	MIMAT0000076	140750.7	1.11	$4.37x10^{-06}$	$4.03x10^{-05}$
hsa-miR-15b-3p	MIMAT0004586	100.6	1.44	$4.61x10^{-06}$	$4.19x10^{-05}$
hsa-miR-9-2-5p	MIMAT0000441_2	118.5	3.01	$6.18x10^{-06}$	$5.49x10^{-05}$
hsa-miR-4524a-5p	MIMAT0019062	4.1	2.34	$6.15x10^{-06}$	$5.49x10^{-05}$
hsa-miR-942-5p	MIMAT0004985	25.3	1.15	$8.36x10^{-06}$	$7.27x10^{-05}$
hsa-miR-3652	MIMAT0018072	8.9	3.05	$9.54x10^{-06}$	$8.19x10^{-05}$
hsa-miR-454-5p	MIMAT0003884	12.7	0.93	$9.83x10^{-06}$	$8.37x10^{-05}$
hsa-miR-155-3p	MIMAT0004658	5.5	2.19	$1.09x10^{-05}$	$9.20x10^{-05}$
hsa-miR-939-5p	MIMAT0004982	4.1	2.27	$1.23x10^{-05}$	0.00010
hsa-miR-4690-5p	MIMAT0019779	2.1	3.85	$1.31x10^{-05}$	0.00011
hsa-miR-3913-1-5p	MIMAT0018187	12.1	1.79	$1.34x10^{-05}$	0.00011
hsa-miR-96-5p	MIMAT0000095	90.2	1.86	$1.40x10^{-05}$	0.00011
hsa-miR-873-3p	MIMAT0022717	7.5	3.79	$1.58x10^{-05}$	0.00013
hsa-miR-19b-1-3p	MIMAT0000074	4447.5	1.03	$1.59x10^{-05}$	0.00013
hsa-miR-1296-3p	MIMAT0026637	3.6	2.35	$1.79x10^{-05}$	0.00014
hsa-miR-3681-3p	MIMAT0018109	1.3	3.63	$2.06x10^{-05}$	0.00016
hsa-miR-18a-5p	MIMAT0000072	359.9	1.55	$2.56x10^{-05}$	0.00019
hsa-miR-151a-3p	MIMAT0000757	4394.7	0.82	$2.61x10^{-05}$	0.00019

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 “fold change” >0) (Jarraipena).

MikroRNA	Ids	“Base Mean”	“Log2 Fold Change”	p-balioa	padj
hsa-miR-503-5p	MIMAT0002874	5,9	1,43	$2{,}63\times10^{-05}$	0,00019
hsa-miR-10393-3p	MIMAT0041618	12,9	1,96	$3{,}60\times10^{-05}$	0,00026
hsa-miR-9-2-3p	MIMAT0000442_2	6,9	2,59	$3{,}73\times10^{-05}$	0,00026
hsa-miR-573	MIMAT0003238	3,2	3,34	$3{,}88\times10^{-05}$	0,00027
hsa-miR-4651	MIMAT0019715	1,5	2,64	$3{,}88\times10^{-05}$	0,00027
hsa-miR-409-3p	MIMAT0001639	244,7	1,68	$4{,}14\times10^{-05}$	0,00029
hsa-miR-7705	MIMAT0030020	13,5	1,03	$4{,}19\times10^{-05}$	0,00029
hsa-miR-3615	MIMAT0017994	274,9	1,37	$4{,}43\times10^{-05}$	0,00031
hsa-miR-589-5p	MIMAT0004799	168,8	1,03	$4{,}70\times10^{-05}$	0,00032
hsa-miR-937-3p	MIMAT0004980	7,5	2,19	$5{,}14\times10^{-05}$	0,00035
hsa-miR-188-3p	MIMAT0004613	3,6	1,20	$5{,}20\times10^{-05}$	0,00035
hsa-miR-141-3p	MIMAT0000432	1540,8	1,80	$5{,}65\times10^{-05}$	0,00038
hsa-miR-301b-3p	MIMAT0004958	99,9	1,43	$5{,}76\times10^{-05}$	0,00038
hsa-miR-7702	MIMAT0030017	2,2	2,12	$6{,}19\times10^{-05}$	0,00041
hsa-miR-33b-3p	MIMAT0004811	4,0	1,55	$6{,}22\times10^{-05}$	0,00041
hsa-miR-30d-3p	MIMAT0004551	159,0	0,90	$6{,}53\times10^{-05}$	0,00043
hsa-miR-183-3p	MIMAT0004560	3,0	2,02	$7{,}00\times10^{-05}$	0,00045
hsa-miR-4491	MIMAT0019026	3,2	1,94	$7{,}00\times10^{-05}$	0,00045
hsa-miR-671-5p	MIMAT0003880	50,7	1,71	$7{,}32\times10^{-05}$	0,00047
hsa-miR-181b-2-5p	MIMAT0000257_1	3292,3	1,00	$7{,}48\times10^{-05}$	0,00048
hsa-miR-3916	MIMAT0018190	4,2	1,62	$8{,}08\times10^{-05}$	0,00051
hsa-miR-3136-5p	MIMAT0015003	4,1	1,04	$8{,}19\times10^{-05}$	0,00051
hsa-miR-592	MIMAT0003260	5,4	1,46	$8{,}83\times10^{-05}$	0,00055
hsa-miR-3195	MIMAT0015079	6,4	4,35	$9{,}65\times10^{-05}$	0,00060
hsa-miR-9901	MIMAT0039321	21,8	5,88	0,00010	0,00064
hsa-miR-6852-5p	MIMAT0027604	2,5	1,30	0,00010	0,00064
hsa-miR-9-3-3p	MIMAT0000442_1	7,0	2,73	0,00011	0,00067
hsa-miR-424-3p	MIMAT0004749	27,9	1,13	0,00011	0,00068
hsa-miR-181d-5p	MIMAT0002821	233,5	0,77	0,00011	0,00068
hsa-miR-25-3p	MIMAT0000081	9225,6	0,80	0,00012	0,00074
hsa-miR-548e-5p	MIMAT0026736	17,8	0,83	0,00014	0,00080
hsa-miR-129b-2-5p	MIMAT0000242	19,0	2,62	0,00014	0,00084
hsa-let-7i-5p	MIMAT0000415	22108,6	0,56	0,00015	0,00087
hsa-miR-181b-3p	MIMAT0022692	11,9	1,19	0,00015	0,00090
hsa-miR-548j-5p	MIMAT0005875	5,3	1,22	0,00016	0,00092
hsa-miR-7977	MIMAT0031180	4065,0	1,39	0,00016	0,00094
hsa-miR-4677-3p	MIMAT0019761	28,0	0,60	0,00016	0,00094

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 “fold change” >0) (Jarraipena).

MikroRNA	Ids	“Base Mean”	“Log2 Fold Change”	p-balioa	padj
hsa-miR-598-5p	MIMAT0026620	1,2	3,21	0,00020	0,00113
hsa-miR-129-1-3p	MIMAT0004548	7,4	2,31	0,00021	0,00121
hsa-miR-2278	MIMAT0011778	1,3	1,88	0,00023	0,00127
hsa-miR-940	MIMAT0004983	9,9	0,94	0,00024	0,00136
hsa-miR-4523	MIMAT0019061	3,1	2,75	0,00029	0,00161
hsa-miR-9-3-5p	MIMAT0000441_1	91,3	2,57	0,00029	0,00162
hsa-miR-219a-1-3p	MIMAT0004567	4,0	1,58	0,00029	0,00162
hsa-miR-760	MIMAT0004957	5,8	1,43	0,00029	0,00162
hsa-miR-3150a-3p	MIMAT0015023	2,0	2,50	0,00030	0,00164
hsa-miR-6762-3p	MIMAT0027425	1,8	2,11	0,00031	0,00170
hsa-miR-3659	MIMAT0018080	1,2	3,77	0,00033	0,00178
hsa-miR-365a-5p	MIMAT0009199	4,3	1,43	0,00034	0,00182
hsa-miR-1976	MIMAT0009451	13,1	0,97	0,00034	0,00182
hsa-miR-4513	MIMAT0019050	2,7	3,20	0,00035	0,00183
hsa-miR-3174	MIMAT0015051	1,6	1,65	0,00035	0,00185
hsa-miR-423-3p	MIMAT0001340	2454,7	0,87	0,00037	0,00191
hsa-miR-4645-3p	MIMAT0019706	7,7	0,84	0,00037	0,00191
hsa-miR-4745-5p	MIMAT0019878	0,7	2,68	0,00038	0,00194
hsa-miR-30c-1-3p	MIMAT0004674	36,4	0,94	0,00039	0,00199
hsa-miR-6842-3p	MIMAT0027587	28,5	0,81	0,00041	0,00211
hsa-miR-612	MIMAT0003280	0,9	2,86	0,00043	0,00218
hsa-miR-127-3p	MIMAT0000446	3977,2	1,54	0,00045	0,00226
hsa-miR-1538	MIMAT0007400	7,3	1,72	0,00046	0,00231
hsa-miR-3144-3p	MIMAT0015015	2,8	3,18	0,00048	0,00240
hsa-miR-4677-5p	MIMAT0019760	2,0	2,06	0,00049	0,00246
hsa-miR-7703	MIMAT0030018	1,5	1,29	0,00050	0,00249
hsa-miR-6751-3p	MIMAT0027403	1,0	2,46	0,00050	0,00250
hsa-miR-873-5p	MIMAT0004953	33,4	2,07	0,00052	0,00259
hsa-miR-19b-2-3p	MIMAT0000074_1	1793,7	1,48	0,00054	0,00264
hsa-miR-548b-5p	MIMAT0004798	5,6	1,98	0,00056	0,00272
hsa-miR-6516-3p	MIMAT0030418	71,5	1,49	0,00059	0,00283
hsa-miR-10397-5p	MIMAT0041625	6,8	0,97	0,00059	0,00283
hsa-miR-3158-1-3p	MIMAT0015032	5,5	1,05	0,00061	0,00293
hsa-miR-4515	MIMAT0019052	0,7	2,36	0,00062	0,00293
hsa-miR-4802-5p	MIMAT0019981	0,9	2,28	0,00062	0,00293
hsa-miR-7-2-5p	MIMAT0000252	3,2	2,79	0,00063	0,00296
hsa-miR-4444-1	MIMAT0018962	10,3	1,85	0,00063	0,00296

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 “fold change” >0) (Jarraipena).

MikroRNA	Ids	“Base Mean”	“Log2 Fold Change”	p-balioa	padj
hsa-miR-3934-5p	MIMAT0018349	5,7	0,83	0,00065	0,00305
hsa-miR-3928-3p	MIMAT0018205	12,2	1,56	0,00070	0,00325
hsa-miR-7704	MIMAT0030019	266,5	3,73	0,00071	0,00329
hsa-miR-1910-5p	MIMAT0007884	1,6	2,76	0,00072	0,00334
hsa-miR-582-3p	MIMAT0004797	68,7	1,27	0,00073	0,00336
hsa-miR-4766-3p	MIMAT0019918	2,2	1,77	0,00074	0,00342
hsa-miR-30d-5p	MIMAT0000245	17897,8	0,67	0,00075	0,00342
hsa-miR-4767	MIMAT0019919	10,4	1,24	0,00078	0,00354
hsa-miR-3940-5p	MIMAT0019229	1,9	2,53	0,00084	0,00376
hsa-miR-6839-5p	MIMAT0027580	0,9	2,52	0,00084	0,00376
hsa-miR-148a-5p	MIMAT0004549	431,0	1,15	0,00085	0,00381
hsa-miR-664b-5p	MIMAT0022271	4,3	1,44	0,00093	0,00411
hsa-miR-548l	MIMAT0005889	2,1	1,26	0,00093	0,00412
hsa-miR-92a-3p	MIMAT0000092	52252,0	0,85	0,00100	0,00441
hsa-miR-4444-2	MIMAT0018962_1	11,4	1,88	0,00105	0,00459
hsa-miR-6850-3p	MIMAT0027601	0,7	2,76	0,00106	0,00462
hsa-miR-449a	MIMAT0001541	12,7	1,43	0,00107	0,00465
hsa-miR-146b-5p	MIMAT0002809	15631,5	0,96	0,00107	0,00465
hsa-miR-3678-5p	MIMAT0018102	0,9	1,85	0,00110	0,00474
hsa-miR-181b-1-5p	MIMAT0000257	495,1	1,00	0,00110	0,00474
hsa-miR-135b-5p	MIMAT0000758	8,6	1,38	0,00113	0,00484
hsa-miR-5009-5p	MIMAT0021041	0,5	2,34	0,00114	0,00489
hsa-miR-1270	MIMAT0005924	5,0	1,15	0,00117	0,00498
hsa-miR-378a-5p	MIMAT0000731	103,4	0,81	0,00121	0,00512
hsa-miR-6515-5p	MIMAT0025486	1,4	2,36	0,00123	0,00518
hsa-miR-3611	MIMAT0017988	8,3	1,68	0,00127	0,00535
hsa-miR-370-3p	MIMAT0000722	16,1	1,95	0,00129	0,00541
hsa-miR-17-5p	MIMAT0000070	1127,3	1,20	0,00130	0,00542
hsa-miR-324-3p	MIMAT0000762	74,5	0,60	0,00133	0,00551
hsa-miR-4668-5p	MIMAT0019745	4,4	2,47	0,00139	0,00573
hsa-miR-10394-5p	MIMAT0041619	2,6	2,54	0,00151	0,00615
hsa-miR-103a-1-5p	MIMAT0037306	0,9	1,74	0,00151	0,00615
hsa-miR-6818-3p	MIMAT0027537	2,1	1,93	0,00154	0,00628
hsa-miR-1226-5p	MIMAT0005576	0,5	2,43	0,00158	0,00640
hsa-miR-20a-5p	MIMAT0000075	1664,1	1,19	0,00159	0,00642
hsa-miR-1295a	MIMAT0005885	4,9	1,85	0,00188	0,00743
hsa-miR-4529-3p	MIMAT0019068	1,2	2,44	0,00195	0,00766

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 “fold change” >0) (Jarraipena).

MikroRNA	Ids	“BaseMean”	“Log2 Fold Change”	p-balioa	padj
hsa-miR-3064-5p	MIMAT0019864	2,2	1,47	0,00196	0,00770
hsa-miR-597-5p	MIMAT0003265	2,9	1,47	0,00218	0,00846
hsa-miR-219b-3p	MIMAT0019748	1,3	1,53	0,00225	0,00868
hsa-miR-7111-3p	MIMAT0028120	1,4	2,35	0,00232	0,00885
hsa-miR-632	MIMAT0003302	2,2	1,94	0,00251	0,00948
hsa-miR-4524a-3p	MIMAT0019063	1,1	1,83	0,00251	0,00948
hsa-miR-324-5p	MIMAT0000761	63,5	0,49	0,00281	0,01047
hsa-miR-6821-3p	MIMAT0027543	1,7	1,61	0,00328	0,01214
hsa-miR-542-3p	MIMAT0003389	36,7	0,73	0,00342	0,01258
hsa-miR-4687-3p	MIMAT0019775	0,7	2,10	0,00346	0,01266
hsa-miR-6840-5p	MIMAT0027582	1,0	2,15	0,00356	0,01300
hsa-miR-12135	MIMAT0049031	14,0	3,48	0,00372	0,01351
hsa-miR-5001-5p	MIMAT0021021	2,3	1,20	0,00371	0,01351
hsa-miR-219a-5p	MIMAT0000276	14,3	0,75	0,00400	0,01440
hsa-miR-138-1-3p	MIMAT0004607	18,2	1,63	0,00403	0,01445
hsa-miR-421	MIMAT0003339	186,1	0,73	0,00407	0,01453
hsa-miR-1286	MIMAT0005877	0,8	2,39	0,00410	0,01463
hsa-miR-193b-5p	MIMAT0004767	9,9	1,02	0,00412	0,01464
hsa-miR-182-3p	MIMAT0000260	1,0	2,16	0,00419	0,01481
hsa-miR-2861	MIMAT0013802	8,0	5,40	0,00428	0,01502
hsa-miR-100-3p	MIMAT0004512	9,8	0,95	0,00430	0,01506
hsa-miR-3181	MIMAT0015061	2,1	1,94	0,00433	0,01514
hsa-miR-425-5p	MIMAT0003393	1019,7	0,61	0,00438	0,01526
hsa-miR-663a	MIMAT0003326	42,3	3,20	0,00452	0,01570
hsa-miR-3661	MIMAT0018082	1,1	1,30	0,00470	0,01626
hsa-miR-3198	MIMAT0015083	1,3	3,43	0,00482	0,01657
hsa-miR-7848-3p	MIMAT0030423	0,5	2,30	0,00494	0,01681
hsa-miR-5094	MIMAT0021086	1,0	1,60	0,00495	0,01681
hsa-miR-4707-5p	MIMAT0019807	0,6	2,48	0,00549	0,01830
hsa-miR-3158-2-3p	MIMAT0015032_1	5,3	0,90	0,00548	0,01830
hsa-miR-191-5p	MIMAT0000440	31969,0	0,89	0,00549	0,01830
hsa-miR-135a-5p	MIMAT0000428	0,7	3,68	0,00583	0,01926
hsa-miR-3187-3p	MIMAT0015069	1,2	1,14	0,00594	0,01953
hsa-miR-331-5p	MIMAT0004700	18,7	0,74	0,00599	0,01961
hsa-miR-2355-3p	MIMAT0017950	6,3	1,15	0,00645	0,02094
hsa-miR-33a-5p	MIMAT0000091	277,1	0,77	0,00644	0,02094
hsa-miR-491-5p	MIMAT0002807	3,6	1,00	0,00716	0,02294

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 “fold change” >0) (Jarraipena).

MikroRNA	Ids	“BaseMean”	“Log2 Fold Change”	p-balioa	padj
hsa-miR-545-5p	MIMAT0004785	3,6	0,85	0,00730	0,02333
hsa-miR-4421	MIMAT0018934	1,3	2,28	0,00734	0,02343
hsa-miR-454-3p	MIMAT0003885	318,1	0,80	0,00767	0,02438
hsa-miR-6502-5p	MIMAT0025460	2,4	1,19	0,00792	0,02507
hsa-miR-625-3p	MIMAT0004808	41,4	0,71	0,00853	0,02694
hsa-miR-4539	MIMAT0019082	0,8	3,44	0,00866	0,02727
hsa-miR-1243	MIMAT0005894	0,4	2,20	0,00878	0,02759
hsa-miR-6854-5p	MIMAT0027608	0,5	1,97	0,00891	0,02786
hsa-miR-501-5p	MIMAT0002872	33,9	0,86	0,00908	0,02831
hsa-miR-6764-3p	MIMAT0027429	0,5	1,80	0,00959	0,02983
hsa-miR-431-5p	MIMAT0001625	5,3	1,21	0,00969	0,03005
hsa-miR-4703-3p	MIMAT0019802	1,2	1,96	0,00980	0,03034
hsa-miR-1306-3p	MIMAT0005950	2,2	1,27	0,01022	0,03153
hsa-miR-1229-3p	MIMAT0005584	1,1	1,13	0,01032	0,03176
hsa-miR-181a-5p	MIMAT0000256	45360,1	0,76	0,01043	0,03203
hsa-miR-2116-3p	MIMAT0011161	5,4	1,08	0,01123	0,03423
hsa-miR-147a	MIMAT0000251	0,6	3,15	0,01142	0,03474
hsa-miR-3155a	MIMAT0015029	1,4	1,13	0,01156	0,03506
hsa-miR-1181	MIMAT0005826	1,7	2,22	0,01164	0,03523
hsa-miR-5706	MIMAT0022500	0,9	1,29	0,01198	0,03617
hsa-miR-5582-3p	MIMAT0022280	0,6	1,48	0,01256	0,03766
hsa-miR-4479	MIMAT0019011	0,6	1,98	0,01282	0,03834
hsa-miR-6881-3p	MIMAT0027663	0,8	1,95	0,01302	0,03871
hsa-miR-1537-5p	MIMAT0026765	0,8	1,51	0,01301	0,03871
hsa-miR-3940-3p	MIMAT0018356	6,7	0,84	0,01304	0,03871
hsa-miR-222-5p	MIMAT0004569	7,3	1,22	0,01308	0,03875
hsa-miR-4647	MIMAT0019709	1,0	2,72	0,01370	0,04039
hsa-miR-127-5p	MIMAT0004604	30,8	1,08	0,01384	0,04071
hsa-miR-3654	MIMAT0018074	4,7	1,61	0,01397	0,04089
hsa-miR-651-5p	MIMAT0003321	23,8	0,62	0,01423	0,04155
hsa-miR-636	MIMAT0003306	2,0	0,88	0,01476	0,04279
hsa-miR-2277-5p	MIMAT0017352	9,5	0,81	0,01479	0,04279
hsa-miR-4496	MIMAT0019031	2,2	2,04	0,01504	0,04319
hsa-miR-4504	MIMAT0019040	0,4	1,87	0,01507	0,04319
hsa-miR-301a-5p	MIMAT0022696	1,7	0,93	0,01502	0,04319
hsa-miR-550a-5p	MIMAT0004800	2,1	0,97	0,01547	0,04413
hsa-miR-580-5p	MIMAT0026617	0,5	1,69	0,01566	0,04457

5. eranskin taula: Gure kohortean gain-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 “fold change” >0) (Jarraipena).

MikroRNA	Ids	“Base Mean”	“Log2 Fold Change”	p-balioa	padj
hsa-miR-580-3p	MIMAT0003245	8,5	0,50	0,01624	0,04603

6. eranskin taula: Gure kohortean azpi-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 "fold change" <0).

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-215-5p	MIMAT0000272	53,8	-4,42	$6,74x10^{-35}$	$8,39x10^{-32}$
hsa-miR-150-5p	MIMAT0000451	6310,7	-3,22	$3,46x10^{-25}$	$1,44x10^{-22}$
hsa-miR-224-5p	MIMAT0000281	47,7	-3,30	$5,11x10^{-21}$	$1,27x10^{-18}$
hsa-miR-194-1-5p	MIMAT0000460	37,8	-4,33	$6,19x10^{-17}$	$7,00x10^{-15}$
hsa-miR-452-3p	MIMAT0001636	7,2	-2,33	$2,10x10^{-16}$	$1,87x10^{-14}$
hsa-miR-335-5p	MIMAT0000765	127,6	-2,74	$6,20x10^{-16}$	$5,14x10^{-14}$
hsa-miR-145-5p	MIMAT0000437	2060,5	-2,16	$2,65x10^{-15}$	$1,94x10^{-13}$
hsa-miR-101-5p	MIMAT0004513	30,2	-1,69	$5,46x10^{-15}$	$3,57x10^{-13}$
hsa-miR-139-5p	MIMAT0000250	48,8	-2,26	$5,40x10^{-15}$	$3,57x10^{-13}$
hsa-miR-126-3p	MIMAT0000445	3182,5	-1,44	$6,54x10^{-15}$	$3,88x10^{-13}$
hsa-miR-497-5p	MIMAT0002820	332,5	-2,07	$3,53x10^{-14}$	$1,91x10^{-12}$
hsa-miR-212-5p	MIMAT0022695	14,4	-1,54	$1,60x10^{-12}$	$6,88x10^{-11}$
hsa-miR-10b-3p	MIMAT0004556	14,0	-1,60	$5,28x10^{-12}$	$1,99x10^{-10}$
hsa-miR-10a-3p	MIMAT0004555	10,2	-2,29	$7,45x10^{-12}$	$2,73x10^{-10}$
hsa-miR-95-3p	MIMAT0000094	18,1	-2,13	$3,79x10^{-11}$	$1,22x10^{-09}$
hsa-miR-125a-5p	MIMAT0000443	4941,1	-1,34	$3,92x10^{-10}$	$1,04x10^{-08}$
hsa-miR-125b-2-5p	MIMAT0000423_1	1975,9	-1,66	$3,89x10^{-10}$	$1,04x10^{-08}$
hsa-miR-151b	MIMAT0010214	24,8	-2,21	$6,67x10^{-10}$	$1,60x10^{-08}$
hsa-miR-874-5p	MIMAT0026718	10,1	-1,94	$8,02x10^{-10}$	$1,81x10^{-08}$
hsa-miR-10a-5p	MIMAT0000253	21228,4	-1,71	$1,05x10^{-10}$	$2,33x10^{-08}$
hsa-let-7c-5p	MIMAT0000064	764,1	-1,92	$1,22x10^{-09}$	$2,66x10^{-08}$
hsa-miR-504-5p	MIMAT0002875	9,1	-1,93	$1,69x10^{-09}$	$3,56x10^{-08}$
hsa-miR-551b-3p	MIMAT0003233	7,9	-2,25	$2,97x10^{-09}$	$5,78x10^{-08}$
hsa-miR-194-2-5p	MIMAT0000460_1	234,5	-2,52	$4,25x10^{-09}$	$8,01x10^{-08}$
hsa-miR-101-1-3p	MIMAT0000099	1463,8	-1,26	$4,34x10^{-09}$	$8,07x10^{-08}$
hsa-miR-642a-5p	MIMAT0003312	9,2	-1,50	$1,02x10^{-08}$	$1,76x10^{-07}$
hsa-miR-29a-3p	MIMAT0000086	7934,1	-1,14	$1,38x10^{-08}$	$2,35x10^{-07}$
hsa-miR-135a-1-5p	MIMAT0000428_1	4,3	-3,49	$1,92x10^{-08}$	$3,07x10^{-07}$
hsa-miR-99a-5p	MIMAT0000097	1513,3	-1,70	$2,68x10^{-08}$	$4,23x10^{-07}$
hsa-miR-339-3p	MIMAT0004702	159,2	-1,24	$1,03x10^{-07}$	$1,51x10^{-06}$
hsa-miR-549a-3p	MIMAT0003333	1,6	-2,39	$2,28x10^{-07}$	$3,02x10^{-06}$
hsa-miR-99a-3p	MIMAT0004511	15,8	-1,67	$2,48x10^{-07}$	$3,25x10^{-06}$
hsa-miR-335-3p	MIMAT0004703	253,9	-1,81	$4,39x10^{-07}$	$5,46x10^{-06}$
hsa-miR-549a-5p	MIMAT0037328	3,4	-2,44	$4,93x10^{-07}$	$5,96x10^{-06}$
hsa-miR-224-3p	MIMAT0009198	4,9	-1,60	$5,92x10^{-07}$	$6,76x10^{-06}$

6. eranskin taula: Gure kohortean azpi-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 "fold change" <0) (Jarraipena).

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-452-5p	MIMAT0001635	67,8	-1,44	$7{,}92\times10^{-07}$	$8{,}77\times10^{-06}$
hsa-miR-140-3p	MIMAT0004597	2321,3	-1,01	$1{,}42\times10^{-06}$	$1{,}50\times10^{-05}$
hsa-miR-195-5p	MIMAT0000461	747,6	-1,72	$1{,}47\times10^{-06}$	$1{,}53\times10^{-05}$
hsa-let-7b-5p	MIMAT0000063	2879,4	-1,12	$1{,}55\times10^{-06}$	$1{,}61\times10^{-05}$
hsa-let-7g-5p	MIMAT0000414	8351,5	-1,24	$1{,}63\times10^{-06}$	$1{,}65\times10^{-05}$
hsa-miR-483-3p	MIMAT0002173	4,0	-2,00	$3{,}40\times10^{-06}$	$3{,}18\times10^{-05}$
hsa-miR-451a	MIMAT0001631	6085,0	-2,07	$4{,}55\times10^{-06}$	$4{,}16\times10^{-05}$
hsa-miR-125b-2-3p	MIMAT0004603	68,8	-1,39	$4{,}73\times10^{-06}$	$4{,}26\times10^{-05}$
hsa-miR-342-3p	MIMAT0000753	4801,5	-1,03	$6{,}23\times10^{-06}$	$5{,}49\times10^{-05}$
hsa-miR-204-5p	MIMAT0000265	78,2	-1,92	$6{,}34\times10^{-06}$	$5{,}55\times10^{-05}$
hsa-miR-670-3p	MIMAT0026640	0,9	-2,72	$8{,}61\times10^{-06}$	$7{,}44\times10^{-05}$
hsa-miR-1271-5p	MIMAT0005796	53,8	-1,61	$1{,}07\times10^{-05}$	$9{,}06\times10^{-05}$
hsa-miR-505-3p	MIMAT0002876	105,1	-0,53	$1{,}17\times10^{-05}$	$9{,}77\times10^{-05}$
hsa-miR-193a-3p	MIMAT0000459	115,1	-1,17	$1{,}50\times10^{-05}$	0,00012
hsa-miR-197-3p	MIMAT0000227	316,1	-0,94	$1{,}60\times10^{-05}$	0,00013
hsa-miR-6511b-3p	MIMAT0025848	1,3	-1,18	$1{,}68\times10^{-05}$	0,00013
hsa-miR-29c-3p	MIMAT0000681	3703,5	-0,93	$1{,}72\times10^{-05}$	0,00013
hsa-miR-4636	MIMAT0019693	1,1	-1,88	$1{,}95\times10^{-05}$	0,00015
hsa-miR-190a-5p	MIMAT0000458	20,4	-1,20	$2{,}08\times10^{-05}$	0,00016
hsa-miR-99b-5p	MIMAT0000689	7335,7	-1,01	$2{,}44\times10^{-05}$	0,00018
hsa-miR-95-5p	MIMAT0026473	2,6	-1,28	$2{,}43\times10^{-05}$	0,00018
hsa-miR-6803-3p	MIMAT0027507	2,7	-1,19	$2{,}69\times10^{-05}$	0,00020
hsa-miR-135a-3p	MIMAT0004595	0,6	-2,46	$2{,}89\times10^{-05}$	0,00021
hsa-miR-502-3p	MIMAT0004775	26,3	-0,84	$3{,}04\times10^{-05}$	0,00022
hsa-miR-199a-1-3p	MIMAT0000232_1	72,7	-1,85	$3{,}07\times10^{-05}$	0,00022
hsa-miR-585-3p	MIMAT0003250	2,3	-1,66	$3{,}24\times10^{-05}$	0,00023
hsa-miR-216a-5p	MIMAT0000273	3,0	-2,19	$5{,}48\times10^{-05}$	0,00037
hsa-miR-144-5p	MIMAT0004600	157,2	-1,91	$6{,}25\times10^{-05}$	0,00041
hsa-miR-26a1-5p	MIMAT0000082_1	20323,2	-1,92	$6{,}66\times10^{-05}$	0,00043
hsa-miR-101-2-3p	MIMAT0000099_1	8966,6	-1,11	$8{,}04\times10^{-05}$	0,00051
hsa-miR-217-5p	MIMAT0000274	1,1	-2,15	$9{,}96\times10^{-05}$	0,00062
hsa-miR-4662a-5p	MIMAT0019731	2,9	-1,37	0,00013	0,00077
hsa-let-7b-3p	MIMAT0004482	51,0	-0,79	0,00014	0,00080
hsa-miR-497-3p	MIMAT0004768	4,3	-1,13	0,00016	0,00093
hsa-miR-338-5p	MIMAT0004701	33,2	-1,44	0,00016	0,00093
hsa-miR-331-3p	MIMAT0000760	139,0	-0,73	0,00030	0,00164
hsa-miR-484	MIMAT0002174	480,5	-0,69	0,00031	0,00166
hsa-miR-676-3p	MIMAT0018204	1,1	-1,46	0,00034	0,00180
hsa-miR-143-5p	MIMAT0004599	81,1	-1,02	0,00034	0,00182
hsa-miR-23b-3p	MIMAT0000418	547,8	-1,11	0,00037	0,00193
hsa-miR-28-5p	MIMAT0000085	525,6	-1,00	0,00041	0,00211
hsa-let-7e-5p	MIMAT0000066	470,3	-0,93	0,00054	0,00264

6. eranskin taula: Gure kohortean azpi-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 "fold change" <0) (Jarraipena).

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-320b-2	MIMAT0005792_1	6,5	-1,22	0,00057	0,00279
hsa-miR-181b-2-3p	MIMAT0031893	0,7	-1,45	0,00058	0,00279
hsa-miR-2114-5p	MIMAT0011156	1,7	-1,15	0,00062	0,00293
hsa-miR-1260a	MIMAT0005911	72,7	-1,75	0,00080	0,00361
hsa-miR-143-3p	MIMAT0000435	92690,9	-0,78	0,00083	0,00376
hsa-miR-190a-3p	MIMAT0026482	1,1	-1,45	0,00087	0,00390
hsa-miR-30c-2-5p	MIMAT0000244_1	1209,7	-1,14	0,00098	0,00433
hsa-miR-3622a-5p	MIMAT0018003	0,9	-1,63	0,00115	0,00489
hsa-miR-181a-2-5p	MIMAT0000256_1	8231,0	-0,94	0,00130	0,00542
hsa-miR-126-5p	MIMAT0000444	12938,0	-0,93	0,00145	0,00597
hsa-miR-376c-3p	MIMAT0000720	25,7	-1,13	0,00146	0,00598
hsa-miR-5571-3p	MIMAT0022258	3,7	-1,70	0,00160	0,00645
hsa-miR-551a	MIMAT0003214	1,2	-1,15	0,00162	0,00649
hsa-miR-628-5p	MIMAT0004809	14,3	-0,53	0,00167	0,00667
hsa-miR-150-3p	MIMAT0004610	62,6	-1,04	0,00167	0,00667
hsa-miR-144-3p	MIMAT0000436	576,9	-1,44	0,00171	0,00679
hsa-miR-500a-3p	MIMAT0002871	388,1	-0,56	0,00181	0,00717
hsa-miR-338-3p	MIMAT0000763	179,8	-1,10	0,00203	0,00794
hsa-miR-130a-3p	MIMAT0000425	677,2	-0,91	0,00208	0,00812
hsa-miR-409-5p	MIMAT0001638	11,0	-1,60	0,00219	0,00847
hsa-miR-561-5p	MIMAT0022706	4,9	-1,05	0,00222	0,00859
hsa-miR-31-5p	MIMAT0000089	94,3	-1,38	0,00232	0,00885
hsa-miR-5683	MIMAT0022472	24,0	-1,69	0,00231	0,00885
hsa-miR-142-5p	MIMAT0000433	61555,7	-0,81	0,00233	0,00888
hsa-miR-5193	MIMAT0021124	0,7	-1,21	0,00243	0,00921
hsa-miR-6737-3p	MIMAT0027376	1,6	-1,10	0,00256	0,00963
hsa-miR-26a-2-3p	MIMAT0004681	14,1	-0,75	0,00263	0,00985
hsa-miR-365b-3p	MIMAT0022834	35,7	-2,01	0,00274	0,01025
hsa-let-7a-3-5p	MIMAT0000062_1	4442,2	-1,05	0,00300	0,01115
hsa-miR-26b-5p	MIMAT0000083	10400,2	-0,86	0,00329	0,01214
hsa-miR-192-3p	MIMAT0004543	3,9	-1,79	0,00346	0,01266
hsa-let-7a-2-5p	MIMAT0000062	3740,8	-1,41	0,00380	0,01375
hsa-miR-628-3p	MIMAT0003297	8,5	-0,71	0,00387	0,01396
hsa-miR-675-3p	MIMAT0006790	1,8	-1,37	0,00420	0,01481
hsa-miR-514a-2-3p	MIMAT0002883_1	0,3	-1,89	0,00419	0,01481
hsa-miR-342-5p	MIMAT0004694	133,8	-0,68	0,00456	0,01580
hsa-miR-100-5p	MIMAT0000098	10399,0	-0,77	0,00480	0,01655
hsa-miR-652-5p	MIMAT0022709	14,9	-0,56	0,00487	0,01671
hsa-miR-32-5p	MIMAT0000090	219,0	-0,80	0,00490	0,01676
hsa-miR-202-5p	MIMAT0002810	0,6	-1,86	0,00499	0,01692
hsa-miR-5690	MIMAT0022482	3,2	-0,99	0,00507	0,01713
hsa-miR-212-3p	MIMAT0000269	17,4	-0,75	0,00525	0,01770

6. eranskin taula: Gure kohortean azpi-adierazitako mikroRNA guztiak BZHLH pazienteetan kontrolekin alderatuz (p<0,05 eta log2 “fold change” <0) (Jarraipena).

MikroRNA	Ids	“Base Mean”	“Log2 Fold Change”	p-balioa	padj
hsa-miR-4526	MIMAT0019065	0,4	-1,49	0,00539	0,01811
hsa-miR-152-3p	MIMAT0000438	257,5	-0,77	0,00565	0,01880
hsa-miR-3912-3p	MIMAT0018186	12,7	-0,69	0,00584	0,01926
hsa-miR-509-3-3p	MIMAT0002881_1	1,3	-2,18	0,00584	0,01926
hsa-miR-145-3p	MIMAT0004601	403,6	-0,98	0,00595	0,01953
hsa-miR-29b-2-3p	MIMAT0000100_1	189,5	-1,09	0,00634	0,02070
hsa-miR-29b-1-3p	MIMAT0000100	811,1	-0,82	0,00668	0,02155
hsa-miR-199a-2-3p	MIMAT0000232	271,3	-1,07	0,00668	0,02155
hsa-let-7c-3p	MIMAT0026472	0,8	-1,45	0,00669	0,02155
hsa-let-7e-3p	MIMAT0004485	9,5	-0,68	0,00715	0,02294
hsa-miR-153-2-3p	MIMAT0000439_1	12,2	-1,46	0,00768	0,02438
hsa-miR-584-5p	MIMAT0003249	16,6	-0,67	0,00882	0,02764
hsa-miR-24-1-5p	MIMAT0000079	3,1	-0,74	0,01056	0,03236
hsa-miR-151a-5p	MIMAT0004697	2051,2	-0,83	0,01074	0,03284
hsa-miR-3613-3p	MIMAT0017991	16,1	-0,48	0,01220	0,03676
hsa-miR-195-3p	MIMAT0004615	25,2	-0,50	0,01256	0,03766
hsa-let-7a-3p	MIMAT0004481	39,1	-0,53	0,01334	0,03942
hsa-let-7d-5p	MIMAT0000065	613,2	-0,66	0,01390	0,04078
hsa-miR-31-3p	MIMAT0004504	4,1	-1,28	0,01453	0,04233
hsa-miR-26a-2-5p	MIMAT0000082	29705,3	-0,76	0,01475	0,04279
hsa-miR-376a-5p	MIMAT0003386	5,9	-1,03	0,01488	0,04296
hsa-miR-6513-3p	MIMAT0025483	0,7	-0,94	0,01543	0,04413
hsa-miR-488-3p	MIMAT0004763	4,8	-1,49	0,01605	0,04560
hsa-miR-574-3p	MIMAT0003239	266,7	-0,80	0,01707	0,04826
hsa-miR-361-5p	MIMAT0000703	364,4	-0,66	0,01749	0,04935
hsa-miR-215-5p	MIMAT0000272	53,8	-4,42	$6{,}74\times10^{-35}$	$8{,}39\times10^{-32}$

7. eranskin taula: Gure kohortean modu esangarrian adierazitako mikroRNA guztiak BZHLH ez-ZGB eta ZGB azpimoten artean (p<0,05 eta log2 "fold change" <0).

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-625-3p	MIMAT0004808	39,4	-1,34	$1{,}19\text{x}10^{-07}$	0,00006
hsa-miR-625-5p	MIMAT0003294	19,3	-1,13	$5{,}40\text{x}10^{-06}$	0,00135
hsa-miR-511-5p	MIMAT0002808	7,6	-2,01	$9{,}92\text{x}10^{-06}$	0,00142
hsa-miR-185-3p	MIMAT0004611	40,4	-1,80	$7{,}70\text{x}10^{-06}$	0,00142
hsa-miR-205-5p	MIMAT0000266	20,3	-3,30	$4{,}10\text{x}10^{-05}$	0,00342
hsa-miR-1307-3p	MIMAT0005951	422,0	-1,18	$8{,}68\text{x}10^{-05}$	0,00620
hsa-miR-296-3p	MIMAT0004679	31,6	-1,94	0,00020	0,01033
hsa-miR-4453	MIMAT0018975	1,2	-1,93	0,00036	0,01707
hsa-miR-451a	MIMAT0001631	5979,3	-1,74	0,00038	0,01707
hsa-miR-144-3p	MIMAT0000436	572,9	-1,70	0,00043	0,01809
hsa-miR-6821-3p	MIMAT0027543	1,6	-1,97	0,00065	0,02583
hsa-miR-320a-3p	MIMAT0000510	2407,6	-1,35	0,00078	0,02904
hsa-miR-3652	MIMAT0018072	8,9	-2,44	0,00088	0,03137
hsa-miR-7706	MIMAT0030021	81,1	-1,17	0,00102	0,03414
hsa-miR-144-5p	MIMAT0004600	154,1	-1,66	0,00136	0,03996
hsa-miR-27a-5p	MIMAT0004501	37,7	-0,85	0,00135	0,03996
hsa-miR-3610	MIMAT0017987	20,3	-1,89	0,00157	0,04483

8. eranskin taula: Gure kohortean modu esangarrian adierazitako mikroRNA guztiak BZHLH ZGB eta ez-ZGB azpimoten artean (p<0,05 eta log2 "fold change" >0).

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-28-3p	MIMAT0004502	3415,2	1,45	$3,36x10^{-11}$	$3,36x10^{-08}$
hsa-miR-129-2-3p	MIMAT0004605	4,9	4,12	$1,50x10^{-06}$	0,00050
hsa-miR-181a-2-5p	MIMAT0000256_1	6688,9	1,33	$8,83x10^{-06}$	0,00142
hsa-miR-4464	MIMAT0018988	2,9	3,91	$1,21x10^{-05}$	0,00151
hsa-miR-3150b-3p	MIMAT0018194	49,0	2,00	$1,90x10^{-05}$	0,00211
hsa-miR-181a-5p	MIMAT0000256	39940,6	1,23	$2,40x10^{-05}$	0,00240
hsa-miR-28-5p	MIMAT0000085	475,7	1,24	$3,85x10^{-05}$	0,00342
hsa-miR-181b-2-5p	MIMAT0000257_1	3046,9	1,06	$4,75x10^{-05}$	0,00366
hsa-miR-138-1-5p	MIMAT0000430_1	568,1	2,00	$9,92x10^{-05}$	0,00661
hsa-miR-486-2-3p	MIMAT0004762_1	27,6	1,75	0,00015	0,00891
hsa-miR-129-5p	MIMAT0000242	18,2	2,90	0,00014	0,00891
hsa-miR-181a-2-3p	MIMAT0004558	166,9	1,02	0,00017	0,00951
hsa-miR-181d-5p	MIMAT0002821	213,7	0,71	0,00024	0,01176
hsa-miR-151b	MIMAT0010214	22,0	1,56	0,00040	0,01761
hsa-miR-181a-3p	MIMAT0000270	1066,6	0,97	0,00067	0,02583
hsa-miR-4677-3p	MIMAT0019761	25,5	0,57	0,00093	0,03219
hsa-miR-582-3p	MIMAT0004797	65,7	1,28	0,00114	0,03663
hsa-miR-200b-3p	MIMAT0000318	15,7	1,56	0,00128	0,03996
hsa-miR-151a-5p	MIMAT0004697	1783,9	1,09	0,00166	0,04618

9. eranskin taula: Gure kohortean tratamenduari emandako erantzun onarekin modu esangarrian erlazionatutako mikroRNAk (p<0,05).

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-12136	MIMAT0049032	120,0	25,73	$1,29x10^{-13}$	$9,70x10^{-11}$
hsa-miR-129a-5p	MIMAT0000242_1	42,7	5,09	$2,86x10^{-09}$	$1,08x10^{-06}$
hsa-miR-129-1-3p	MIMAT0004548	7,4	4,08	$1,86x10^{-06}$	0,00035
hsa-miR-3150b-3p	MIMAT0018194	50,7	2,33	$1,60x10^{-05}$	0,00241
hsa-miR-409-3p	MIMAT0001639	244,7	1,91	$4,36x10^{-05}$	0,00547
hsa-miR-127-3p	MIMAT0000446	3977,2	2,01	$6,14x10^{-05}$	0,00661
hsa-miR-3681-5p	MIMAT0018108	75,3	2,34	0,00016	0,01507
hsa-miR-28-3p	MIMAT0004502	3740,9	1,02	0,00021	0,01729
hsa-miR-136-5p	MIMAT0000448	60,2	1,34	0,00033	0,02251
hsa-miR-127-5p	MIMAT0004604	30,8	1,92	0,00036	0,02279
hsa-miR-370-3p	MIMAT0000722	16,1	2,59	0,00041	0,02380
hsa-miR-145-5p	MIMAT0000437	2060,5	1,07	0,00074	0,03719
hsa-miR-4464	MIMAT0018988	3,3	3,55	0,00117	0,04641
hsa-miR-129b-5p	MIMAT0000242	27,5	2,88	0,00102	0,04641
hsa-miR-3928-3p	MIMAT0018205	16,5	2,03	0,00113	0,04641

10. eranskin taula: Gure kohortean tratamenduari emandako erantzun txarrarekin modu esangarrian erlazionatutako mikroRNAk (p<0,05)

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-192-5p	MIMAT0000222	10375,5	-2,41	$1,60x10^{-07}$	$4,01x10^{-05}$
hsa-miR-222-3p	MIMAT0000279	1984,9	-1,10	0,00027	0,02010
hsa-miR-221-3p	MIMAT0000278	5082,8	-1,14	0,00061	0,03302
hsa-miR-4454	MIMAT0018976	183,5	-1,05	0,00107	0,04641

11. eranskin taula: Gure kohortean pronostiko onarekin modu esangarrian erlazionatutako mikroRNAk (p<0,05)

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-4444	MIMAT0018962	10,3	2,26	$6{,}20\text{x}10^{-05}$	0,00897
hsa-miR-449c-5p	MIMAT0010251	9,8	2,22	$4{,}75\text{x}10^{-05}$	0,00897
hsa-miR-3615	MIMAT0017994	322,3	1,55	$5{,}32\text{x}10^{-05}$	0,00897
hsa-miR-3681-5p	MIMAT0018108	75,3	2,03	0,00022	0,01427
hsa-miR-3928-3p	MIMAT0018205	16,5	2,03	0,00018	0,01427
hsa-miR-449b-5p	MIMAT0003327	7,2	2,22	0,00037	0,02168
hsa-miR-1287-5p	MIMAT0005878	19,7	1,75	0,00039	0,02168
hsa-miR-146a-3p	MIMAT0004608	27,7	1,49	0,00053	0,02495
hsa-miR-423-3p	MIMAT0001340	3193,5	1,15	0,00049	0,02495
hsa-miR-370-3p	MIMAT0000722	16,1	2,12	0,00071	0,02954
hsa-miR-184	MIMAT0000454	14,6	1,98	0,00071	0,02954
hsa-miR-4424	MIMAT0018939	110,8	2,32	0,00107	0,04174

12. eranskin taula: Gure kohortean pronostiko txarrarekin modu esangarrian erlazionatutako mikroRNAk (p<0,05)

MikroRNA	Ids	"Base Mean"	"Log2 Fold Change"	p-balioa	padj
hsa-miR-133a-2-3p	MIMAT0000427_1	470,9	-3,81	$1{,}53\text{x}10^{-06}$	0,00102
hsa-miR-133a-1-3p	MIMAT0000427	69,3	-2,53	$6{,}78\text{x}10^{-05}$	0,00897
hsa-miR-338-3p	MIMAT0000763	179,8	-1,39	0,00011	0,01186
hsa-miR-208b-3p	MIMAT0004960	12,9	-3,09	0,00014	0,01329
hsa-miR-205-5p	MIMAT0000266	62,5	-3,37	0,00021	0,01427

13. eranskin taula: Gain-adierazitako mikroRNAen itu diren 138 azpi-adierazitako gene.

CXCR4	*SOX4*	*CYLD*	*ELAVL1*	*ATF1*	*CUL4A*	*TUG1*
REST	*EZR*	*CCND2*	*DICER1*	*TP53BP1*	*RUNX2*	*PRKAR1A*
SNCA	*MCL1*	*MAPK1*	*PDCD2*	*FBXW7*	*SUZ12*	*FANCM*
RAD52	*MNT*	*WASF2*	*PTBP3*	*CNOT6L*	*BCLAF1*	*FNDC3A*
KRAS	*BTRC*	*RECK*	*MALAT1*	*DRD2*	*CPM*	*CTNNB1*
IRAK2	*JUND*	*STAT1*	*ALDH5A1*	*SIKE1*	*BAX*	*SPAG9*
POU2F2	*TSC22D3*	*SRC*	*FOXN3*	*PRKCE*	*KLF8*	*TIA1*
ETS1	*NOTCH1*	*ILF3*	*INPP5A*	*CUL5*	*PTP4A1*	*TRIM8*
FOXO3	*TP53*	*KMT5A*	*PSMA3*	*SIAH1*	*REL*	*HES1*
IRAK1	*IL24*	*ARPP19*	*PTPN2*	*HOXD10*	*MTDH*	*NFAT5*
ROCK1	*SMAD4*	*FLOT1*	*ZFAND4*	*NFATC3*	*YWHAZ*	*WNK1*
FOXO1	*CREB1*	*PTEN*	*ITGB3*	*PIK3CG*	*RHOA*	*FGFR1*
RARG	*FGFR2*	*BMPR2*	*ACSL4*	*PPP2CB*	*PDPK1*	*ATM*
BRCA2	*CASP8*	*YY1*	*RAC1*	*TGFBR2*	*CACNB2*	*QKI*
APC	*MTSS1*	*ERN1*	*FOXP3*	*MTHFD1*	*IGF2BP3*	*MSH3*
PTPN1	*EGR1*	*SKP2*	*HIF3A*	*BCL2L11*	*DMD*	*RBAK*
XIST	*AP3B1*	*AKT2*	*FOXP1*	*STAT3*	*DLG1*	*CCNJ*
FAF1	*EGLN2*	*CDK6*	*TP53INP1*	*SOX2*	*CAMKK2*	*RAP2A*
E2F5	*TGFB1*	*DYRK1A*	*XIAP*	*SOS1*	*PRKAA1*	
ITGB1	*BCL2*	*GSK3B*	*SP1*	*LMNA*	*STAT5B*	

14. eranskin taula: Azpi-adierazitako mikroRNAen itu diren 76 gain-adierazitako gene.

KLF4	*SOX4*	*EDNRA*	*HOXA10*	*ZEB2*	*ITSN1*	*CEMIP*
MYO6	*AP2M1*	*SRGAP1*	*JAG1*	*BMPR2*	*FAM162A*	*MTDH*
ROBO1	*EGFR*	*CDH2*	*BRCA1*	*EPAS1*	*RHOT1*	*NID1*
PTEN	*NR4A2*	*HBEGF*	*ID4*	*RREB1*	*CD28*	*DLC1*
STAT1	*SMAD4*	*PTPN12*	*SERINC5*	*IGF1*	*IL10*	*PTPN14*
YES1	*VEGFA*	*ITGA9*	*MEST*	*DPYSL2*	*YAP1*	*TCEAL9*
TNC	*WNK1*	*SOCS2*	*MMP1*	*MTSS1*	*ACTC1*	*PRKCA*
PARP8	*FZD7*	*CD44*	*MMP12*	*CELF2*	*ADGRL4*	*CHD1*
MIF	*MMP2*	*SERPINE1*	*APH1A*	*SRCIN1*	*SPTLC1*	*PLAUR*
APC	*MMP9*	*ITGB8*	*ABHD17C*	*CHEK1*	*CEBPD*	*Reck*
MERTK	*BCL2*	*KRAS*	*MYO5A*	*TRIB1*	*MYOCD*	

15. eranskin taula: BZHLHn gain-adierazitako mikroRNAk eta azpi-adierazitako gene ituak minbiziko bidezidorrean.

mikroRNA	*Entrez-gene izena*
hsa-miR-612 **hsa-miR-2861**	*AKT2*
hsa-miR-135a-5p **hsa-miR-663a** **hsa-miR-129-5p**	*APC*
hsa-miR-7-5p **hsa-miR-182-5p** **hsa-miR-205-5p** **hsa-miR-135a-5p** **hsa-miR-9-5p**	*BCL2*
hsa-miR-182-5p **hsa-miR-146a-5p**	*CCND2*
hsa-miR-129-5p **hsa-miR-320a**	*CDK6*
hsa-miR-146a-5p **hsa-miR-146a-3p** **hsa-miR-9-5p** **hsa-miR-663a**	*CXCR4*
hsa-miR-182-5p **hsa-miR-183-5p** **hsa-miR-9-5p** **hsa-miR-9-3p** **hsa-miR-135a-5p**	*FOXO1*
hsa-miR-183-5p **hsa-miR-182-5p** **hsa-miR-9-5p** **hsa-miR-129-1-3p** **hsa-miR-1246**	*GSK3B*
hsa-miR-9-3p **hsa-miR-9-5p**	*HES1*
hsa-miR-183-5p **hsa-miR-9-3p**	*ITGB1*
hsa-miR-320a **hsa-miR-9-3p** **hsa-miR-129-5p**	*MAPK1*
hsa-miR-129-5p **hsa-miR-9-5p** **hsa-miR-146a-5p**	*NOTCH1*
hsa-miR-182-5p **hsa-miR-155-3p** **hsa-miR-205-5p** **hsa-miR-320a**	*PTEN*
hsa-miR-320a **hsa-miR-146a-5p**	*RAC1*
hsa-miR-146a-5p **hsa-miR-135a-5p**	*ROCK1*
hsa-miR-146a-5p **hsa-miR-182-5p** **hsa-miR-205-5p** **hsa-miR-183-5p**	*SMAD4*
hsa-miR-129-5p **hsa-miR-612**	*SP1*
hsa-miR-663a **hsa-miR-146a-5p**	*TGFB1*
hsa-miR-612 **hsa-miR-663a** **hsa-miR-155-3p**	*TP53*
hsa-miR-7-5p	*BAX*
hsa-miR-9-5p	*BCL2L11*
hsa-miR-146a-5p	*BRCA2*
hsa-miR-19b-1-5p	*CASP8*
hsa-miR-320a	*CTNNB1*
hsa-miR-205-5p	*EGLN2*
hsa-miR-9-5p	*ETS1*
hsa-miR-573	*FGFR1*
hsa-miR-19b-1-5p	*FGFR2*
hsa-miR-18a-3p	*KRAS*
hsa-miR-7-5p	*MSH3*
hsa-miR-146a-5p	*RHOA*
hsa-miR-7-5p	*SKP2*
hsa-miR-146a-5p	*SOS1*
hsa-miR-146a-5p	*STAT1*
hsa-miR-1181	*STAT3*
hsa-miR-9-5p	*TGFBR2*
hsa-miR-182-3p	*STAT5B*
hsa-miR-7-5p	*XIAP*

16. eranskin taula: BZHLHn azpi-adierazitako mikroRNAk eta gain-adierazitako gene ituak.

mikroRNA	*Entrez-gene izena*
hsa-miR-451a	*BCL2*
hsa-miR-497-5p	*BCL2*
hsa-miR-135a-5p	*BCL2*
hsa-miR-224-5p	*BCL2*
hsa-miR-139-5p	*BCL2*
hsa-miR-145-5p	*EGFR*
hsa-miR-135a-5p	*EGFR*
hsa-miR-217	*KRAS*
hsa-miR-224-5p	*KRAS*
hsa-miR-217	*PTEN*
hsa-miR-216a-5p	*PTEN*
hsa-miR-483-3p	*SMAD4*
hsa-miR-224-5p	*SMAD4*
hsa-miR-145-5p	*STAT1*
hsa-miR-150-5p	*STAT1*
hsa-miR-150-5p	*VEGFA*
hsa-miR-145-5p	*VEGFA*
hsa-miR-135a-5p	*APC*
hsa-miR-224-5p	*EDNRA*
hsa-miR-145-5p	*EPAS1*
hsa-miR-145-5p	*FZD7*
hsa-miR-483-3p	*IGF1*
hsa-miR-145-5p	*JAG1*
hsa-miR-145-5p	*MMP1*
hsa-miR-451a	*MMP2*
hsa-miR-451a	*MMP9*
hsa-miR-150-5p	*PRKCA*

17. eranskin taula: Minbizi bidean azpi-adierazitako geneen ituak.

Entrez-gene ID	***Entrez-gene name***
2113	*ETS1:* ETS proto-oncogene 1, transcription factor
208	*AKT2:* AKT serine/threonine kinase 2
2260	*FGFR1:* fibroblast growth factor receptor 1
2263	*FGFR2:* fibroblast growth factor receptor 2
2308	*FOXO1:* forkhead box O1
324	*APC:* APC, WNT signaling pathway regulator
331	*XIAP:* X-linked inhibitor of apoptosis
4437	*MSH3:* mutS homolog 3
6502	*SKP2:* S-phase kinase associated protein 2
387	*RHOA:* ras homolog family member A
6654	*SOS1:* SOS Ras/Rac guanine nucleotide exchange factor 1
6667	*SP1:* Sp1 transcription factor
581	*BAX:* BCL2 associated X, apoptosis regulator
596	*BCL2:* BCL2, apoptosis regulator
6772	*STAT1:* signal transducer and activator of transcription 1
6774	*STAT3:* signal transducer and activator of transcription 3
6777	*STAT5B:* signal transducer and activator of transcription 5B
675	*BRCA2:* BRCA2, DNA repair associated
4851	*NOTCH1:* notch 1
1499	*CTNNB1:* catenin beta 1
841	*CASP8:* caspase 8
2932	*GSK3B:* glycogen synthase kinase 3 beta
894	*CCND2:* cyclin D2
7048	*TGFBR2:* transforming growth factor beta receptor 2
7157	*TP53:* tumor protein p53
1021	*CDK6:* cyclin dependent kinase 6
3280	*HES1:* hes family bHLH transcription factor 1
7040	*TGFB1:* transforming growth factor beta 1
5594	*MAPK1:* mitogen-activated protein kinase 1
5728	*PTEN:* phosphatase and tensin homolog
3688	*ITGB1:* integrin subunit beta 1
7852	*CXCR4:* C-X-C motif chemokine receptor 4
5879	*RAC1:* Rac family small GTPase 1
3845	*KRAS:* KRAS proto-oncogene, GTPase
112398	*EGLN2:* egl-9 family hypoxia inducible factor 2
10018	*BCL2L11:* BCL2 like 11
6093	*ROCK1:* Rho associated coiled-coil containing protein kinase 1
4089	*SMAD4:* SMAD family member 4

17. eranskin taula: Minbizi bidean gain-adierazitako geneen ituak.

Entrez-gene ID	*Entrez-gene name*
8324	FZD7: frizzled class receptor 7
182	JAG1: jagged 1
4312	MMP1: matrix metallopeptidase 1
4313	MMP2: matrix metallopeptidase 2
4318	MMP9: matrix metallopeptidase 9
324	APC: APC, WNT signaling pathway regulator
596	BCL2: BCL2, apoptosis regulator
6772	STAT1: signal transducer and activator of transcription 1
7422	VEGFA: vascular endothelial growth factor A
3479	IGF1: insulin like growth factor 1
5578	PRKCA: protein kinase C alpha
5728	PTEN: phosphatase and tensin homolog
3845	KRAS: KRAS proto-oncogene, GTPase
1909	EDNRA: endothelin receptor type A
1956	EGFR: epidermal growth factor receptor
2034	EPAS1: endothelial PAS domain protein 1
4089	SMAD4: SMAD family member 4

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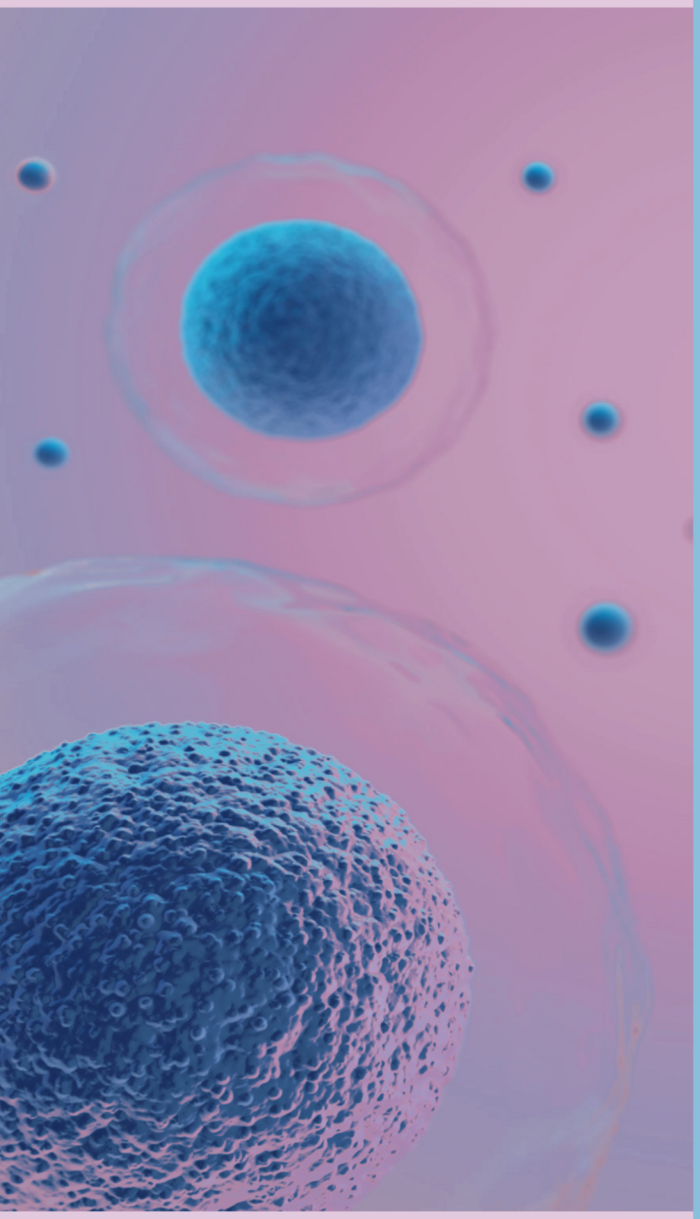

Potential of microRNAs in the diagnosis, classification and prognosis of Diffuse Large B Cell Lymphoma

Doctoral thesis

Ane Larrabeiti Etxebarria

Leioa 2021

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Universidad del País Vasco
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Departamento de Genética, Antropología Física y Fisiología Animal / Genetika, Antropologia Fisikoa eta Animalien Fisiologia Saila

Tesis doctoral / Doktorego tesia

Potential of microRNAs in the diagnosis, classification and prognosis of Diffuse Large B Cell Lymphoma.

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Leioa, 2021

Nire bi izartxuei, Eneko eta Malen

Ama, zuretzat.

Acknowledgements

Quiero aprovechar estas líneas para agradecer el apoyo de todas las personas que han hecho posible esta tesis y que han estado a mi lado durante esta etapa.

En primer lugar, a África, gracias por brindarme la oportunidad de realizar la tesis y haber confiado en mí para este proyecto.

Eli, muchas gracias por tu gran apoyo y sobre todo por toda la paciencia que has tenido conmigo. Ha sido un camino duro en el que a pesar de todo el trabajo que tenías siempre has estado dispuesta a ayudarme y tirar para que esta tesis saliera adelante. Angela, desde la distancia has conseguido trasmitirme tu gran positividad y motivarme para no tirar la toalla.

¡Muchísimas gracias a las dos! Indudablemente sois unas maravillosas investigadoras y bellísimas personas que trasmitís pasión con vuestro trabajo. Siempre estaré agradecida por vuestro apoyo.

Agradecer a Maitane, Borja, Nerea e Idoia, por formar parte del equipo, guiarme y ayudarme a dar forma a este trabajo.

Santi, en ti encontré la motivación para embarcarme en este proyecto y siempre has estado ahí, me has entendido y ayudado.

Eskerrik asko nire familiari, bereziki, Ama, Eneko eta Malentxu. Amatxu gracias por estar siempre a mi lado, creer en mi incondicionalmente y ayudarme a superar todos los baches que me he encontrado. Eneko eskerrik asko zure pazientziagaitik, zu barik hau ez litzateke posible izango. Durante estos años me has animado y en los momentos más difíciles me has hecho sacar fuerzas de donde creía que no las tenía. Zuri Malen, gure izarra. Llegaste al final de este proyecto iluminando nuestras vidas. Nunca olvidaré esas horas de trabajo contigo en el pecho como un koalita. Jesus, zuri ere mila esker, urte hauetan zehar hain ondo zaintzeagaitik.

Eskerrik asko denoi, bihotz-bihotzez!

Publications

The work of this thesis is reflected in the following publications:

Systematic Review of the Potential of MicroRNAs in Diffuse Large B Cell Lymphoma. Larrabeiti-Etxebarria A, Lopez-Santillan M, Santos-Zorrozua B, Lopez-Lopez E, Garcia-Orad A. Cancers (Basel). 2019 Jan 26;11(2). pii: E144.

Potential of microRNAs in the diagnosis, classification and prognosis of Diffuse Large B Cell Lymphoma though miRNA-Sequencing. In preparation.

Furthermore, during the development of this thesis, I have also published the following articles:

Circulating miRNAs as biomarkers in diffuse large B-cell lymphoma: a systematic review. Lopez-Santillan M, Larrabeiti-Etxebarria A, Arzuaga-Mendez J, Lopez-Lopez E, Garcia-Orad A. Oncotarget. 2018 Apr 27;9(32):22850-22861.

Abbreviation	**Explanation**
(aa)-IPI	age-adjusted IPI
ACVR2B	activin receptor type 2B
ALL	acute lymphoblastic leukemia
allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	acute myeloid leukemia
AML	acute myeloid leukemia
auto-HSCT	autologous-hematopoietic stem cell transplantation
B-CLL	B cell chronic lymphocytic leukemia
B2MG	beta-2 microglobulin
BEAM	BCNU, etoposide, cytarabine and melphalan
BM	Bone marrow
CHOP	cyclophosphamide, doxorubicin, vincristine and prednisone
CLL	chronic lymphocytic leukemia
CMR	complete metabolic remission
CNS	Central Nervous System
COO	Cell of origin
Cox PH	Cox proportional hazards
CRG	Centre for Genomic Regulation
CTCL	cutaneous T-cell lymphoma
CTL	cytotoxic T lymphocytes
DA-EPOCH-R	dose-adjusted-(etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin-rituximab)
DE	differential expression
DEG	Differentially Expressed Genes
DLBCL	Diffuse Large B Cell Lymphoma
dsRNA	double-stranded RNA
EBV	Epstein Barr Virus
ECOG	Eastern Cooperative Oncology Group
EMT	epithelial mesenchymal transition
EMT	epithelial mesenchymal transition
ENCODE	Encyclopedia of DNA Elements
FFPE	formalin-fixed paraffin-embedded
GCB	Germinal center B-cell-like
GELTAMO	Spanish Lymphoma Group
GEO	Gene Expression Omnibus
GEP	Gene expression profile
GO	Gene Ontology
HCC	hepatocellular carcinoma
HGP	Human Genome Project
HIF1α	hypoxia inducible factor 1α
HIV	Human Immunodeficiency Virus
IFRT	involved-field radiation therapy
IHC	Immunohistochemistry
IPI	International Prognostic Index
LDH	Lactate dehydrogenase

logFC	log2 fold change
mRNA	messenger RNAs
MTIs	microRNA-target interactions
MTX	methotrexate
NCCN	The National Comprehensive Cancer Network
ncRNA	non-coding RNAs
NGS	Next Generation Sequencing
NHL	Non-Hodgkin Lymphoma
NK	Natural killer
non-GCB or ABC	Activated B-cell-like
NOS	Not Otherwise Specified
ORR	overall response rates
OS	Overall survival
padj	P-values adjusted using False discovery rate
PCA	Principal component analysis
PDCD4	Programmed Cell Death 4
PFS	progression free survival
PPI	protein-protein interaction
PR	partial remission
PTEN	Phosphatase and Tensin Homolog
R	Rituximab
R-ACVBP	rituximab, doxorubicin, cyclophosphamide, vindesine, bleomycin and prednisone
R-CHOP	rituximab-CHOP
R-DHAP	rituximab-dexamethasone, cytosine arabinoside, and cisplatin
R-GDP	rituximab, gemcitabine, cisplatin, and dexamethasone
R-GemOx	rituximab-gemcitabine, oxaliplatin
R-ICE	rituximab-ifosfamide, carboplatin, and etoposide
R-ICE	rituximab-ifosfamide, carboplatin, etoposide
RISC	RNA-inducing silencing complex
RT-qPCR	reverse transcription quantitative PCR
ssRNA	single-stranded RNAs
TGF	transforming growth factor
WHO	World Health Organization
XPO5	Exportine5

Summary

Diffuse Large B Cell Lymphoma (DLBCL) is the most common lymphoid malignancy in adults, accounting for 30–40% non-Hodgkin's lymphoma (NHL). DLBCL presents with variable backgrounds in terms of morphology, genetics, and biological behavior, which results in heterogeneous outcomes among patients. Although new tools have been developed for the classification and management of patients, 40% of them still have primary refractory disease or relapse. In addition, multiple factors regarding the pathogenesis of this disease remain unclear and identification of novel biomarkers is needed. In this context, recent investigations point to microRNAs as useful biomarkers in cancer as well as important players in the development of the disease. However, regarding DLBCL, up to date, there is inconsistency in the data reported. Therefore, in this work, the main goals were to determine a microRNA set with utility as biomarkers for DLBCL diagnosis, classification, prognosis and treatment response, as well as to decipher the mechanism of action of deregulated microRNAs in the origin of the disease. To achieve these goals, we first determined whether microRNAs already proposed in the literature were reliable biomarkers in DLBCL in our population. We identified a signature of four microRNAs that were consistently deregulated in DLBCL at diagnosis. However, the limited number of microRNAs analyzed in the literature and the inconsistency in results did not allow us to identify reliable signatures for classification, treatment response or prognosis. Then, we analyzed all the existing microRNAs in our cohort of DLBCL patients using small RNA sequencing and, with this approach, we were able to define new signatures for diagnosis, classification, treatment response and prognosis. Finally, using a microRNA-mRNA interaction network analysis, we identified the mechanism of action by which deregulated microRNAs could be involved in DLBCL pathogenesis. In summary, our study remarks that microRNAs could play an important role as biomarkers in diagnosis, classification, treatment response and prognosis in DLBCL, as well as the pathogenesis of the disease. Moreover, Next Generation Sequencing technology is of great value, since it allows detecting associations even with poorly studied microRNAs.

Index

INTRODUCTION

DIFFUSE LARGE B CELL LYMPHOMA

Introduction

Diffuse Large B Cell Lymphoma (DLBCL) is the most common lymphoid malignancy in adults, accounting for 30–40% of all non-Hodgkin lymphoma (NHL) cases (1). DLBCL is quite heterogeneous in terms of morphology, genetics, and biological behavior, which leads to high differences in treatment response and outcome among patients (2). Despite those differences, most patients with DLBCL are treated with standard chemotherapy regimens, which allow complete remission in 75-80% of patients. Nevertheless, approximately up to 40% of patients have primary refractory disease or relapse (3). Worryingly, those patients tend to respond poorly to additional chemotherapy lines, which remains a major cause of morbidity and mortality (4). Hence, nowadays one of the challenging fields in DLBCL is the investigation of reliable biomarkers and new therapeutic targets to improve the management of these patients.

Definition

DLBCL is an aggressive type of NHL of large B lymphoid cells (with nuclear size equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte) that has a diffuse growth pattern (5).

The diagnostic category of "DLBCL" is quite heterogeneous in terms of morphology, genetics, and biological behavior. A number of clinicopathologic entities are now recognized in the fourth edition of the World Health Organization (WHO) classification that are sufficiently distinct to be considered separate diagnostic categories, as shown in Table 1 (5).

Table 1: WHO Classification of Large B-cell lymphomas (5).

Diffuse large B-cell lymphoma, NOS
Other lymphomas of large B cells
T-cell/histiocyte-rich large B-cell lymphoma
Primary diffuse large B-cell lymphoma of the CNS
Primary cutaneous diffuse large B-cell lymphoma, leg type
EBV-positive diffuse large B-cell lymphoma, NOS
Diffuse large B-cell lymphoma associated with chronic inflammation
Lymphomatoid granulomatosis
Large B-cell lymphoma with IRF4 rearrangement
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK-positive large B-cell lymphoma
Primary effusion lymphoma
High-grade B-cell lymphoma
High-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements
High-grade B-cell lymphoma, NOS
B-cell lymphoma, unclassifiable
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classic Hodgkin lymphoma

Abbreviations: NOS: not otherwise specified; CNS: central nervous system; EBV: Epstein Barr Virus

However, many cases remain biologically heterogeneous with no clear and accepted criteria for subdivision and are classified as DLBCL, not otherwise specified (NOS) (6). Due to the fact that the most frequent B-lymphoma is DLBCL, NOS, a considerable amount of work still needs to be done to better understand the biology and pathogenesis of this type of lymphoma for specific therapeutic development. Therefore, we focused on this entity.

Epidemiology

DLBCL, NOS is primarily a disease of middle-aged or elderly, but it can also affect children and young adults, and it is a bit more frequent in males than in females. DLBCL, NOS constitutes 25−30% of adult NHL in western countries and it is even more frequent in developing countries. In particular, in Europe, the incidence is approximately 4.92 cases per 100,000 people per year (7).

Etiology

DLBCL originates from mature B-cells at different stages of differentiation. Several genetic alterations promote changes in those B-cells, affecting gene expression and leading to tumoral transformation (5) (Figure 1).

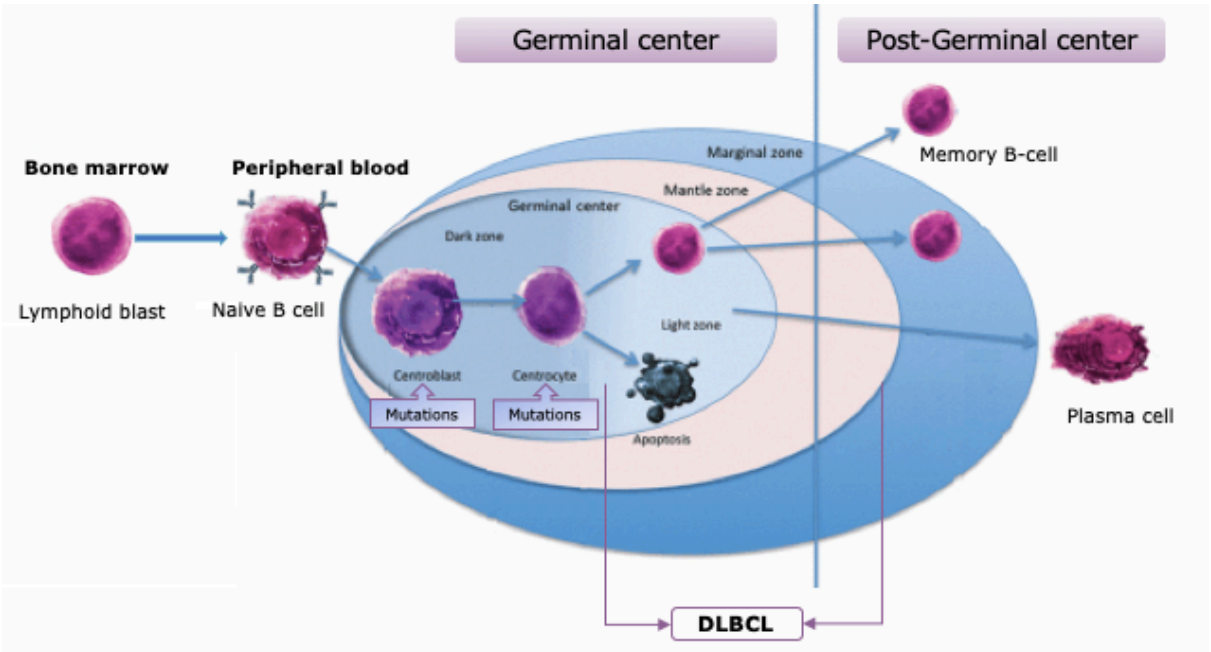

Figure 1: Diagram of the differentiation and maturation of normal B lymphocytes and pathogenesis of diffuse large B-Cell lymphoma (Adapted from Basic medical Key (https://basicmedicalkey.com/cutaneous-b-cell-immunobiology/))

Normal B cell development takes place in the bone marrow and results in the transformation of a B cell progenitor cell into mature B cell. After release from the bone marrow, antigen naïve mature B cells travel to secondary lymphoid tissues where they will be exposed to their respective antigen in the interfollicular area of secondary lymphoid tissues (8).

In the germinal center of the secondary follicle, centroblasts mature into centrocytes as they transition into light zone of the germinal center. In the germinal center, B cell undergoes class-switch recombination and somatic hypermutation. The rearranged immunoglobulin genes in DLBCL, as in other non-Hodgkin's lymphomas, present mutations that are characteristic of somatic hypermutation, an antibody diversification mechanism that normally occurs only within the germinal center of secondary lymphoid organs (9). This evidence suggests that DLBCL arises either from germinal center B cells or from B cells at a later stage of differentiation (10).

DLBCL can occur as primary or *de novo* as well as through the transformation of many different types of low-grade B-cell lymphomas, including B cell chronic lymphocytic leukemia, lymphoplasmacytic lymphoma, follicular lymphoma, marginal zone (MALT) lymphoma, and splenic marginal zone lymphoma (11,12).

Diagnosis

Diagnosis of DLBCL is based on morphological, immunohistochemical, cytogenetic, and molecular analyses. Surgical excision/incision biopsy of a node or affected extra-nodal tissue is the method of choice for diagnosis. This allows assessment of nodal architecture and provides adequate material for phenotypic and molecular studies (12).

Ideally, the biopsy should be sent unfixed to the laboratory to carry out flow cytometry studies and extract high-quality DNA and RNA. Core needle biopsies may be performed if surgical biopsy is excessively risky (12,13).

Clinical and pathological features

The majority of cases occur in lymph nodes and involvement of extranodal sites occurs in 40% of cases, frequently as primary tumor. The most common extranodal site is the gastrointestinal tract, but it can appear in other organs, such as the skin, central nervous system, bone marrow, salivary gland, lung, kidney, and liver. In fact, bone marrow involvement is found in 11% to 27% of all cases but the peripheral blood is rarely affected (14,15).

Patients with DLBCL typically present with a rapidly enlarging symptomatic mass at single or multiple nodal or extranodal sites, the most common presentation being a nodal enlargement in the neck or abdomen (16).

Approximately 60% of patients present with advanced stage DLBCL, while the remaining 40% have more localized disease, usually defined as that which can be contained within one irradiation field (17).

Most patients are asymptomatic but, if they present any symptoms, they tend to be dependent on the site of involvement. Systemic "B" symptoms (i.e., fever, weight loss, drenching night

sweats) are observed in approximately 30% of patients, and the serum lactate dehydrogenase (LDH) is commonly elevated (18).

Morphologically, lymph nodes present a diffuse proliferation of large lymphoid cells totally or partially missing their characteristic architecture. Cases in which medium-sized cells prevail may require additional studies to exclude other diagnosis, such as extramedullary leukemia, Burkitt lymphoma or blastoid mantle cell lymphomas (5).

Classification

Different attempts have been made to classify DLBCL, NOS according to their morphology, molecular characteristics and genetic alterations (Table 2)(5).

Table 2: DLBCL, NOS subtypes according to the 2017 WHO classification(5).

Diffuse large B-cell lymphoma, NOS
Morphological variants
Centroblastic
Immunoblastic
Anaplastic
Other rare variants
Molecular subtypes
Germinal center B-cell subtype
Activated B-cell subtype

I-Morphological variants

Three common morphological variants (centroblastic, immunoblastic and anaplastic) and several rare variants have been recognized. The centroblastic variant is the most common variant and is characterized by the presence of centroblasts, which are medium-sized to large lymphoid cells with vesicular nuclei containing fine chromatin. However, in most cases the tumor is polymorphic with an admixture of centroblasts and immunoblasts (<90%). The immunoblastic variant is defined as a tumor with >90% immunoblasts. The immunoblasts typically contain a large, oval or round nucleus, a single prominent centrally-located nucleolus, and considerable basophilic cytoplasm. The anaplastic variant is characterized by large to very large cells with pleomorphic nuclei (5).

However, attempts to define subgroups based on morphology have failed due to diagnostic discrepancies arising lack of reproducibility among observations (15)

II-Molecular subtypes

Molecular characterization of diffuse large B-cell lymphoma mainly relies on the distinction between subtypes determined by identifying the **"cell of origin" (COO)**, a concept used to classify lymphoma according to the most related non-malignant cell type based on clinical, phenotypic, or genetic characteristics.

II.a. Gene expression profile (GEP)

GEP, is considered the gold standard to assign the molecular subtypes (10). This way, DLBCL can be divided into two main molecular subgroups: the germinal center B-cell-like (GCB) and the activated B-cell-like (ABC or non-GCB). The GCB and non-GCB subgroups differ in their chromosomal alterations, the signaling pathways that are activated, and clinical outcome (19). In addition, approximately 10-15% of cases cannot be included in either of these subtypes and remain unclassified (11).

These two subtypes can be differentiated by the profile of expression of hundreds of genes, using microarrays on RNA from frozen tissue (10). Those genes link each subtype to a different stage of B-cell differentiation and activation (10).

However, GEP is not widely available as a routine clinical test and fresh-frozen lymphoma tissues are not always available, thus, determination of the cell of origin using immunohistochemistry (IHC) algorithms is considered acceptable.

II.b. Immunohistochemistry

Different approaches based on immuhistochemistry with small panels of biomarkers have been developed to translate the information obtained from molecular studies into platforms that can be used in the clinical routine, including Colomo (20), Hans (21), Choi (22), Muris (23) and Tally (24) algorithms (Figure 2). Two of these algorithms were designed in the preimmunotherapy era (Colomo and Hans, Figure 2.A and 2.B) and the other 3 were used in cohorts of patients treated with rituximab (Muris, Choi and Tally, Figure 2.C, 2.D, 2.E). The biomarkers used included the GCB markers CD10, BCL6, GCET1, and LMO2 and antigens related to postgerminal center (non-GCB) differentiation, such as MUM1/IRF4 and FOXP1.

One of the earliest attempts to classify by IHC was the Colomo algorithm, which established non-GCB or GCB phenotype according to three markers (MUM1/IRF4, CD10 and BCL6) as detailed in figure 2.A, but did not predict clinical outcome (20).

In 2004 Hans *et al.,* proposed the most commonly used algorithm based on the expression of three markers to distinguish the GCB from the non-GCB subtype: CD10, BCL6, and IRF4/MUM1 (Figure 2.B), which has an accuracy of 85-93% in comparison with the GEP (12,21).

Additional proposed methods included Muris algorithm, which assesses BCL2, CD10 and MUM1 to classify GCB or non-GCB phenotype and two prognostic groups (1 and 2) (23).

On the other hand, two additional indicators were added to the Hans algorithm by Choi *et al.*: FOXP1 and GCET1. FOXP1 expression has been described in around 20% of DLBCL cases without the germinal center phenotype (22) and GCET1 was highly correlated with the GCB type (25).

Lastly, the Tally algorithm, proposed by Meyer *et al.,* (2011), uses LMO2, a marker of germinal center B-cells, instead of BCL6 from the Choi algorithm (24). This algorithm included the same number of GCB markers (GCET1 and CD10) and non-GCB markers (FOXP1 and MUM1/IRF4) to determine the phenotype (GCB or non-GCB) (26).

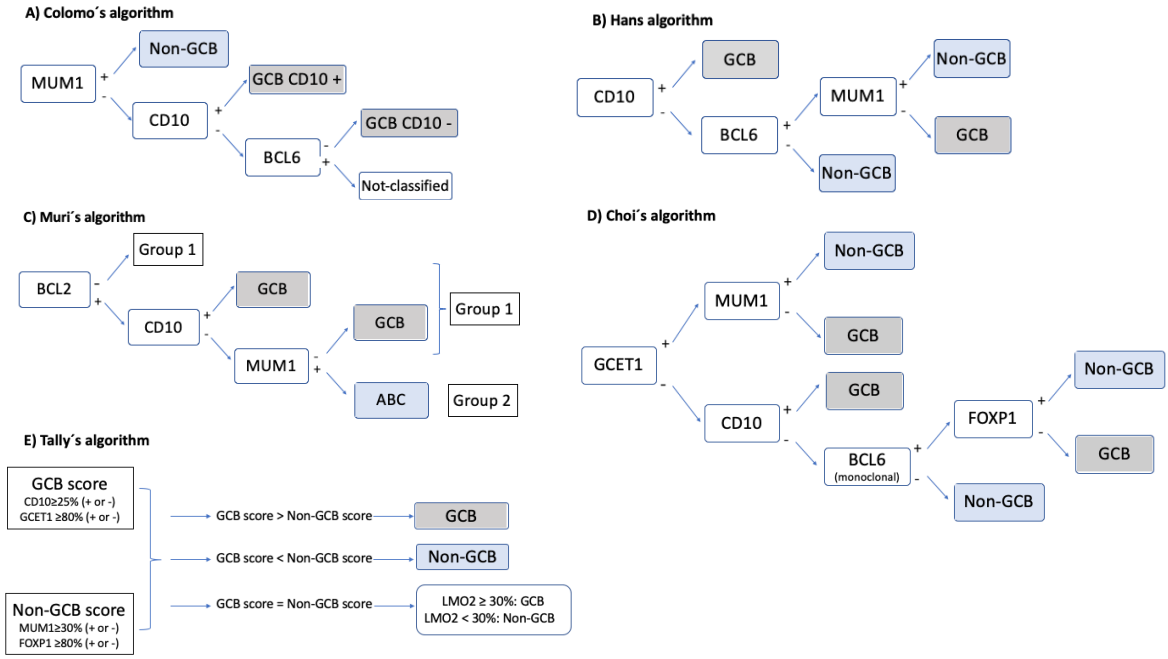

Figure 2: Algorithms to approximate Molecular Subtypes based on Immunohistochemical patterns. (A) Colomo´s algorithm, (B) Hans' algorithm, (C) Muri´s algorithm, (D) Choi´s algorithm, and (E) Tally´s algorithm.

However, there were discrepancies in the clinical outcomes predicted by these different classifiers, and ultimately, none of them was robust enough to classify DLBCL compared with the gold standard GEP classifier. It is recognized that the IHC algorithms do not recognize the 10%-15% tumors that are also unclassified by GEP, and, in addition, they have reproducibility issues, and their prognostic utility is controversial (1).

II.c. Other techniques to determine the COO

Most recently, DLBCL COO was identified by using GEP methods applied to formalin-fixed paraffin-embedded (FFPE) tissue sections and a more simplified digital gene-expression signature (Lymph2Cx) using a nanostring platform (27).

On the other hand, quantification of RNA expression in FFPE tissues newer technologies such as digital PCR have been shown to provide concordant results with conventional microarray GEP, are more reproducible between laboratories, and capture the prognostic impact of the COO classification (28). However, these methods are still not accessible to most laboratories, although they may be a promising alternative to the IHC algorithms (1).

Risk assessment and treatment options

The protocol used up in Spain for DLBCL treatment follows the Spanish Lymphoma Group (GELTAMO) guidelines. It has several versions, with slight differences among them. The last recommendations are described below (12) .

Since one of the main characteristics of this pathology is its great heterogeneity in clinical behavior and response to chemotherapeutic agents, nowadays, treatment strategies are stratified based on risk factors, including the extent of disease (limited or advanced stage, depending on whether the tumor can be contained within one irradiation field), the age of the patient, and their International Prognostic Index (IPI) score.

Risk assessment

The tumor stage of patients with aggressive non-Hodgkin's lymphoma is currently determined with the Ann Arbor classification (29).This classification was originally developed for Hodgkin's disease and, thus, it is less accurate for stablishing prognostic subgroups in non-Hodgkin's lymphoma (30).

A complete clinical history, including Eastern Cooperative Oncology Group (ECOG) functional status and the presence of B symptoms, and a complete physical examination, with particular emphasis on identifying lymphadenopathy and visceromegaly, are performed. Moreover, the following analyses are performed: blood count; biochemical analysis including lactate dehydrogenase (LDH), beta2 microglobulin, urate, liver and renal function; proteinogram; and serology for hepatitis B (HBs antigen, anti-HBc), hepatitis C and human immunodeficiency virus (HIV) (12).

The IPI is still the most commonly used tool for prognostication in DLBCL patients based on clinical parameters. This indicator is bases on five clinical factors: age, stage, the number of extranodal sites, performance status, and LDH (Table 3). The IPI segregated patients into 4 outcome groups with differentiated 5-year overall survival (OS) (26%-73%). A low IPI score (0-2) identified patients with better OS in comparison with patients with high IPI score (3-5). Age-adjusted IPI is used for patient ≤60 years, where all the prognostic factors in main IPI are used except for age and extranodal involvement to calculate the score (31).

Table 3: The International Prognostic Index (IPI) and age-adjusted IPI for Non-Hodgkin's Lymphoma (aa-IPI) (6,31)

Risk Factor	Points	Risk Group	5 years OS (%)
IPI			
Age		• Low (0 or 1)	73
≤ 60 years	0	• Low-intermediate (2)	51
> 60 years	1	• High-intermediate (3)	43
Ann Arbor stage		• High (4 or 5)	26
Stage I or II	0		
Stage III or IV	1		
ECOG performace status			
0 or 1	0		
≥ 2	1		
Serum LDH			
≤ 1 x normal	0		
> 1 x normal	1		
Extranodal sites			
≤ 1	0		
> 1	1		
aaIPI (≤ 60 years)			
Ann Arbor stage		• Low (0)	83
Stage I or II	0	• Low-intermediate (1)	69
Stage III or IV	1	• High-intermediate (2)	46
ECOG performace status		• High (3)	32
0 or 1	0		
≥ 2	1		
Serum LDH			
≤ 1 x normal	0		
> 1 x normal	1		

In order to better stratify the patients in risk groups, especially patients with the worst prognosis, other prognostic indexes have been developed, such as R-IPI, NCCN-IPI and GELTAMO-IPI.

Since the end of the last century, the addition of rituximab (R) to conventional CHOP or CHOP-like regimens has resulted in a major improvement in survival across all risk groups of DLBCL (32). For this reason, the IPI has been reevaluated in patients treated with rituximab-based therapy and has been shown to retain its prognostic utility. The R-IPI identifies 3 differentiated prognostic groups with a very good (4-year OS 94%), good (4-year OS 79%), and poor (4-year OS 55%) outcome, respectively (33).

Recently, The National Comprehensive Cancer Network (NCCN)-IPI and GELTAMO-IPI were developed to enable better risk prediction of patients with DLBCL. The NCCN-IPI incorporates the same 5 variables, although further refines the categorization of age and normalized lactate dehydrogenase and the identification of disease involvement at specific extranodal sites. Compared with the IPI, the NCCN-IPI was able to better discriminate a high-risk group with 5-

year OS of 33%, although it represented only 8% to 14% of the patients (34). The GELTAMO-IPI incorporated the elevation of beta-2 microglobulin (B2MG) above the upper limit of normality and they reported a better discrimination of high-risk DLBCL patients compared to the NCCN-IPI (5-year OS 39% vs. 49%) (35).

Treatment options

The GELTAMO treatment protocol is presented as collected from their recently published guidelines (12):

I) First-line therapy

First-line treatment can be stratified based on the extent of disease (limited stage or disseminated), the age of the patient, and their IPI score.

Ia) Limited stage disease (Ann Arbor I-II)

The current standard is combination chemotherapy with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) treatment combined with rituximab every 21 days. Patients with non-bulky (<7 cm) limited-stage DLBCL are treated with four cycles of rituximab-CHOP (R-CHOP) and patients with high risk factors are treated with two additional cycles of R-CHOP. In addition, those patients who did not achieve complete remission after four cycles of R-CHOP underwent two additional cycles of R-CHOP, and involved-field radiation therapy (IFRT) is recommended (36).

Patients with bulky (>7 cm) stage II disease have a less favorable prognosis than those with non-bulky stage II disease and, in those cases, R-CHOP×6 + IFRT is recommended. Unfortunately, despite the addition of radiation therapy, the presence of bulky disease remained a poor prognostic factor and survival rates remain worse than those of patients without bulky disease (37).

Ib) Disseminated disease (Ann Arbor III-IV)

- Patients aged 60–80 years

The standard treatment is six to eight cycles of R-CHOP every 21 days. Other options have also been explored; however, the addition of bortezomib or the substitution of rituximab with obinutuzumab (a type II anti-CD20 antibody) did not improvesurvival (38,39). In addition, there is controversy regarding the use of radiotherapy to treat extranodal lesions or bulky disease (40).

- Low or low-intermediate risk patients (IPI score, 0–2) aged <60 years

The standard treatment in these patients is also six to eight cycles of R-CHOP every 21 days (41,42). Furthermore, patients with bulky disease received radiotherapy. Although other more intensive regimens have been shown to increase survival inyoung patients with DLBCL and an age-adjusted (aa)-IPI=1, they have also been associated with higher toxicity (43,44).

- High-risk patients (IPI score, 3–5) aged <60 years

While there is no standard treatment for this group of patients, they are usually treated with six to eight cycles of R-CHOP every 21 days. Regimen based on DA-EPOCH-R [dose-adjusted-(etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin-rituximab)] (45) could be an alternative for these patients (46). On the other hand, the use of high-dose chemotherapy followed by transplantation of autologous hematopoietic stem cells has been investigated but contradictory results were obtained (47).

- Patients with comorbidities or aged >80 years

There is no standard treatment for patients of over 80 years of age. A geriatric assessment is recommended to identify "fit" patients who can undergo chemotherapy. The combination of rituximab and polychemotherapy with doxorubicin induces complete remission and prolongs survival. Therefore, it is recommended to use conventional treatments such as R-CHOP, or an attenuated immunochemotherapy regimen (48). In patients with cardiac pathologies, adriamycin can be omitted (R-COP) or substituted with liposomal doxorubicin or other agents such as mitoxantrone, etoposide, or gemcitabine (49–51)

II) Second-line and subsequent therapy

About 30-40% of patients are resistant to treatment or relapse after receiving first-line treatment and, consequently, salvage therapy would be required.

IIa) High-dose chemotherapy in hematopoietic stem cell transplantation (HSCT) candidates

Salvage immunochemotherapy is the treatment of choice in young patients (<60–70 years) without comorbidities. In chemosensitive patients, the response should be consolidated with high-dose chemotherapy and auto-HSCT (52). However, no specific salvage regimen has demonstrated superiority over others; R-DHAP (rituximab-dexamethasone, cytosine arabinoside and cisplatin), R-ICE (rituximab-ifosfamide, carboplatin and etoposide) (53) or R-GDP (rituximab, gemcitabine, cisplatin and dexamethasone) showed similar efficacy (54), with a very poor progression free survival (PFS) of around 20% (55). As a result, the best option for these patients appears to be the enrollment in clinical trials with regimens that include new drugs(12).

The main prognostic factor at the time of transplantation is lymphoma status because patients with complete remission have significantly better survival than patients in partial remission (PFS: 72%–87% vs. 18%–49%) (56,57). The BEAM (BCNU, etoposide, cytarabine and melphalan) conditioning regimen is the most widely used in Europe (12). Allogeneic hematopoietic stem cell transplantation (allo-HSCT) may be effective in patients with relapse or progression after multiple lines of treatment, even after auto-HSCT has failed, and it could be also an alternative to auto-HSCT for patients with very high-risk features, such as primary refractory disease (58,59).

IIb) Non-auto-HSCT candidates

There is no standard regimen established for these patients and durable remissions are uncommon.

R-GemOx (rituximab-gemcitabine and oxaliplatin) results in overall response rates of about 60%, with a 1-year OS rate of 45% and a PFS of around 25% (60). However, whenever possible these patients should be enrolled in clinical trials (12).

III) Third-line and later lines of therapy

Third-line therapy is an option in patients who do not respond to second-line therapy. In some cases, patients may be treated with second-line polychemotherapy regimens not previously used or with monotherapy with drugs such as pixantrone (61) or lenalidomide, which can also be combined with rituximab (62,63). There are no clinical trial data to indicate which other drugs in monotherapy (gemcitabine, oxaliplatin, etoposide, mitoxantrone and vinorelbine) are most appropriate in each case. On the other hand, two treatment regimens at metronomic doses (chronic, equally spaced administration of low doses) have been analyzed: celecoxib, methotrexate, and cyclophosphamide, , and the PEP-C (prednisone, etoposide, procarbazine-cyclophosphamide) regimen (64). However, limited response rates were obtained with these different approaches and inclusion of these patients in clinical trials is recommended whenever possible (12).

New proposals for prognosis stratification

Taking into account the bad outcome and limited therapeutic options for 30-40 % of patients, which are refractory or relapse, a better understanding of lymphoma biology and identification of new molecular markers with potential prognostic significance has led to multiple attempts to improve prognostication of patients with DLBCL.

A number of **laboratory parameters** such as serum albumin or β2M were combined with the IPI variables (35,65) . This parameter was also combined with other clinical parameters (66) and/or laboratory parameters (67), as well as IHC (68) for a better prediction of prognosis.

On the other hand, multiple signatures based on **gene expression** that are predictive for survival outcomes have been identified in diffuse large B-cell lymphoma. The lymphoma/leukemia molecular profiling project reported a 17-gene predictor (69) , and a 3-component signature (~ 400 genes) (70). Lossos *et al.,* built a predictive model based on 6 genes (71). Alizadeh *et al*., further simplified this model to a 2-gene model as risk predictor (72). Although gene-based predictors have good discrimination ability, when used alone, the IPI remains the most powerful predictor of the clinical outcome of patients with DLBCL (73).

The cell of origin phenotype determined by GEP is also a major prognostic factor in DLBCL (74). GCB and non-GCB DLBCLs were associated with statistically significant differences in OS and in event free survival (EFS). While GCB DLBCL patients has an average five-year OS of 76%, the OS was 16% in the group of non-GCB DLBCL patients. Thus, the molecular differences between these two kinds of lymphoma were also accompanied by a great divergence in clinical behavior, suggesting that GCB and non-GCB DLBCL should be considered as distinct diseases (14).

On the other hand, regardless of translocations, expression of *MYC* and *BCL2* in DLBCL appears to independently predict inferior overall and progression-free survival in DLBCL (75,76).

More recently, Next Generation Sequencing (NGS) studies have identified common somatic mutations that are present in all subgroups of DLBCL, but also a specific profiles of mutations that only appear in either GCB or non-GCB subtypes. Somatic mutations that can be found in both DLBCL subtypes include inactivating mutations of *TP53* and genes involved in immunosurveillance (*B2M*, *CD58*), changes in epigenetic regulators (*CREBBP/EP300*, *KMT2D/C*, *MEF2B*), and activating mutations in *BCL6*. On the other hand, GCB DLBCL present common alterations in the histone methyl transferase *EZH2*, and in the cell motility regulator *GNA13*, while non-GCB DLBCL have mutations in genes (*MYD88*, *CD79A*, *CARD11*, *TNFAIP3*) activating the B-cell receptor/Toll-like receptor and NF-κB pathways. However, the clinical implications of these mutations are not fully understood (1).

Although many models have been described as useful to assess prognosis, there is no model that accurately predicts which patients would be refractory or relapse. The utility of these factors in clinical practice is, thus, limited, and the therapeutic decisions based on these markers are restricted to clinical trials at the moment.

Therefore, since early detection of treatment resistance is of pivotal importance for successful cancer therapy, it is important to search for new therapeutic markers for DLBCL treatment.

NON CODING REGIONS

The Human Genome Project (HGP) revealed in 2001 that the genes encoding proteins are about 20.000-25.000, which represents a very small fraction of the genome (only 1.5% approximately), a proportion that increases to 2% if untranslated regions are considered (77).

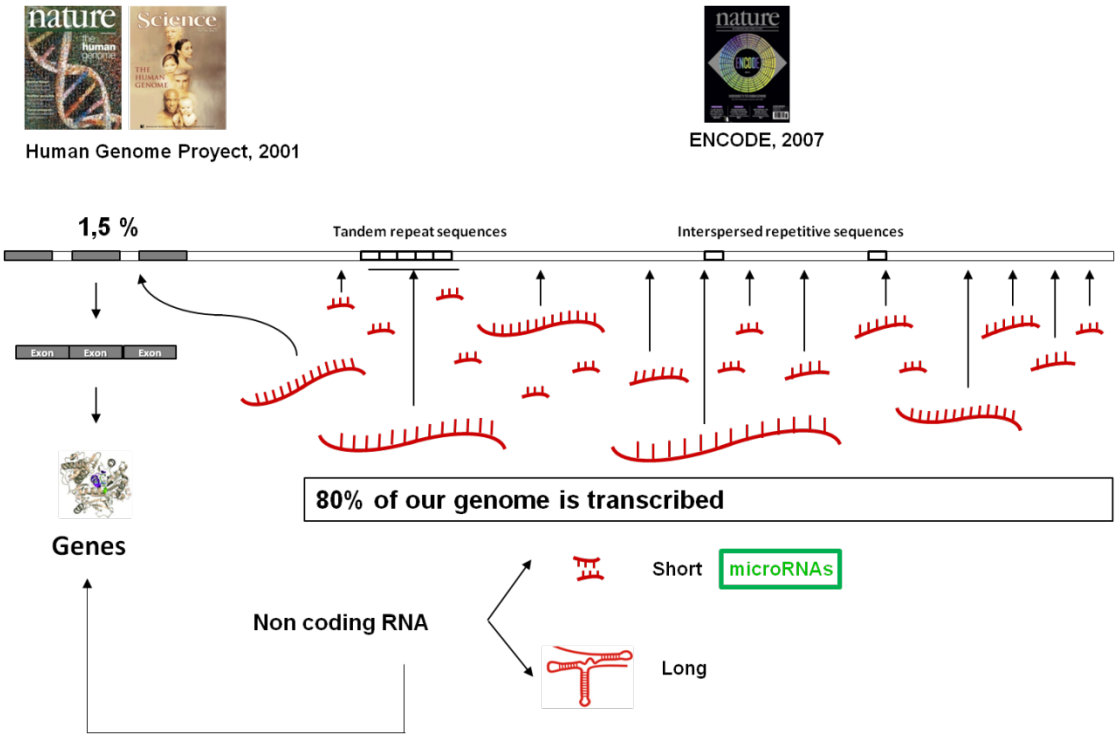

Figure 3: Transcription of our genome

However, new challenges arise in order to interpret the data generated by the HGP, and a subsequent Project called ENCODE (Encyclopedia of DNA Elements) was launched. The Project aimed to prepare a complete catalog which contained all functional elements codified in the human genome, and one of the main conclusions of the first phase was that about 80% of our genome is transcribed as elements that do not encode proteins. Early considered "junk DNA", it was determined that a large part of the non-protein coding regions was functional. These elements are known as non-coding RNAs (ncRNA) (Figure 3) (78).

NcRNAs are primarily classified by their length. Among the small non coding RNAs, microRNAs are the most studied and their involvement in cancer has already been confirmed (79).

MicroRNAs

MicroRNAs comprise a large family of single stranded RNAs of about 18-22 nucleotides in length that have emerged as key negative regulators of gene expression at the post-transcriptional level (80). MicroRNAs are transcribed from different locations in the genome by RNA polymerase II into long primary transcripts called pri-microRNAs (300-5000 bp). The pri-microRNAs are characterized by a central region of double-stranded RNA (dsRNA) of about 30-40 nucleotides, a terminal loop and two single-stranded RNAs (ssRNA) opposite among each other. These pri-microRNAs are processed in the nucleus by the complex formed by DROSHA RNase and DGCR8, which contains dsRNA binding domains. The dsRNA sequence determines its secondary structure and its capacity to bind RNA processing proteins (81).

Processing of pri-microRNAs results in smaller molecules (about 70 nucleotides) known as pre-microRNAs. Pre-microRNAs are exported from the nucleus to the cytoplasm through Exportine5 (XPO5) and RAN GTPase protein (82,83). In the cytoplasm, pre-microRNAs are processed by Dicer (84,85) and TARBP2, which eliminate the loop, generating a dsRNA molecule known as microRNA duplex (86). The two strands in the microRNA duplex are separated to form the mature microRNA as a single strand. The selected strand of the microRNA duplex is incorporated into a multiprotein complex known as RISC (RNA-inducing silencing complex), composed by EIF2C1 (AGO1), EIF2C2 (AGO2), SND1, GEMIN3, GEMIN4 and CCR-NOT complex (87). The mature microRNA is transported by the RISC complex to the messenger RNAs (mRNAs), which are the target of this mechanism of regulation (88) (Figure 4).

For target recognition, microRNAs present a specific sequence of approximately 7 bp, known as seed region. The microRNA acts by specific binding of the seed sequence to a complementary target sequence in the 3´ UTR region of the mRNA. The regulation mechanism depends on the degree of complementarity between microRNA and mRNA: direct cleavage and degradation of the mRNA, when the complementarity is perfect; protein translation blocking/inhibition in the case of imperfect base pairing (89).

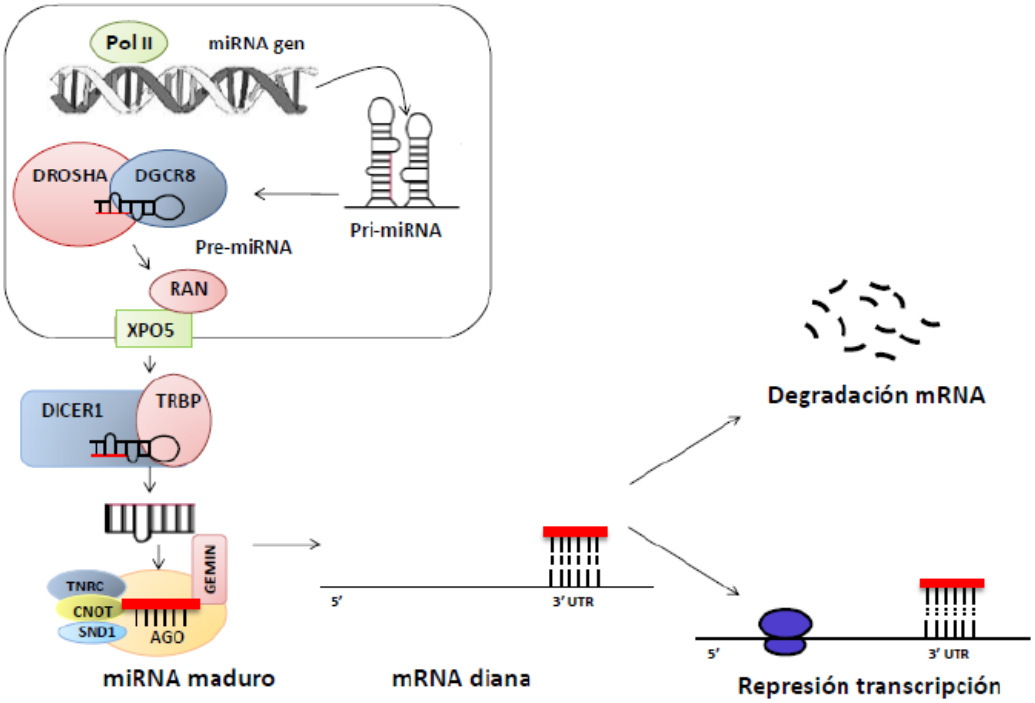

Figure 4. MicroRNA biogenesis and mechanism of action.
Source: Adapted from Ryan *et al.,* 2010 (Ryan, Robles, and Harris 2010)(90).

Since the complementary sequence is short, a microRNA may degrade or repress translation of many target mRNAs containing complementary sequences to its seed region. Additionally, a single gene may also be regulated by multiple microRNAs.

Through this mechanism of regulation, microRNAs could regulate more than 50 % of human genes, including genes involved in cancer (91). However, microRNA-mediated gene regulation can be affected by changes in the levels of microRNAs. Consequently, microRNA expression has shown potential as diagnostic, classification and prognostic predictors in cancer.

MicroRNA expression in human cancer

MicroRNA profiling and deep sequencing provided direct evidences that microRNA expression is deregulated in human cancer. This deregulation occurs through various mechanisms, including amplification or deletion of microRNA genes, abnormal transcriptional control of microRNAs, deregulated epigenetic changes and defects in the microRNA biogenesis machinery. The resulting deregulated microRNAs have been shown to affect pathways of relevance in cancer, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, activating invasion and metastasis, and inducing angiogenesis (92).

Interestingly, microRNA expression signatures could distinguish tumor cells from normal tissues, and allowed different types of cancer to be differentiated very accurately and the identification of the tissue of origin of poorly differentiated tumors (93,94). It is remarkable that microRNAs may function as either oncogenes or tumor suppressors under certain conditions. In addition, distinct microRNA signatures allowed most cancers to be further subclassified into prognostic groups.

Tumor suppressive microRNAs

The first report suggesting the involvement of microRNAs in cancer was provided by Dr. Croce's group attempting to find tumor suppressor genes within a chromosomal region (13q14) that is often deleted in patients with B cell chronic lymphocytic leukemia (B-CLL). Instead of finding a canonical tumor suppressor gene that encodes a protein, they found two microRNAs that acted as tumor suppressors, miR-15 and miR-16-1. This result provided the first evidence that microRNAs could have a role in the pathogenesis of human cancer (95). After this first study a great number of microRNAs have been found to be downregulated in cancer.

Among the microRNAs that are downregulated in cancer and, therefore, exert a tumor suppressive function, the miR-34 family (miR-34a, miR-34b and miR-34c) has received substantial attention. These microRNAs are downregulated in lung, breast and many other cancers (96,97). The transcription of all three microRNAs is activated by the tumor suppressor p53 during the DNA damage response (98). In fact, studies suggest that p53 specifically modulates the tumor immune response by regulating *PDL1* via miR-34 (99).

On the other hand, several studies have demonstrated that let-7 family of microRNAs is lost or downregulated in various cancers such us breast, lung or prostate cancer (100–103). The functional implications are not fully elucidated but downregulation of let-7 in cancer cells results in fast tumor progression through alterations in signaling pathways involving the RAS family of proteins (104).

In addition, miR-506 was shown to be downregulated in ovarian cancer in comparison with normal ovarian tissues and its downregulation was reported to promote metastasis (105). MiR-520 has also been shown to be downregulated in ovarian cancers, as well as in breast cancer. This microRNA could be a tumor suppressor, downregulating the expression of *TGFBR2*, which encodes a TGF signaling receptor protein that promotes metastasis (106,107).

Finally, the miR-200 family, which modulates the expression of proteins involved in tumor metastasis, epithelial mesenchymal transition, and angiogenesis, is also downregulated in cancer (108,109).

Oncogenic microRNAs

As opposed to the role of microRNAs as tumor suppressors, microRNAs have also been identified as oncogenes.

For instance, numerous studies have shown that miR-21 is significantly upregulated in tumors compared with normal tissues (110–112). In fact, an amplification of the chromosomal 17q23.2 region, which includes the gene encoding miR-21, has been reported in several tumors, such as breast, lung, hepatocellular, ovarian and prostate cancers (113).

Other important tumor promoting microRNAs are miR-155, which acts as an oncomiR in lymphoma and solid tumors (113–117), and miR-210, which has been described as a relevant target of the hypoxia inducible factor 1α (HIF1α), promoting cancer cell survival (118).

The miR-17-92 cluster, which includes miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a, has been shown to be upregulated in several tumors (119), which was mediated by the upregulation of its host gene *MIR17HG* by the oncogenic protein MYC. The miR-17-92 cluster has multifaceted roles: for example, it downregulates the pro-apoptotic BIM protein in B lymphocytes, resulting in decreased apoptosis (120).

Other malignancies, such as metastatic breast cancer, glioblastoma and melanoma, show miR-10b significantly upregulated (121–123), and miR-221 is significantly upregulated hepatocellular carcinoma is, targeting key tumor suppressors, such as *CDKN1B*, *PTEN* and *TIMP3* (124,125).

MicroRNAs as prognostic and predictive biomarkers

In addition to their role as tumor suppressors or oncogenes, several groups have reported the potential of microRNAs as prognostic markers to predict cancer outcome. For example, miR-155 overexpression and let-7a downregulation in lung cancer were linked to poor disease outcome (126). Likewise, low miR-191 and high miR-193a expression were associated with shorter survival in melanoma (127). In gastric cancer, a signature comprised of seven microRNAs could predict OS and EFS (128).

Furthermore, some microRNAs could be of use to predict response to specific therapies, which could be of translational value to the clinic. Some microRNAs can be upregulated in drug resistance, such as miR-19 and miR-21 in breast cancer and miR-221/222 cluster in lung and liver cancer (124,129,130).

Others, like miR-130a and miR-298, are downregulated, suggesting an important biological role of microRNAs in this process (131,132). In other cases, the effect of microRNAs in drug resistance is tissue specific. For instance, miR-27a is indirectly related to drug sensitivity in ovarian cancer, but it can be directly involved in drug resistance in leukemia (133).

On this basis, microRNA profiling for patient prognosis and treatment response are now underway. In addition to their use as prognostic biomarkers, the correlation between microRNA expression and response to specific therapies has also suggested their promising potential as therapeutic adjuvant (134). The ability of microRNAs to target multiple mRNAs altered in disease conditions points them out as interesting candidate therapeutics (in the form of pre-microRNAs, mature microRNAs, microRNA mimics…) or as promising targets of therapy (in the form of antimiRs, microRNA inhibitors…)(135).

MicroRNAs in hematologic malignancies

Abnormal expression of microRNAs is also common in B cell neoplasms, including lymphoma. It is known that precise gene dose regulation by microRNAs is needed for a proper lineage decision in hematopoietic cells and its disruption leads to malignant transformation. MicroRNAs have critical regulatory roles in the immune system ranging from stem cell maintenance to immune cell functions including B-cell maturation and immunoglobulin production (136).

Regarding microRNAs proposed as biomarkers of leukemia, miR-155 overexpression showed consistency across multiple studies in acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL), and diffuse large B-cell lymphoma (DLBCL),

as well as in other hematologic disorders (137–139). In addition, miR-150 has been shown to be lowly expressed in pediatric patients with ALL compared to healthy controls (140), and several microRNAs, such as miR-720, miR-1246, miR-451, miR-1915, miR-1308, and miR-638, have been found as differentially expressed in multiple myeloma patients compared with healthy individuals (141).

Further, microRNA expression has been used to distinguish between the leukemic types. For example, miR-128a, miR-128b, let-7b and miR-223 were found to be sufficient to discriminate between ALL and AML (142).

Finally, microRNAs have been also associated with prognosis in hematologic malignancies. For instance, increased levels of miR-146a were associated with more favorable prognosis in acute myeloid leukemia (AML) (143), while underexpression of miR-122 had been well studied to be unfavorable prognosis predictor of AML (144).

Taking all of this into account, evaluation of the expression levels of specific microRNAs in DLBCL tissue samples may be used to diagnosis and characterization, predict the cancer prognosis, and as markers for therapy selection and response. However, up to date, there is inconsistency in the data reported. For instance, miR-150-5p was found to be downregulated in DLBCL in four studies (145–148), while it was found to be upregulated (149) in another study and unchanged in another one (150). Similarly, miR-222-3p was associated with good prognosis in four studies (151–154) while no association was observed in other four studies (147,150,155,156). Therefore, future studies need to elucidate further the potential application of microRNAs as specific and sensitive markers for clinical diagnosis, classification, or prognosis in DLBCL.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Despite the current greater understanding of DLBCL pathological mechanisms and the new tools developed for the classification and management of patients, including the addition of rituximab-based chemoimmunotherapy, 40% of them still have primary refractory disease or relapse due to the high heterogeneity and rapid emergence of drug resistance. To improve survival rates, there is an urgent need for an in-depth understanding of DLBCL pathogenesis and drug-resistance mechanisms, as well as the definition of new biomarkers with prognosis potential. Recently, important knowledge is emerging regarding the participation of microRNAs in the regulation of the biological behavior of cancer cells and drug resistance. Moreover, recent investigations point to microRNAs as useful biomarkers in cancer.

Therefore, in this work we propose that microRNAs have a role in DLBCL origin, differences among subtypes and response to treatment, and could be used as biomarkers for diagnosis, classification, prognosis, and treatment response in DLBCL.

OBJECTIVES

The main goals of the present study were to determine a microRNA set with utility in DLBCL diagnosis, classification, prognosis and treatment response, as well as to decipher the mechanism of action of deregulated microRNAs in the origin of the disease.

In order to achieve these goals, we set the following specific objectives:

1. To determine the relevance of microRNAs previously associated with DLBCL diagnosis, classification, treatment response and prognosis:
    a. Identification of microRNAs previously proposed as biomarkers in DLBCL through a systematic review.
    b. Validation of the utility of the identified microRNAs as biomarkers in a new cohort of patients diagnosed and treated according to the GELTAMO guidelines.
2. To define a new signature of microRNAs of use as biomarkers in DLBCL through the analysis of all annotated microRNAs using small RNA sequencing.
3. To identify the mechanism of action of deregulate microRNAs in DLBCL using a microRNA-mRNA interaction network analysis.

MATERIALS AND METHODS

SYSTEMATIC REVIEW

Search strategy

A systematic search with the terms [((“Non-coding RNA”) OR (“miRNA” OR “microRNA” OR “miR”) OR (“exosome”) OR (“extracellular vesicle”) OR (“secretome”)) AND (“Diffuse large B cell lymphoma” OR “DLBCL”)] was performed using PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/), including articles published until March 2020.

Inclusion and exclusion criteria

Independent original studies that evaluated the expression of microRNAs in DLBCL tumor tissue as diagnosis, subtype, prediction of treatment response or prognosis biomarkers in human patient populations were included. Exclusion criteria encompassed: articles not including original data (reviews, meta-analyses, letters, and comments), case reports, abstracts, articles not published in English, studies that did not include microRNA data on human populations, and studies on diseases other than DLBCL. After full text revision, articles that included other diseases, analyzed circulating microRNAs, were focused on non-primary DLBCL, did not assess the role of microRNAs in diagnosis, subtype, treatment response, or prognosis, or did not analyze microRNA expression, were excluded. References within the identified studies were reviewed to identify additional matches. Study selection was performed by two researchers independently and disagreements were resolved by consensus.

Data extraction

The following information was extracted from each study: publication year, type of tissue sample analyzed, characteristics of the study population, methodology, number of microRNAs studied, and the list of differentially expressed microRNAs provided. Only the microRNAs that were reported as statistically significant in more than two studies with consistent results were selected.

MICRORNA EXPRESSION ANALYSIS

Population of study

The study included FFPE samples from a total of 78 adult patients diagnosed with DLBCL and treated with R-CHOP or similar chemotherapy regimens and 17 adult controls. The study was approved by the Clinical Research Ethical Committee of the Basque Country (P2016121). Signed informed consent was obtained from each participant and the study was carried out according to the Declaration of Helsinki.

Patient samples were obtained at diagnosis from tumor specimens of 78 DLBCL patients (50 patients with long-term complete remission, 12 patients with refractory disease, and 16 patients that relapsed within 10 years after diagnosis). Samples were collected from 1999 to 2018 at the Hematology Units of 3 Spanish reference hospitals (Cruces University Hospital, Donostia University Hospital, and Araba University Hospital). Control samples were obtained from non-tumoral ganglia from individuals without DLBCL, which were collected at Araba University Hospital.

Demographic and clinical data were collected from patient's medical files by two independent clinical researchers. For each patient the collected data included: age, sex, clinical stage (I-IV), B symptoms (yes/no), IPI score (0-5), subtype (GCB or non-GCB), response to treatment (Complete response (CR), not complete response (not CR)), LDH (normal or high), β-2 microglobulin (normal or high), outcome, PFS and OS at 5 years (Table 4). Demographic information was also obtained for the controls (Table 4).

Table 4: Demographic and clinical characteristics of DLBCL patients at diagnosis (n=78)

Variables	Patients with DLBCL (n=78)	Controls (n=17)
Age		
Mean (range)	58.66 (21-81)	67.76 (31-86)
≥60	43	4
<60	34	13
NA	1	0
Sex		
Male	41	9
Female	37	8
Stage		
I	7	
II	11	
III	23	
IV	27	
NA*	10	
B symptoms		
Yes	31	
No	31	
NA*	18	
IPI scores		
Low risk (0-1)	15	
Low to intermediate risk (2)	13	
Intermediate to high risk (3)	15	
High risk (4-5)	20	
NA*	15	
Subtype IHC or molecular		
GCB	32	
Non-GCB	21	
NA*	25	
Therapy response		
CR	50	
not CR	28	
LDH		
Normal	23	
Augmented	40	
NA*	17	
β2-MG, β 2 microglobulin		
Normal	22	
Augmented	39	
NA*	17	
5 years PFS		
Median, months	39.68	
5 years OS		
Median (range), months	46.63	

Abbreviations: CR, complete response; DLBCL, diffuse large B-cell lymphoma; GCB, germinal center B cell; IHC, immunohistochemistry; IPI, International Prognostic Index; β2-MG, β 2 microglobulin; LDH, lactate dehydrogenase; PFS, progression free survival; OS, Overall survival; NA, not available; *Several variables contain missing values due to the lack of information in patient´s medical files

Sample processing and RNA isolation

Samples and data from patients included in this study were provided by the Basque Biobank (www.biobancovasco.org) and were processed following standard operation procedures.

RNA was isolated using column-based RNA extraction methods that co-isolate small and large RNA molecules with the miRNeasy FFPE Kit (Qiagen, Hilden, Germany).

Small RNA-seq library preparation and sequencing

TruSeq small RNA Sample Prep Kit (ref. RS-200-0012, Illumina) was used according to the manufacturer's protocol. Briefly, 1 µg of total RNA was used. First, 3' adapters and subsequently 5' adapters were ligated to the RNA. cDNA was synthesized using reverse transcriptase (SuperScript II, ref. 18064-014, Invitrogen) and a specific primer (RNA RT Primer) complementary to the 3' RNA adapter. cDNA was further amplified by PCR using indexed adapters supplied in the kit. Finally, libraries were size selected using 6% Novex® TBE Gels (ref. EC6265BOX, Life Technologies). Fragments with insert sizes of 18 to 36 bp were cut from the gel, and DNA was precipitated and eluted in 10 µl EB.

Final libraries were analyzed using Agilent DNA High Sensitivity chip to estimate the quantity and check size distribution and were then quantified by qPCR using the KAPA Library Quantification Kit (ref. KK4835, KapaBiosystems) prior to amplification with Illumina's cBot. Libraries were sequenced Single Read, 50nts (v4) on Illumina's HiSeq 2500. The sequencing was carried out with a depth of approximately 10 million reads per sample. All the process was carried out at the Centre for Genomic Regulation (CRG).

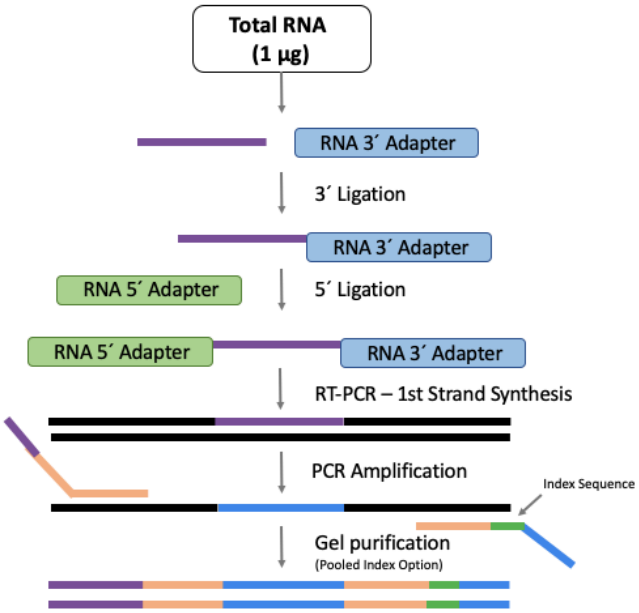

Figure 5: Truseq RNA sample preparation (Adapted from Truseq sample preparation guide. https://support.illumina.com

Bioinformatic analysis and differential microRNA expression

Reads were trimmed for the presence of the small RNA adapter using Skewer and filtered removing every sequence shorter than 15 and longer than 40 bases. The remaining ones were then aligned to the reference genome (GRCh38) using ShortStack and the resulting alignments used by htseq-count for determining the number of tags per gene. We used the annotation v27 from Gencode consortium. Finally, the count matrix was reduced to only microRNA genes.

Every small RNA with less than 10 reads considering the sum of the reads in every condition was excluded and we used the remaining genes for differential expression (DE) analysis with DESeq2. DESeq2 takes care of normalizing and transforming the read counts, estimates the dispersion and runs a Wald Test for detecting the significant DE genes. P-values were adjusted using False discovery rate (padj).

Principal component analysis (PCA) was conducted to reduce the dimensions of large data sets and to explore the naturally arising sample classes based on the expression profile. This allows the projection of the initial data set into a reduced space spanned by mutually independent axes (principal components) explaining the relevant part of total variance of the system. By including the expression of all microRNAs, we can obtain an overview of how the samples cluster based on this variance. The normalized log-transformed values were used for the analysis. The largest component in the variation is plotted along the X-axis, and the second largest is plotted on the Y-axis. PCA was performed using the prcomp package from R to analyze the transformed read counts.

Survival analysis

Survival analyses were conducted to estimate the effect on PFS and OS of those microRNAs differentially expressed (adjusted p-value <0.05; $\log_2$ fold change > 2 or <-2) between samples at diagnosis from patients with long-term remission and those that relapsed. The median value of the expression of deregulated microRNAs (miR-4444, miR-449c-5p, miR-3681-5p, miR-3928-3p, miR-449b-5p, miR-370-3p and miR-4424 as good prognosis biomarkers, and miR-133a-3p, miR-208b-3p and miR-205-5p associated with bad prognosis) was considered to categorize patients into low or high expression groups. Kaplan–Meier analysis was used to define the survival curves and the log-rank test was used to assess significance. For the multivariate analysis, we also considered the subtype and IPI status using the Cox proportional hazards (Cox PH) method. All calculations were performed using the Survival R package. Significant associations with survival were those with p-value <0.05.

MicroRNA-mRNA interaction network

Using our own data on microRNA expression (DLBCL patients vs controls) and mRNA expression data contained in the Gene Expression Omnibus (GEO) database (157), we constructed a microRNA-mRNA interaction network to elucidate the mechanism DLBCL developing.

Identification of target genes

For those microRNA deregulated at diagnosis (DLBCL patients vs controls), target genes were identified. MirTarbase (158)(http://mirtarbase.cuhk.edu.cn/php/index.php) was used to search for experimentally validated microRNA-mRNA target interactions. MiRTarBase has accumulated more than three hundred and sixty thousand microRNA-target interactions (MTIs), which are collected by manually surveying pertinent literature. Generally, the collected MTIs are validated experimentally by reporter assay, western blot, microarray and next-generation sequencing experiments. Targets validated by qRT-PCR, ELISA, immunohistochemistry, Western blot reporter assay are the most reliable method to demonstrate direct interaction between a microRNA and its targets (strong interactions). Methods like microarray or high-throughput sequencing provide indirect relationships between microRNAs and their targets (weak interactions). For the current study, only strong microRNA-mRNA interactions were selected.

Datasets from GEO

GEO database was used to find gene expression data (mRNA) containing DLBCL tumors with clinical information as well as healthy controls. For a comprehensive search of genetic information, we used “DLBCL and expression profiling by array” terms to identify relevant research publications with an unlimited starting publication date until September 2020. We examined 77 datasets and of the 15 articles that met the criteria for inclusion, only one contained control data and was included in our analysis (Figure 6). The workflow of the microRNA and mRNA co-expression network analysis is shown in Figure 7.

One microarray dataset from the GPL570 platform ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array) was downloaded from the GEO (http://www.ncbi.nlm.nih.gov/geo/) to analyze differentially expressed genes in DLBCL compared to controls. The expression profile contains 55 DLBCL samples and 33 human healthy tonsil samples derived from Alborg University Hospital (159,160).

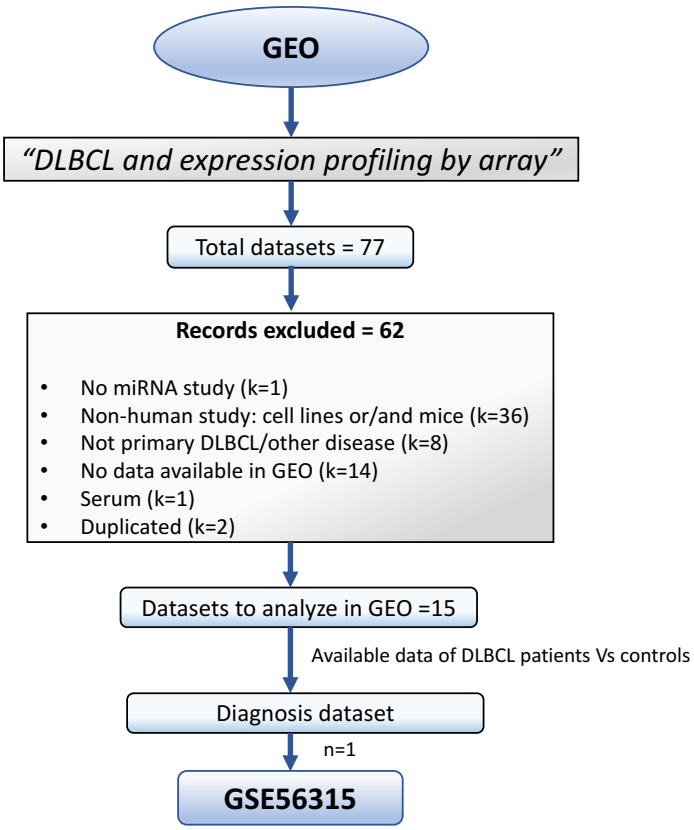

Figure 6: Flow chart diagram of dataset selection

Identification of the Differentially Expressed Genes (DEGs)

GEO2R is an interactive web tool that allows to compare two or more groups of samples in a GEO Series in order to identify genes that are differentially expressed across experimental conditions using arrays datasets. Results are presented as a table of genes ordered by significance, and as a collection of graphic plots to help visualize differentially expressed genes and assess data set quality. After classifying the dataset into patients and controls, the analysis was run with default parameters. We established the following inclusion criteria for the DEGs: upregulated genes must have a log2 fold change (logFC) ≥ 2 and an adjusted p-value < 0.05, while downregulated genes must have a logFC ≤ –2 and an adjusted p-value < 0.05.

Data Integration

Experimentally validated targets of differentially deregulated microRNAs were overlapped with mRNAs differentially expressed obtained from GEO databases as represented in Figure 7. As an example, target genes of upregulated microRNAs were overlapped with downregulated genes from GEO data, according to the mechanism of action of microRNAs. The intersection of the upregulated and downregulated genes was mapped using the Venn package (http://bioinformatics.psb.ugent.be/webtools/Venn/). For genes resulting upregulated or downregulated depending on the probe analyzed within the array, only those probes showing

an enrichment in B-cells according to bioinformatic tools were selected (http://xavierlab2.mgh.harvard.edu/EnrichmentProfiler/help.html).

Bioinformatic analysis and functional prediction

Gene Ontology (GO) functional annotations, Protein ANalisis THroug Evolutionary Relationships (PANTHER) Classification System and signaling pathway enrichment analysis (Consensus Pathway database http://cpdb.molgen.mpg.de/) were conducted with overlapping genes. To explore the protein-protein interactions of DEGs of miR-205, we submitted the DEGs to the Search Tool for the Retrieval of Interacting Genes (STRING http://string.embl.de/) database.

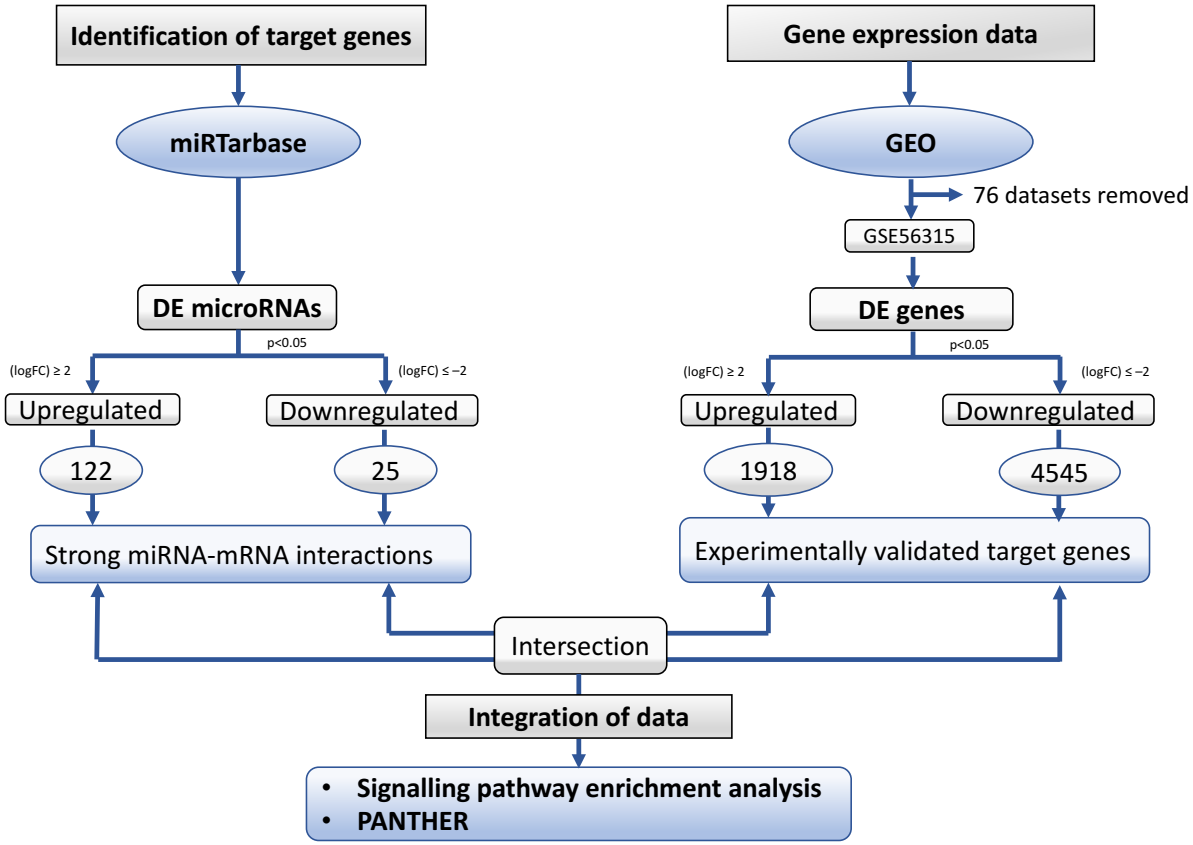

Figure 7: Workflow of microRNA-mRNA network construction

RESULTS

SELECTION OF POTENTIAL MICRORNAS IN DIFFUSE LARGE B CELL LYMPHOMA THROUGH A SYSTEMATIC REVIEW OF THE LITERATURE

The search strategy provided a total of 677 records in PubMed database (Figure 8). Once the duplicated articles were removed, 438 remained. Of these 438, 303 were excluded after reading the abstract because they did not meet the inclusion criteria. Then, the full texts of the remaining 135 studies, focused on microRNAs in DLBCL, were read carefully. Additionally, other 84 articles were excluded because there were other coexisting pathologies, microRNAs were not analyzed in the tumor sample, not primary DLBCL was considered, they did not assess the role of microRNAs in diagnosis, subtype, prediction of treatment response or prognosis, or microRNA expression changes were not considered. A total of 51 studies investigating the role of microRNA expression changes as biomarkers in DLBCL tumor samples were included. Thirty-three of them considered microRNAs as putative DLBCL diagnosis biomarkers, twenty-two in subtype classification, five in treatment response and twenty-five of the studies searched for markers for their role in prognosis.

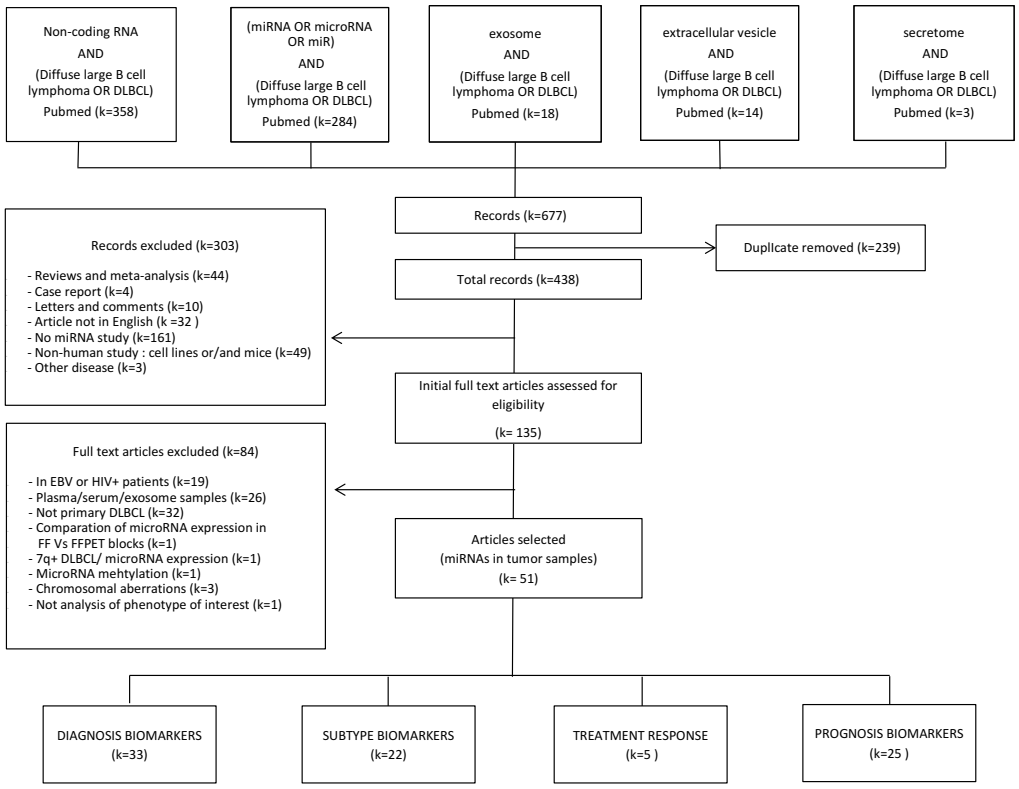

Figure 8: Flow-chart diagram of study selection.

Tumor tissue microRNAs as biomarkers for diagnosis in DLBCL

Thirty-three studies analyzed the expression of microRNAs comparing DLBCL cases vs. healthy controls (145–150,154,161–186). These 33 studies provided a total of 152 differentially

expressed microRNAs in DLBCL patients compared with healthy control individuals, which are shown in Annex Table 1.

Regarding the microRNAs that were concordantly deregulated in more than two studies, we identified three microRNAs that were mainly repeatedly reported to be up-regulated in DLBCL patients (miR-155-5p (145,147,167,170,174,176,178–181), miR-21-5p (145,161,171,174,178,180,183), and miR-146a-5p (169,174,179)), although some studies did not find a significant association (miR-155-5p (149,150), miR-21-5p (147,150), miR-146a-5p ((145,150). We also identified one microRNA with contradictory results: miR-150-5p was found to be down-regulated in DLBCL patients in four studies (145–148) and contradictorily up-regulated in DLBCL patients in another study (149), while no significant association was reported in the remaining study (150) (Table 5).

Tumor tissue microRNAs as biomarkers for DLBCL subtype classification

Twenty two studies analyzed the role of tumor tissue microRNAs to distinguish between GCB and non-GCB DLBCL subtypes (145,147,150,151,155,171,173,175–181,187–194). These studies found 80 microRNAs differentially expressed between GCB and non-GCB DLBCL samples, which are shown in Annex Table 2. Among these 80 differentially expressed microRNAs, four microRNAs were concordantly reported in more than two studies. Three of them were reported as down-regulated (miR-155-5p (145,150,155,178–181,187,192,194), miR-221-3p (150,151,155,180), and miR-222-3p (150,151,155)) or unchanged (miR-155-5p (147), miR-221-3p (145,191), miR-222-3p (145,147,187,191)) in GCB samples, whereas miR-28-5p was found to be up-regulated (150,151,187,191) or unchanged (145,155) in the same subtype (Table 6).

Table 5: microRNAs significantly associated with DLBCL diagnosis in more than two studies.

Significant microRNAs	Result	n DLBCL	n Control	Sample source	Method	Nº microRNAs	Reference
miR-155-5p	Up	29	32 (RLH)	Tissue	qRT-PCR	1	Li 2017
		22	6 (NLN)	Biopsy	qRT-PCR	1	Huskova 2015
		200	11 (NT)	FFPE	qRT-PCR	3	Go 2015
		45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
		90	31 (RLN)	FFPE	qRT-PCR	2	Zhong 2012
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle 2008
		48	6 (NBC)	FF and FFPE	qRT-PCR	3	Lawrie 2007
		23	2	FF	Semi RT-PCR	1	Eis 2005
		24	14 (NLN)	FFPE	Array	3100 probes	Tamaddon 2016
		84	4	FFPE	Array	1	Wu 2018
	NS	92	15	FF	sequencing	miRNAome	Lim 2015
		12	7	FFPE	qRT-PCR	4	Handal 2013
miR-21-5p	Up	55	20 (NLN)	FF and FFPE	qRT-PCR	1	Liu 2017
		26	10 (NLN)	FFPE	qRT-PCR	1	Song 2017
		200	11 (NT)	FFPE	qRT-PCR	3	Go 2015
		45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
		48	6 (NBC)	FF and FFPE	qRT-PCR	3	Lawrie 2007
		24	14 (NLN)	FFPE	Array	3100 probes	Tamaddon 2016
		45	23(RLNH)	Tissue	qRT-PCR	1	Chen 2020
	NS	92	15	FF	sequencing	miRNAome	Lim 2015
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle 2008
miR-146a-5p	Up	90	31 (RLN)	FFPE	qRT-PCR	2	Zhong 2012
		24	14 (NLN)	FFPE	Array	3100 probes	Tamaddon 2016
		56	28(RLNH)	Tissue	qRT-PCR	1	Zhuang 2014
	NS	45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
		92	15	FF	sequencing	miRNAome	Lim 2015
miR-150-5p	Up	12	7	FFPE	qRT-PCR	4	Handal 2013
	Down	45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
		36	5 (NLN)	Tissue	qRT-PCR	8	Fassina 2012
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle 2008
		5	4 (RLH)	Tissue	nanostring	800	Jia 2018
	NS	92	15	FF	sequencing	miRNAome	Lim 2015

Abbreviations: FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; NA: not available; Up: statistically significantly upregulated in DLBCL patients; NS: no significant difference between patients and controls; Down: significantly downregulated in DLBCL patient; RLH: Reactive lymphoid hyperplasia; NLN: normal lymph node tissues; NT: normal tonsil; DC: discovery cohort; VC: validation cohort; NBC: normal B cell samples.

Table 6: microRNAs significantly associated with DLBCL subtype in more than two studies.

Significant microRNA	Result	n GCB	n non-GCB	Sample source	Method	Nº microRNAs	Reference
miR-155-5p	Down GCB	53	95	FFPE	qRT-PCR	8	Go 2015
		32	27	FFPE	qRT-PCR/array	377	Iqbal 2015
		20	34	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
		36	31	FF	qRT-PCR	1	Huang 2012
		21	69	FFPE	qRT-PCR	2	Zhong 2012
		32	28	FFPE	Array	464	Lawrie 2009
		16	18	FF and FFPE	qRT-PCR	3	Lawrie 2007
		4	19	FF	Semiq. RT-PCR	1	Eis 2005
		41	30	FF	Sequencing	miRNAome	Lim 2015
		32	32	biopsy	qRT-PCR	1	Due 2019
	NS	25	25	FFPE	qRT-PCR	157	Roehle 2008
miR-221-3p	Down GCB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno 2011
		32	28	FFPE	Array	464	Lawrie 2009
		16	18	FF and FFPE	qRT-PCR	3	Lawrie 2007
		41	30	FF	sequencing	miRNAome	Lim 2015
	NS	20	20	Tissue	Array	113	Zhang 2009
		20	34	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
miR-222-3p	Down GCB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno 2011
		32	28	FFPE	Array	464	Lawrie 2009
		41	30	FF	sequencing	miRNAome	Lim 2015
	NS	25	25	FFPE	qRT-PCR	157	Roehle 2008
		20	20	Tissue	Array	113	Zhang 2009
		32	27	FFPE	qRT-PCR/array	377	Iqbal 2015
		20	34	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
miR-28-5p	Up GCB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno 2011
		32	27	FFPE	qRT-PCR/array	377	Iqbal 2015
		41	30	FF	sequencing	miRNAome	Lim 2015
		20	20	Tissue	Array	113	Zhang 2009
	NS	20	34	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
		32	28	FFPE	Array	464	Lawrie 2009

Abbreviations: GCB: Germinal center B-cell like; non-GCB: Activated B-cell-like; FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; NA: not available; Up: statistically significantly upregulated in GCB patients; NS: no significant difference between GCB and non-GCB patients; Down: significantly downregulated in GCB patients

Tumor tissue microRNAs as biomarkers for prediction of treatment response in DLBCL

Five studies were focused in the role of microRNAs in DLBCL tissue as predictive biomarkers of response to R-CHOP treatment (148,175,176,179,190). The characteristics of each study are shown in Annex Table 3. A total of five microRNAs were differentially expressed between good and poor responders. Three microRNAs were found to be associated with favorable response to therapy (miR-27b-3p (148), miR-34a-5p (176) and miR-224-5p (175)) whereas miR-155-5p (179) and miR-146-5p (179) were found to be associated with chemoresistance. However, each microRNA was analyzed in only one study, without any of the results being replicated.

Tumor tissue microRNAs as biomarkers for DLBCL prognosis

The relevance of tumor tissue microRNAs for prognosis in DLBCL patients was analyzed in twenty five studies (147,148,150–156,167,172,173,175,177–180,184,186,187,190,193–196). A total of 50 microRNAs with significant reported associations with patient survival were found, which are shown in Annex Table 4.

Considering the microRNAs with concordant significant results in more than two studies, miR-222-3p, and miR-155-5p were identified. Up-regulation of miR-222-3p (151–154) and miR-155-5p (167,179,187,195) has been associated with worse outcome in four different studies in each case, in one of them (154) only in the non-GCB subgroup. However, four and eight studies respectively did not find any association with prognosis for miR-222-3p (147,150,155,156) and miR-155-5p (147,150,152,153,155,156,178,180) and one study found contradictory results referring lower OS and PFS in GCB patient with miR-155 low expression (194) (Table 7).

Table 7: microRNAs significantly associated with DLBCL prognosis in more than two studies.

Significant microRNAs	Result	n DLBCL	Sample source	Method	Nº microRNAs	Reference
miR-222-3p	Up: ↓OS	176	FFPE	qRT-PCR	11	Alencar 2011
	Up: ↓PFS and OS	36/240	FFPE	qRT-PCR/array	470/9	Montes-Moreno 2011
	Up: ↓OS and PFS	106	FFPE	qRT-PCR	3	Malumbres 2009
	Up: ↓OS in non-GCB	74	Biopsy	qRT-PCR	1	Shanshan 2019
	NS	64	FFPE	Array	464	Lawrie 2009
		92	FF	sequencing	miRNAome	Lim 2015
		58	Biopsy	qRT-PCR	157	Roehle 2008
		83	FFPE	qRT-PCR/array	±900	Shepshelovich 2015
miR-155-5p	Up: lower survival	118	FF	qRT-PCR	1	Zhu 2016
	Up: ↓ OS	79	FFPE	qRT-PCR	8	Iqbal 2015
	Down: ↑PFS	90	FFPE	qRT-PCR	1	Zhong 2012
	Up: ↓PFS	82	FFPE	qRT-PCR	1	Wu 2018
	Down: ↓ OS and PFS	array	Biopsy	qRT-PCR	Array	Due 2019
	NS	176	FFPE	qRT-PCR	11	Alencar 2011
		200	FFPE	qRT-PCR	3	Go 2015
		35	FF and FFPE	qRT-PCR	3	Lawrie 2007
		64	FFPE	Array	464	Lawrie 2009
		92	FF	sequencing	miRNAome	Lim 2015
		106	FFPE	qRT-PCR	3	Malumbres 2009
		58	Biopsy	qRT-PCR	157	Roehle 2008
		83	FFPE	qRT-PCR/array	±900	Shepshelovich 2015

Abbreviations: FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; OS: overall survival; PFS: progression-free survival; EFS: event free survival; RFS: relapse free survival; Down: significantly downregulated in relapsed DLBCL patients; Up: statistically significantly upregulated in relapsed DLBCL patients; NS: no significant difference in microRNA expression between patients that relapsed and patients with long term remission.

VALIDATION OF CANDIDATE MICRORNAS IN THE STUDY POPULATION

Analysis of the literature diagnostic model in the study population

The four deregulated microRNAs detected in the literature where analyzed in our study population to confirm the discriminatory capability as diagnostic biomarkers.

As shown in Table 8 we found that all the deregulated microRNAs exhibited similar expression patterns in the population of study compared to what was observed in the previous literature (miR-150-5p, padj = $1.44x10^{-22}$; miR-146a-5p, padj = $1.16x10^{-12}$; miR-155-5p, padj = $1.67x10^{-08}$; miR-21-5p, padj = $4.03x10^{-05}$).

Table 8: Expression levels in the study population of deregulated microRNAs deregulated in DLBCL according to the literature.

microRNA	Base Mean	Log2 Fold Change	p-value	padj
hsa-miR-150-5p	6310.7	-3.22	$3.46x10^{-25}$	$1.44x10^{-22}$
hsa-miR-146a-5p	33085.6	2.11	$2.05x10^{-14}$	$1.16x10^{-12}$
hsa-miR-155-5p	20773.1	1.99	$7.26x10^{-10}$	$1.67x10^{-08}$
hsa-miR-21-5p	140750.7	1.11	$4.37x10^{-06}$	$4.03x10^{-05}$

Positive or negative Log2 Fold change represented the overexpression or downregulation respectively of microRNAs in DLBCL patients compared with healthy control individuals; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Evaluation of the literature subtype model in the study population

The four deregulated microRNAs detected in the literature where analyzed in our study population to confirm their discriminatory capability as subtype biomarkers.

Only one of the four microRNAs showed statistically significant differences in our population (miR-28-5p, padj = 0.00342) (Table 9).

Table 9: Expression levels in the study population of microRNAs differentially expressed in DLBCL subtypes according to the literature.

MicroRNA	Base Mean	Log2 Fold Change	p-value	padj
hsa-miR-28-5p	475.7	1.24	$3.85x10^{-05}$	0.00342
hsa-miR-222-3p	1897.9	-0.79	0.00456	0.09895
hsa-miR-221-3p	4859.2	-0.37	0.22993	0.55006
hsa-miR-155-5p	19078.2	0.12	0.74670	0.91329

Positive Log2 Fold change represented the overexpression of microRNAs at GCB subtype and negative represented overexpression in Non-GCB subtype; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Analysis of the literature treatment response prediction model in the study population

Since the five microRNAs detected in the literature were only studied in a single study, we analyzed their expression in our population to confirm if the reported results could be validated.

As shown in Table 10, none of them presented significant differences in their expression between patients with complete remission and refractory patients.

Table 10: Expression levels in the validation cohort of microRNAs associated with treatment response in the literature.

MicroRNA	Base Mean	Log2 Fold Change	p-value	padj
hsa-miR-224-5p	47.7	0.72	0.11013	0.46446
hsa-miR-155-5p	20773.1	-0.49	0.20472	0.59557
hsa-miR-146a-5p	33085.6	-0.12	0.72557	0.91006
hsa-miR-34a-5p	759.4	-0.02	0.91785	0.97574
hsa-miR-27b-3p	14074.6	0.21	0.50219	0.50218

Positive Log2 Fold change represented the overexpression of microRNAs at diagnosis in patients with complete remission compared with refractory patients, and negative represented the opposite; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Evaluation of the literature prognosis model in the study population

The two deregulated microRNAs detected in the literature where analyzed in the study population to confirm the discriminatory capability as prognostic biomarkers.

These microRNAs did not present differences in their expression between patients that relapsed and those that presented long-term remission in our population (Table 11).

Table 11: Expression levels in the study population of microRNAs associated with prognosis according to the literature.

MicroRNA	Base Mean	Log2 Fold Change	p-value	padj
hsa-miR-222-3p	1984.9	-0.08	0.77088	0.93065
hsa-miR-155-5p	20773.1	-0.03	0.92503	0.97641

Positive Log2 Fold change represented the overexpression of microRNAs at diagnosis in patients with long term remission compared with patients that relapsed, and negative represented the opposite; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

IDENTIFICATION OF A NEW SIGNATURE OF MICRORNAS OF RELEVANCE IN DLBCL THROUGH THE ANALYSIS OF ALL THE MICRORNAS USING SMALL RNA SEQUENCING

Global analysis of microRNAs

A total number of 1584 microRNAs were identified through the miRNA sequencing analysis. A principal component analysis (PCA) was performed including the expression of all the microRNAs to obtain an overview of the grouping of samples according to their variance (Figure 9). The first component (PC 1) explains the far major part of system variation (14.9% of total variance explained), while the second accounts for 5.7% of total variance. This led to the separation of the samples in different regions of a PCA plot corresponding to complete response, refractory, relapsed patients or control group of patients. The samples seem to cluster with little difference between complete response, refractory and relapsed groups, whereas the control group samples cluster as a clearly separate group.

The PCA plot showed that the global microRNA portrait was able to separate the majority of DLBCL cancers from the controls. Thus, DLBCL patients presented a differentiated microRNA expression profile from that of the control group. No significant differences were observed among groups of DLBCL patients according to their response and prognosis.

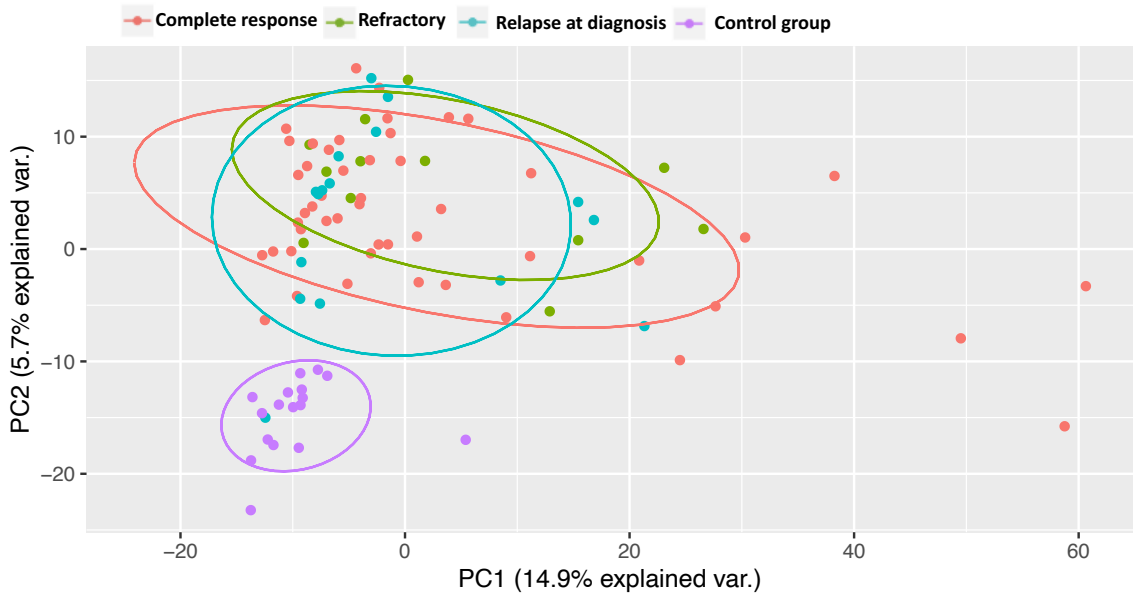

Figure 9: Graphical representation of principal component analysis of the expression of microRNAs among DLBCL patients and controls.

Biomarkers of diagnosis

To obtain a comprehensive list of candidate novel biomarkers of diagnosis characteristic of DLBCL, we compared the expression of each microRNA in 78 DLBCL samples at diagnosis with 17 non-tumoral ganglia from individuals without DLBCL. We noted that 146 microRNAs exhibited

statistically significant expression between samples of DLBCL patients at diagnosis and control samples (padj <0.05; log_2 fold change > 2/<-2). Of these microRNAs, 122 exhibited increased abundance in DLBCL while 24 microRNAs exhibited decreased abundance in DLBCL. All the results of upregulated and downregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) are listed Annex Table 5 and 6. The 20 most discriminatory up- and down-regulated microRNAs are listed in Table 12 and Table 13.

Table 12: The 20 most upregulated microRNAs in DLBCL patients vs. controls.

MicroRNA	Base Mean	log2FoldChange	p-value	padj
hsa-miR-210-3p	842.9	3.51	$2.33x10^{-29}$	$1.45 x10^{-26}$
hsa-miR-944	60.9	4.10	$2.19x10^{-23}$	$6.80 x10^{-21}$
hsa-miR-12136	76.05	26.94	$3.64x10^{-20}$	$6.47 x10^{-18}$
hsa-miR-3681-5p	75.3	5.16	$3.33x10^{-20}$	$6.47 x10^{-18}$
hsa-miR-378i	24.9	3.01	$3.01x10^{-17}$	$4.16 x10^{-15}$
hsa-miR-4454	183.4	2.35	$1.01x10^{-16}$	$1.04 x10^{-14}$
hsa-miR-1291	354.7	4.02	$1.84x10^{-16}$	$1.76 x10^{-14}$
hsa-miR-7974	111.8	3.46	$1.87x10^{-15}$	$1.45 x10^{-13}$
hsa-miR-183-5p	891.1	3.40	$5.77x10^{-15}$	$3.59 x10^{-13}$
hsa-miR-146a-5p	33085.5	2.11	$2.05x10^{-14}$	$1.16 x10^{-12}$
hsa-miR-2467-5p	25.56	2.33	$7.35x10^{-14}$	$3.81 x10^{-12}$
hsa-miR-4420	8.09	4.63	$2.02x10^{-13}$	$1.01 x10^{-11}$
hsa-miR-1248	202.5	2.69	$1.03x10^{-12}$	$4.73 x10^{-11}$
hsa-miR-18a-3p	54.06	2.05	$1.21x10^{-12}$	$5.36 x10^{-11}$
hsa-miR-129-5p	25.1	4.62	$3.03x10^{-12}$	$1.22 x10^{-10}$
hsa-miR-147b-3p	92.5	3.41	$3.78x10^{-12}$	$1.47 x10^{-10}$
hsa-miR-3691-5p	6.6	2.59	$1.09x10^{-11}$	$3.88 x10^{-10}$
hsa-miR-1246	4.6	4.47	$2.64x10^{-11}$	$8.86x10^{-10}$
hsa-miR-205-5p	67.1	6.11	$3.81x10^{-11}$	$1.22x10^{-09}$
hsa-miR-769-3p	21.5	2.53	$4.00x10^{-11}$	$1.25x10^{-09}$

Positive Log2Fold change represented the overexpression of microRNAs in DLBCL patients compared with healthy control individuals; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Table 13: The 20 most downregulated microRNAs in DLBCL patients vs. controls

MicroRNA	Base Mean	log2FoldChange	p-value	padj
hsa-miR-215-5p	53.8	-4.42	6.74×10^{-35}	8.39×10^{-32}
hsa-miR-150-5p	6310.7	-3.22	3.46×10^{-25}	1.44×10^{-22}
hsa-miR-224-5p	47.7	-3.30	5.11×10^{-21}	1.27×10^{-18}
hsa-miR-194-5p	37.8	-4.33	6.19×10^{-17}	7.00×10^{-15}
hsa-miR-452-3p	7.2	-2.33	2.10×10^{-16}	1.87×10^{-14}
hsa-miR-335-5p	127.6	-2.74	6.20×10^{-16}	5.14×10^{-14}
hsa-miR-145-5p	2060.5	-2.16	2.65×10^{-15}	1.94×10^{-13}
hsa-miR-139-5p	48.8	-2.26	5.40×10^{-15}	3.57×10^{-13}
hsa-miR-497-5p	332.5	-2.07	3.53×10^{-14}	1.91×10^{-12}
hsa-miR-10a-3p	10.2	-2.29	7.45×10^{-12}	2.73×10^{-10}
hsa-miR-95-3p	18.1	-2.13	3.79×10^{-11}	1.22×10^{-09}
hsa-miR-151b	24.8	-2.21	6.67×10^{-10}	1.60×10^{-08}
hsa-miR-551b-3p	7.9	-2.25	2.97×10^{-09}	5.78×10^{-08}
hsa-miR-194-5p	234.5	-2.52	4.25×10^{-09}	8.01×10^{-08}
hsa-miR-135a-5p	4.3	-3.49	1.92×10^{-08}	3.07×10^{-07}
hsa-miR-549a-3p	1.6	-2.39	2.28×10^{-07}	3.02×10^{-06}
hsa-miR-549a-5p	3.4	-2.44	4.93×10^{-07}	5.96×10^{-06}
hsa-miR-451a	6085.0	-2.07	4.55×10^{-06}	4.16×10^{-05}
hsa-miR-670-3p	0.9	-2.72	8.61×10^{-06}	7.44×10^{-05}
hsa-miR-135a-3p	0.6	-2.46	2.89×10^{-05}	0.00021

Negative Log2 Fold change represented the downregulation of microRNAs in DLBCL patients compared with healthy control individuals; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Biomarkers of subtype classification

By analyzing expression profiling of 32 GCB and 21 Non-GCB samples at diagnosis, eight microRNAs were differentially expressed (padj <0.05; $\log_2$ fold change > 2/<-2). Five of them were upregulated in GCB DLBCL patients (miR-129-2-3p, miR-4464, miR-3150b-3p, miR-138-5p and miR-129-5p) and three microRNAs were upregulated in Non-GCB subtype (miR-511-5p, miR-205-5p, and miR-3652). These results are listed in Table 14. All the results of upregulated and downregulated microRNAs in GCB DLBCL patients vs. Non-GCB DLBCL patients (p<0.05 and log2foldchange >0) are listed in Annex Table 7 and 8.

Table 14. MicroRNAs differentially expressed between GCB and Non-GCB DLBCL subtypes

MicroRNA	Base Mean	log2FoldChange	p-value	padj
hsa-miR-129-2-3p	4.9	4.12	$1.50x10^{-06}$	0.00050
hsa-miR-4464	2.9	3.91	$1.21x10^{-05}$	0.00151
hsa-miR-3150b-3p	49.0	2.00	$1.90x10^{-05}$	0.00211
hsa-miR-138-5p	568.1	2.00	$9.92x10^{-05}$	0.00661
hsa-miR-129-5p	18.2	2.90	0.00014	0.00891
hsa-miR-511-5p	7.6	-2.01	$9.92x10^{-06}$	0.00142
hsa-miR-205-5p	20.3	-3.30	$4.10x10^{-05}$	0.00342
hsa-miR-3652	8.9	-2.44	0.00088	0.03137

Positive Log2 Fold change represented the upregulation of microRNAs in GCB DLBCL patients compared with Non-GCB individuals; and negative, upregulation in Non-GCB DLBCL; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Treatment response biomarkers.

Eleven microRNAs were differentially expressed (padj <0.05; $\log_2$ fold change > 2 or <-2) between samples at diagnosis from patients with complete remission (n=50) and refractory patients (n=12), of which, ten microRNAs were upregulated in patients with complete remission (miR-12136, miR-129-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129-5p and miR-3928-3p) and one microRNA was downregulated in the same group of patients (miR-192-5p). These results are listed in Table 15. All the results of microRNAs associated with good and bad response to treatment (p<0.05) are listed in Annex Table 9 and 10.

Table 15. MicroRNAs significantly associated with good or poor response to treatment.

MicroRNA	Base Mean	log2FoldChange	pvalue	padj
hsa-miR-12136	120.0	25.73	$1.29x10^{-13}$	$9.70x10^{-11}$
hsa-miR-129a-5p	42.7	5.09	$2.86x10^{-09}$	$1.08x10^{-06}$
hsa-miR-129-1-3p	7.4	4.08	$1.86x10^{-06}$	0.00035
hsa-miR-3150b-3p	50.7	2.33	$1.60x10^{-05}$	0.00241
hsa-miR-127-3p	3977.2	2.01	$6.14x10^{-05}$	0.00661
hsa-miR-3681-5p	75.3	2.34	0.00016	0.01507
hsa-miR-370-3p	16.1	2.59	0.00041	0.02380
hsa-miR-4464	3.3	3.55	0.00117	0.04641
hsa-miR-129b-5p	27.5	2.88	0.00102	0.04641
hsa-miR-3928-3p	16.5	2.03	0.00113	0.04641
hsa-miR-192-5p	10375.5	-2.41	$1.60x10-^{07}$	$4.01x10^{-05}$

Positive Log2 Fold change represented the overexpression of microRNAs at diagnosis in patients with complete remission compared with refractory patients; and negative, overexpression in refractory patients; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Prognostic biomarkers

MicroRNA expression at diagnosis of 50 patients with long term remission and 16 patients that relapsed was compared. The results revealed seven microRNAs (miR-4444, miR-449c-5p, miR-3681-5p, miR-3928-3p, miR-449b-5p, miR-370-3p, miR-4424) significantly upregulated in patients with long term remission (padj <0.05; $\log_2$ fold change > 2). On the other hand, four

microRNAs (miR-133a-2-3p, miR-133a-1-3p, miR-208b-3p, miR-205-5p) were upregulated in relapsed patients (padj <0.05; log_2 fold change < -2) (Table 16). All microRNAs associated with good and bad prognosis (p<0.05) are listed in Annex Table 11 and 12.

Table 16. MicroRNAs significantly associated with prognosis.

MicroRNA	Base Mean	log2FoldChange	pvalue	padj
hsa-miR-4444	10.3	2.25	$6.20x10^{-05}$	0.0089
hsa-miR-449c-5p	9.8	2.22	$4.75x10^{-05}$	0.0089
hsa-miR-3681-5p	75.3	2.03	0.00021	0.014
hsa-miR-3928-3p	16.5	2.02	0.00018	0.014
hsa-miR-449b-5p	7.23	2.22	0.00036	0.021
hsa-miR-370-3p	16.1	2.12	0.00071	0.029
hsa-miR-4424	110.8	2.32	0.0010	0.041
hsa-miR-133a-2-3p	470.9	-3.8	$1.53x10^{-06}$	0.001
hsa-miR-133a-1-3p	69.29	-2.52	$6.78x10^{-05}$	0.0089
hsa-miR-208b-3p	12.94	-3.08	0.00014	0.013
hsa-miR-205-5p	62.4	-3.36	0.0002	0.014

Positive Log2 Fold change represented the overexpression of microRNAs in patients with long term remission compared with relapsed patients; and negative, overexpression in relapsed patients. Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Impact of microRNAs expression level on survival

The patient survival curve was generated according to the 5-year PFS and OS from diagnosis, dividing the patients into high-expression and low-expression groups according to the mean expression of the seven microRNAs associated with good prognosis: miR-4444 (2.83), miR-449c-5p (2.75), miR-3681-5p(4.98), miR-3928-3p (3.02), miR-449b-5p (2.75), miR-370-3p (2.73), miR-4424 (3.92); and the four microRNAs associated with bad prognosis: miR-133a-1-3p (3.73), miR-133a-2-3p (3.83), miR-208b-3p (1.97), miR-205-5p (2.32).

Among the microRNAs associated with good prognosis, significant differences in OS and PFS were identified between miR-4444 high- and low-expression level groups (OS p-value=0.0169, and PFS p-value=0.0162) (Figure 10). Additionally, considering the microRNAs previously associated with bad prognosis, we identified significant differences in OS between the miR-205-5p high- and low-expression level groups (p=0.0444) (Figure 10).

We performed Cox proportional hazards (Cox PH) multivariate analysis to determine if these microRNAs were associated with OS and PFS independently of the two established indicators of DLBCL patient outcome (Subtype and IPI). The results of this analysis revealed that miR-205-5p was associated with worse OS (p=0.02) and PFS (p=0.02) independently of IPI and subtype.

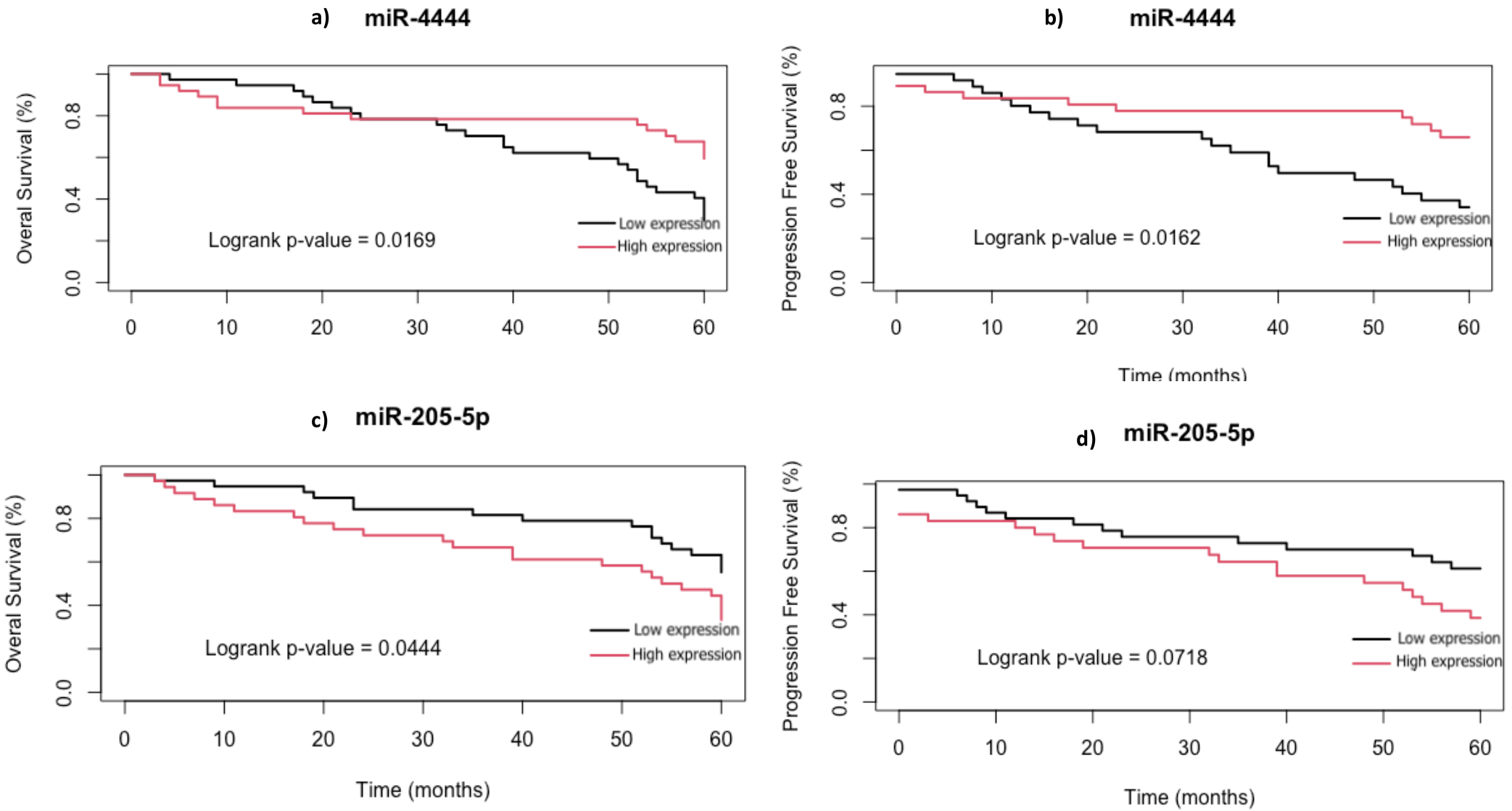

Figure 10: Survival analysis of the microRNAs significantly associated with prognosis.

MicroRNA-mRNA INTERACTION NETWORK ANALYSIS

Identification of experimentally validated targets

Experimentally validated targets of the microRNAs differentially expressed in our diagnostic analysis (DLBCL vs controls) were identified using mirTarbase. For the 122 upregulated microRNAs found in our study, we identified a total of 482 experimentally validated targets. Regarding the 25 downregulated microRNAs from our study, they targeted a total of 375 genes (Figure 11).

Identification of Differentially Expressed Genes in DLBCL

From the total of 15 studies found in GEO that contained data from DLBCL patients, only one contained data of healthy controls and was eligible to include in the analysis (Figure 6). The GSE56315 expression profile dataset contains 55 DLBCL samples and 33 human healthy samples. A total of 1918 significantly upregulated genes and 4545 downregulated genes were found in DLBCL patients compared with healthy control individuals in GEO dataset.

Integration, functional annotation and signaling pathway enrichment analysis

Upregulated microRNAs targeted a total of 138 genes among those lowly expressed in DLBCL (Annex Table 13) and downregulated microRNAs a total of 76 targets among those genes highly expressed in DLBCL (Annex Table 14) (Figure 11). Some genes showed interactions with several microRNAs (Table 17 and Table 18). For instance, *FOXO1*, *BCL2*, *GS3KB* and *PTEN*, downregulated in DLBCL, are targeted by 7, 5 ,5 and 4 microRNAs, respectively.

Figure 11: a) Venn diagram representing overlapping downregulated genes in DLBCL and targets of upregulated microRNAs. b) Venn diagram representing overlapping upregulated genes in DLBCL and targets of downregulated microRNAs.

Table 17: Downregulated target gene showing interactions with several upregulated microRNAs

Target genes	microRNA
AKT2	hsa-miR-612; hsa-miR-2861
ALDH5A1	hsa-miR-210-3p; hsa-miR-147b
APC	hsa-miR-135a-5p; hsa-miR-663a; hsa-miR-129-5p
BCL2	hsa-miR-7-5p; hsa-miR-182-5p; hsa-miR-205-5p; hsa-miR-135a-5p; hsa-miR-9-5p
CCND2	hsa-miR-182-5p; hsa-miR-146a-5p
CDK6	hsa-miR-129-5p; hsa-miR-320a
CREB1	hsa-miR-182-5p; hsa-miR-9-5p
CXCR4	hsa-miR-146a-5p; hsa-miR-9-5p; hsa-miR-663a; hsa-miR-146a-3p
ELAVL1	hsa-miR-9-5p; hsa-miR-146a-5p
EZR	hsa-miR-183-5p; hsa-miR-205-5p
FBXW7	hsa-miR-182-5p; hsa-miR-182-3p; hsa-miR-155-3p
FOXO1	hsa-miR-182-5p; hsa-miR-183-5p; hsa-miR-183-5p; hsa-miR-9-5p; hsa-miR-9-3p; hsa-miR-135a-5p; hsa-miR-135a-5p
FOXO3	hsa-miR-182-5p; hsa-miR-9-5p
GSK3B	hsa-miR-183-5p; hsa-miR-182-5p; hsa-miR-9-5p; hsa-miR-129-1-3p; hsa-miR-1246
HES1	hsa-miR-9-3p; hsa-miR-9-5p
HIF3A	hsa-miR-210-3p; hsa-miR-147a
IGF2BP3	hsa-miR-9-5p; hsa-miR-129-5p
ITGB1	hsa-miR-183-5p; hsa-miR-9-3p
MAPK1	hsa-miR-320a; hsa-miR-9-3p; hsa-miR-129-5p
MTSS1	hsa-miR-182-5p; hsa-miR-135a-5p
NOTCH1	hsa-miR-129-5p; hsa-miR-9-5p; hsa-miR-146a-5p
PTEN	hsa-miR-182-5p; hsa-miR-155-3p; hsa-miR-205-5p; hsa-miR-320a
RAC1	hsa-miR-320a; hsa-miR-146a-5p
RECK	hsa-miR-182-5p; hsa-miR-183-5p
ROCK1	hsa-miR-146a-5p; hsa-miR-135a-5p
RUNX2	hsa-miR-205-5p; hsa-miR-320a; hsa-miR-135a-5p
SIAH1	hsa-miR-135a-5p; hsa-miR-944
SMAD4	hsa-miR-146a-5p; hsa-miR-182-5p; hsa-miR-205-5p; hsa-miR-183-5p
SOX2	hsa-miR-1181; hsa-miR-146a-5p
SP1	hsa-miR-129-5p; hsa-miR-612
TGFB1	hsa-miR-663a; hsa-miR-146a-5p
TP53	hsa-miR-612; hsa-miR-663a; hsa-miR-155-3p
YY1	hsa-miR-7-5p; hsa-miR-205-5p

Table 18: Upregulated target genes showing interactions with several down regulated microRNAs

Target genes	microRNA
BCL2	hsa-miR-451a; hsa-miR-497-5p; hsa-miR-135a-5p; hsa-miR-224-5p; hsa-miR-139-5p
CD44	hsa-miR-216a-5p; hsa-miR-145-5p
CDH2	hsa-miR-194-5p; hsa-miR-145-5p
CEBPD	hsa-miR-95-3p; hsa-miR-135a-5p
EGFR	hsa-miR-145-5p; hsa-miR-135a-5p
KLF4	hsa-miR-145-5p; hsa-miR-135a-5p
KRAS	hsa-miR-217; hsa-miR-224-5p
MTDH	hsa-miR-145-5p; hsa-miR-217
PTEN	hsa-miR-217; hsa-miR-216a-5p
SMAD4	hsa-miR-483-3p; hsa-miR-224-5p
STAT1	hsa-miR-145-5p; hsa-miR-150-5p
VEGFA	hsa-miR-145-5p; hsa-miR-150-5p
ZEB2	hsa-miR-215-5p; hsa-miR-335-5p

On the other hand, some microRNAs showed interactions with several targets (Table 19 and Table 20). For instance, miR-9-5p could be involved in the downregulation of up to 25 target genes, and miR-146a-5p and miR-182-5p could regulated up to 24 and 20 genes downregulated in DLBCL, respectively.

Table 19: Upregulated microRNAs showing interaction with several downregulated target genes

microRNA	Target genes
hsa-miR-1181	*SOX2; STAT3*
hsa-miR-1246	*GSK3B; DYRK1A; PRKAR1A*
hsa-miR-129-1-3p	*GSK3B; PDCD2*
hsa-miR-129-5p	*APC; CDK6; IGF2BP3; MAPK1; NOTCH1; SP1; FNDC3A; PDPK1; SOX4*
hsa-miR-135a-5p	*APC; BCL2; FOXO1; MTSS1; ROCK1; RUNX2; SIAH1; BMPR2; KLF8; RBAK*
hsa-miR-141-5p	*SPAG9; WNK1*
hsa-miR-146a-5p	*CCND2; CXCR4; ELAVL1; NOTCH1; RAC1; ROCK1; SMAD4; SOX2; TGFB1; BCLAF1; BRCA2; CNOT6L; CPM; FAF1; FANCM; HOXD10; IRAK2; NFAT5; PRKCE; RHOA; SIKE1; SOS1; STAT1; WASF2*
hsa-miR-147a	*HIF3A; PSMA3*
hsa-miR-155-3p	*FBXW7; PTEN; TP53*
hsa-miR-182-3p	*FBXW7; STAT5B*
hsa-miR-182-5p	*BCL2; CCND2; CREB1; FBXW7; FOXO1; FOXO3; GSK3B; MTSS1; PTEN; RECK; SMAD4; ATF1; CYLD; FLOT1; RARG; TP53BP1; TP53INP1; TRIM8; TSC22D3; ZFAND4*
hsa-miR-183-5p	*EZR; FOXO1; GSK3B; ITGB1; RECK; SMAD4; BTRC; EGR1; PPP2CB*
hsa-miR-18a-3p	*ATM; KRAS*
hsa-miR-19b-1-5p	*CASP8; FGFR2*
hsa-miR-205-5p	*BCL2; EZR; PTEN; RUNX2; SMAD4; YY1; ACSL4; CCNJ; E2F5; EGLN2; IL24; LMNA; SRC*
hsa-miR-208b-3p	*CACNB2; QKI*
hsa-miR-210-3p	*ALDH5A1; HIF3A; FOXN3; FOXP3; INPP5A; MNT; PTBP3; PTPN1; PTPN2; RAD52; XIST*
hsa-miR-944	*SIAH1; PTP4A1*
hsa-miR-320a	*CDK6; MAPK1; PTEN; RAC1; RUNX2; ARPP19; CTNNB1; ITGB3; MCL1; MTDH; NFATC3; SUZ12; YWHAZ*
hsa-miR-612	*AKT2; SP1; TP53*
hsa-miR-663a	*APC; CXCR4; TGFB1; TP53; JUND*
hsa-miR-7-5p	*BCL2; YY1; BAX; CUL5; ILF3; KMT5A; MSH3; PIK3CG; REL; SKP2; SNCA; XIAP*
hsa-miR-9-3p	*FOXO1; HES1; ITGB1; MAPK1; DLG1; DMD; TUG1*
hsa-miR-9-5p	*BCL2; CREB1; CXCR4; ELAVL1; FOXO1; FOXO3; GSK3B; HES1; IGF2BP3; NOTCH1; TGFBR2; AP3B1; BCL2L11; CAMKK2; CUL4A; DICER1; DRD2; ETS1; FOXP1; MALAT1; MTHFD1; POU2F2; PRKAA1; RAP2A; REST*

Table 20: Downregulated microRNAs showing interactions with upregulated target genes

microRNA	Target genes
hsa-miR-135a-5p	*BCL2; CEBPD; EGFR; KLF4; APC; BMPR2; HOXA10; MTSS1*
hsa-miR-139-5p	*BCL2; ACTC1; ADGRL4; FAM162A; RHOT1*
hsa-miR-145-5p	*CD44; CDH2; EGFR; KLF4; MTDH; STAT1; VEGFA; ABHD17C; APH1A; CD28; EPAS1; FZD7; ITGB8; JAG1; MEST; MMP1; MMP12; MYO5A; MYO6; MYOCD; PARP8; RREB1; SERINC5; SERPINE1; SPTLC1; SRGAP1; YES1*
hsa-miR-150-5p	*STAT1; VEGFA; PRKCA; SRCIN1*
hsa-miR-194-5p	*CDH2; CHD1; HBEGF; IL10; ITGA9; ITSN1; PTPN12; SOCS2*
hsa-miR-215-5p	*ZEB2; NID1; WNK1*
hsa-miR-216a-5p	*CD44; PTEN; CEMIP*
hsa-miR-95-3p	*CEBPD; CELF2*
hsa-miR-217	*KRAS; MTDH; PTEN; NR4A2; PTPN14; ROBO1*
hsa-miR-224-5p	*BCL2; KRAS; SMAD4; AP2M1; DPYSL2; EDNRA; TRIB1*
hsa-miR-335-5p	*ZEB2; BRCA1; ID4; MERTK; PLAUR; SOX4; TCEAL9; TNC*
hsa-miR-451a	*BCL2; MIF; MMP2; MMP9*
hsa-miR-483-3p	*SMAD4; DLC1; IGF1*
hsa-miR-497-5p	*BCL2; CHEK1; Reck*

To gain more biological insight, we performed gene oncology (GO) enrichment analysis using PANTHER. The differentially expressed genes (DEGs) were classified into two functional groups: molecular function and protein class. For the 138 downregulated target genes which interact with upregulated microRNAs, the most enriched molecular functions were binding (77 genes), catalytic activity (44 genes) and molecular function regulator (41 genes) (Figure 12a). For protein class analysis, gene-specific transcriptional regulator (24 genes), protein modifying enzyme (24 genes, 17.4) and nucleic acid metabolism protein (12 genes) were the most enriched (Figure 12b).

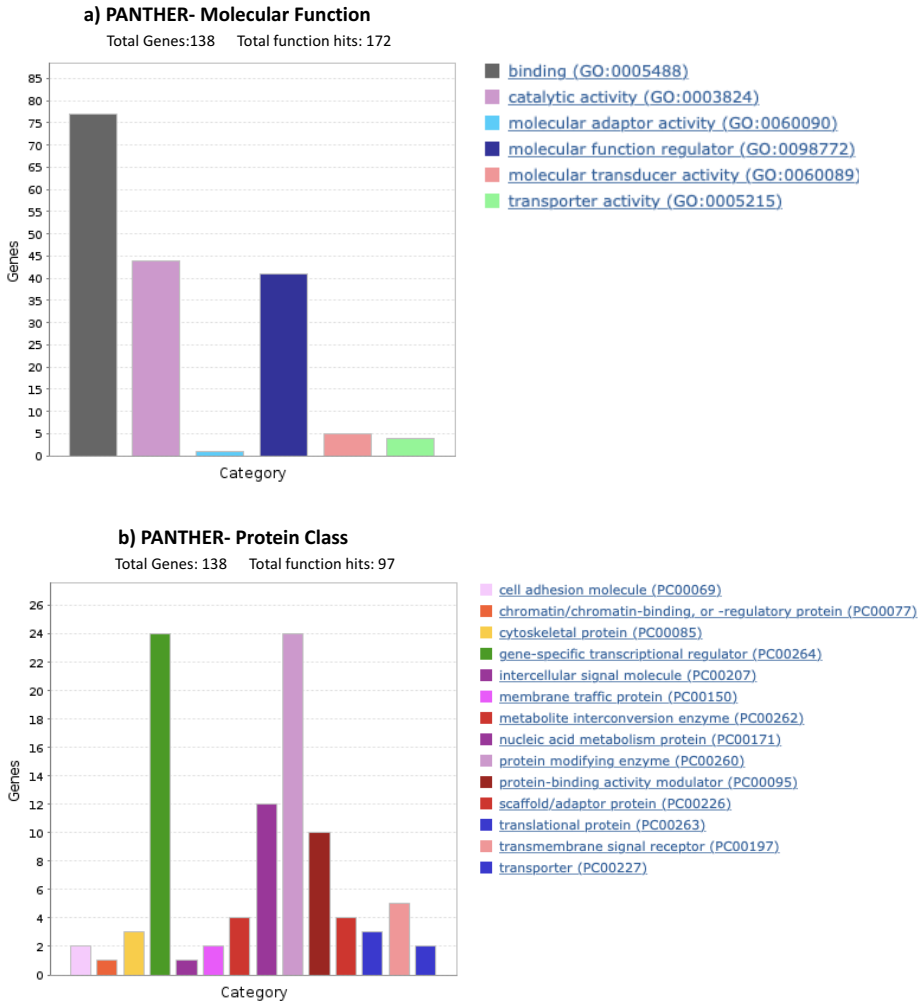

Figure 12: Enrichment analysis for downregulated target genes which interact with upregulated microRNAs regarding a) molecular functions and b) protein class.

Regarding to the 76 upregulated target genes which interact with downregulated microRNAs, the most enriched molecular functions were also binding (32 genes), catalytic activity (27 genes) and molecular function regulator (18 genes) (Figure 13a). For protein class analysis, protein modifying enzyme (14 genes), gene-specific transcriptional regulator (12 genes) and protein-binding activity modulator and transmembrane signal receptor (8 genes) were the most enriched (Figure 13b).

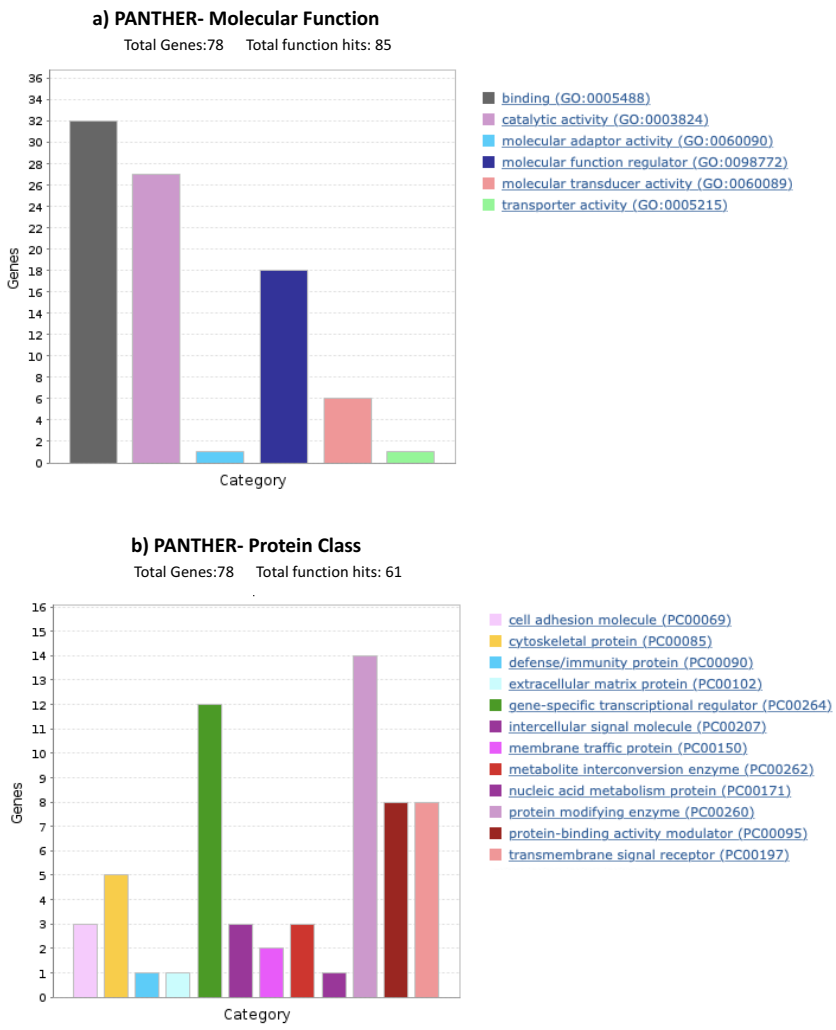

Figure 13: Enrichment analysis for upregulated target genes which interact with downregulated microRNAs regarding a) molecular functions and b) protein class.

In order to evaluate the pathways that could be affected by the microRNAs with the significant target genes, we performed a pathway enrichment analysis using the ConsensusPathDB web tool. Among the ten most significant pathways for downregulated and upregulated target genes, we found Pathways in cancer over-represented in both cases (p-value 3.98×10^{-23} and 1.75×10^{-09}, respectively) (Table 21 and Table 22), represented in Figure 14. In these pathways, upregulated microRNAs targeted up to 38 genes (Annex Table 15) and downregulated microRNAs targeted up to 17 genes (Annex Table 16). Genes of cancer signaling pathways targeted by downregulated and upregulated target genes are listed in Annex Table 17 and 18. Moreover, among the ten most significant pathways, FoxO signaling pathway, signaling by Receptor Tyrosine Kinases and PI3K-Akt signaling pathway were over-represented for downregulated target genes (Table 21). For upregulated target genes, among the ten most significant pathways, was also over-represented signaling by Receptor Tyrosine Kinases pathway, with up to 14 genes (Table 22).

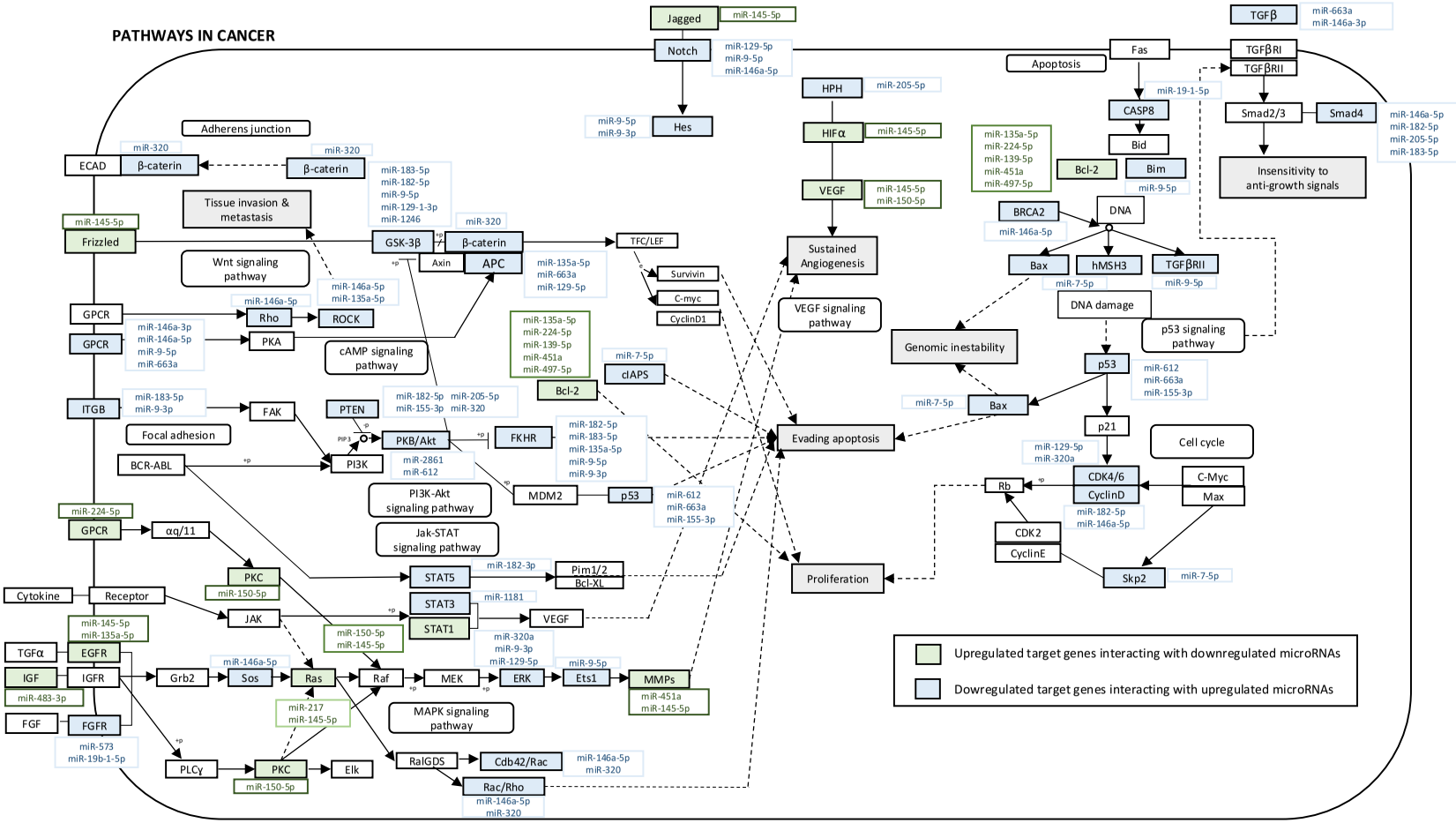

Figure 14: Genes of Pathway in cancer targeted by downregulated and upregulated microRNAs (adapted from KEGG database)

Table 21: The top 10 enriched pathways for downregulated target genes.

Pathway name	Set size	Candidates	P	q-value	Pathway source
Pathways in cancer - Homo sapiens (human)	526	38 (7.2%)	$3.98x10^{-23}$	$2.93x10^{-20}$	KEGG
Colorectal cancer - Homo sapiens (human)	86	17 (19.8%)	$5.11x10^{-18}$	$1.88x10^{-15}$	KEGG
Diseases of signal transduction	248	24 (9.7%)	$1.54x10^{-17}$	$3.8x10^{-15}$	Reactome
MicroRNAs in cancer - Homo sapiens (human)	299	25 (8.4%)	$1.01x10^{-16}$	$1.87x10^{-14}$	KEGG
Hepatitis B - Homo sapiens (human)	144	18 (12.5%)	$2.52x10^{-15}$	$3.72x10^{-13}$	KEGG
Disease	510	29 (5.7%)	$6.92x10^{-15}$	$8.51x10^{-13}$	Reactome
FoxO signaling pathway - Homo sapiens (human)	132	17 (13.0%)	$8.35x10^{-15}$	$8.81x10^{-13}$	KEGG
AGE-RAGE signaling pathway in diabetic complications - Homo sapiens (human)	99	15 (15.2%)	$3.29x10^{-14}$	$3.04x10^{-12}$	KEGG
Signaling by Receptor Tyrosine Kinases	423	26 (6.1%)	$3.83x10^{-14}$	$3.14x10^{-12}$	Reactome
PI3K-Akt signaling pathway - Homo sapiens (human)	354	24 (6.8%)	$4.63x10^{-14}$	$3.33x10^{-12}$	**KEGG**

Table 22: The top 10 enriched pathways for upregulated target genes.

Pathway name	Set size	Candidates	P	q-value	Pathway source
MicroRNAs in cancer - Homo sapiens (human)	299	15 (5.0%)	$4.38x10^{-11}$	1.42x10-08	KEGG
EGFR Transactivation by Gastrin	10	5 (50.0%)	$9.69x10^{-10}$	1.53x10-07	Reactome
Pathways in cancer - Homo sapiens (human)	526	17 (3.2%)	$1.75x10^{-09}$	1.53x10-07	KEGG
Bladder cancer - Homo sapiens (human)	41	7 (17.1%)	$1.88x10^{-09}$	1.53x10-07	KEGG
Proteoglycans in cancer - Homo sapiens (human)	201	11 (5.5%)	$9.01x10^{-09}$	5.84x10-07	KEGG
Gastrin-CREB signaling pathway via PKC and MAPK	19	5 (26.3%)	$4.3x10^{-08}$	2.05x10-06	Reactome
Signaling by Receptor Tyrosine Kinases	423	14 (3.3%)	$4.44x10^{-08}$	2.05x10-06	Reactome
AGE-RAGE signaling pathway in diabetic complications - Homo sapiens (human)	99	8 (8.1%)	$5.46x10^{-08}$	2.21x10-06	KEGG
SHC1 events in ERBB2 signaling	22	5 (22.7%)	$9.63x10^{-08}$	3.32x10-06	Reactome
Signaling by SCF-KIT	43	6 (14.0%)	$1.02x10^{-07}$	3.32x10-06	Reactome

As part of the microRNA-mRNA interaction network analysis strategy, a protein-protein interaction (PPI) network was also constructed for DE expressed genes regulated by miR-205. This network was constructed only with miR-205 as the outcome revealed that miR-205-5p was associated with worse OS and PFS independently of IPI and subtype. A total of 36 DE expressed genes were validated targets of miR-205-5p and were used for the PPI analysis. The two nodes with the highest degree were ERBB3 and PTEN (Figure 15).

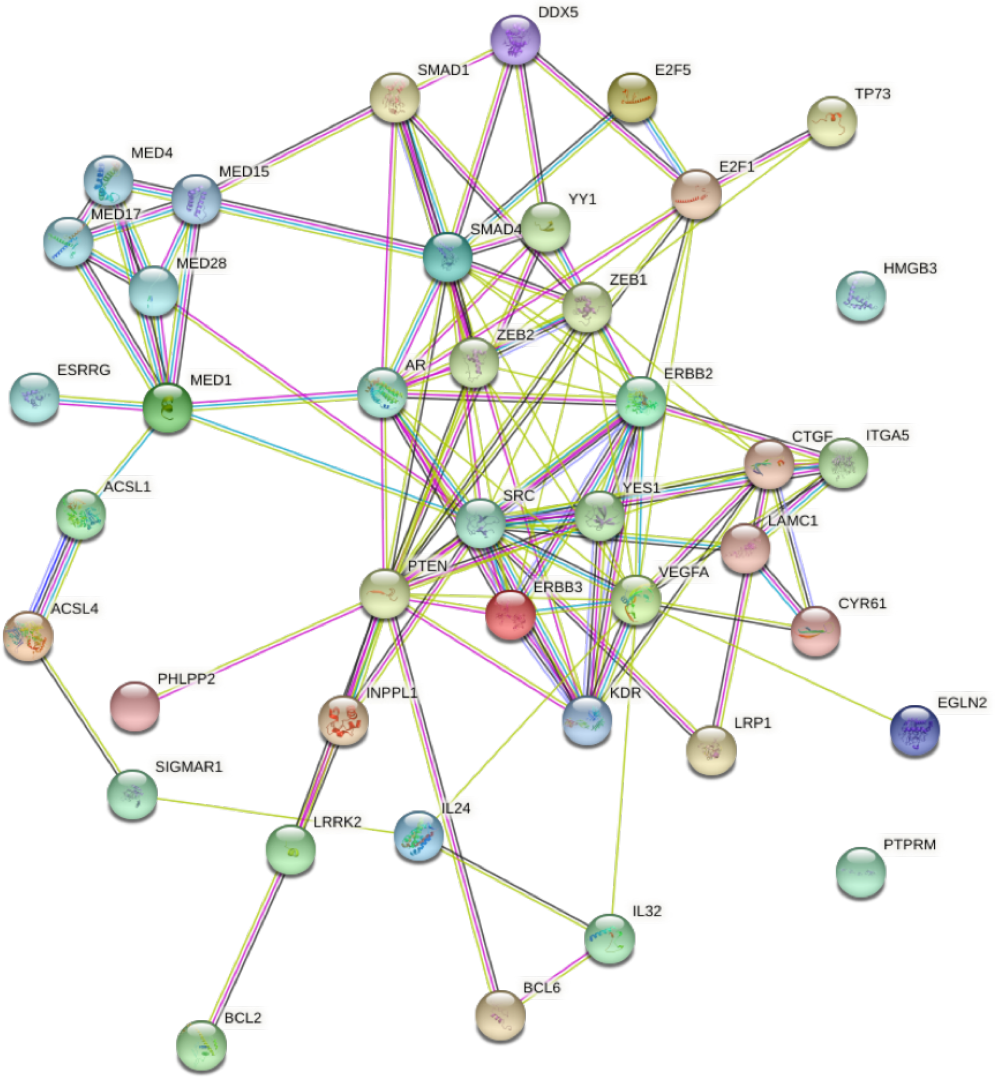

Figure 15: Protein-protein interaction network for 36 target genes of miR-205

DISCUSSION

In this study, our first goal was to determine a microRNA set with utility in DLBCL diagnosis, classification, prognosis, and treatment response. For this, we aimed to identify a new signature of microRNAs of relevance in DLBCL in order to improve the diagnosis, subtype characterization and treatment response. To that end, we conducted, first, a systematic review to identify microRNAs previously proposed as biomarkers in DLBCL, and a validation in a Spanish population. Secondly, a new signature of microRNAs was proposed through small RNA sequencing. Our second goal was to investigate the mechanism of action of microRNAs in the pathogenesis of DLBCL building a microRNA-mRNA interaction network. This will enable to greater understanding of DLBCL pathological mechanisms and improve survival rates.

SELECTION OF POTENTIAL MICRORNAS DESCRIBED IN THE LITERATURE IN DIFFUSE LARGE B CELL LYMPHOMA

We have performed an in-depth analysis of the current literature in relation to the potential role of microRNA expression in tumor biopsies as biomarker for diagnosis, subtype characterization, treatment response and prognosis in patients with DLBCL.

Regarding the suitability of microRNAs as diagnostic biomarkers in DLBCL, 33 articles were identified, in which a total of three microRNAs (miR-155-5p (145,147,167,170,174,176,178–181), miR-21-5p (145,161,171,174,178,180,183), and miR-146a-5p (140,146,154)) were found significantly deregulated in DLBCL patients in more than two studies with concordant results. Among them, miR-155-5p and miR-21-5p presented the most consistent results, being found upregulated in DLBCL patients in most studies.

MiR-155-5p, was the most widely studied microRNA and was found upregulated in DLBCL patients in ten of the studies in which it was analyzed (145,147,167,170,174,176,178–181), while no significant association was found in the other two studies (149,150). Among the two studies that did not find a significant association between mir-155-5p and DLBCL, one presented the smallest sample size with nineteen patients (149), and the other study followed stricter criteria for statistically significant associations (150). Our validation study also supports an upregulation of this microRNA in DLBCL samples, further pointing the expression of this microRNA out as a good biomarker of the disease. In agreement with these results, previous studies have suggested that miR-155 could represent an onco-miR as its expression is activated in many tumors, i.e., prostate cancer, breast cancer, and other tumors, particularly those of the lymphoid tissue (197–199). A possible explanation for its implication in DLBCL is that the validated targets of this microRNA include known hallmarks of DLBCL, such as *SOSC* or *SHIP1* (200).

On the other hand, it is noteworthy that miR-21-5p, which was analyzed in nine independent studies, was significantly upregulated in DLBCL patients in seven of them (157–159,161,164,165,170,173,176,179), while no statistically significant association was found in the other two studies (147,150). The overexpression of this microRNA was also confirmed in our validation study, being upregulated in DLBCL patients, which further supports the role of this microRNA in the disease. In agreement with this observation, miR-21 has been reported to be deregulated in most cancers, such as colorectal cancer, acting as an oncogene (201). Overall,

miR-21 is considered an onco-miR that acts through the inhibition of the expression of different phosphatases, such as PDCD4 (Programmed Cell Death 4) and PTEN (Phosphatase and Tensin Homolog), controlling the activity of signaling pathways like AKT and MAPK (202), which could explain its role in DLBCL.

By contrast, miR-150-5p presented controversial results since it was found down-regulated in DLBCL patients in four studies (145–148) and contradictorily up-regulated in DLBCL patients in another study (149), while no significant association was reported in the remaining study (150). However, this contradiction could be explained by the small sample size of the study by Hans *et al*., (DLBCL patients n = 12 and controls n = 7) finding the microRNA upregulated, which can lead to unreliable results. Indeed, this microRNA was the second most downregulated microRNA in our validation population. In addition, *in vitro* studies also support an important role for this microRNA. In fact, increased expression of miR-150 has been shown to decrease cell proliferation and induce apoptosis in NK/T-cell lymphoma lines (203). Furthermore, miR-150 has been reported to be a key regulator of normal hematopoiesis targeting *MYB* (204). Thus, given its crucial role in hematopoiesis, it is tempting to speculate that miR-150 may function as a general tumor suppressor in DLBCL.

The utility of microRNAs for DLBCL classification was analyzed by 22 studies. A total of four microRNAs (miR-155-5p (145,150,155,178–181,187,192,194), miR-221-3p (150,151,155,180), miR-222-3p (150,151,155) and miR-28-5p (150,151,187,191)) were found deregulated in more than two studies. However, miR-222-3p and miR-28-5p showed contradictory results since they were not significantly related to DLBCL classification in four (145,147,187,191) and two studies (145,155), respectively. Of note, miR-28-5p was the only microRNA in this section that we could validate in our population, which prevents us from excluding it as a potential biomarker. Some of the discrepancies might be due to the fact that subtype classification of the DLBCL patients was performed by GEP or IHC, which makes the studies less comparable due to the variable reproducibility of IHC stains and interpretations. The only microRNAs that showed more consistent results were miR-155-5p and miR-221-3p. MiR-155-5p was found upregulated in the non-GCB subgroup in ten out of eleven studies and only found not associated in a study which used IHC for classification and a more stringent requirement for differentially expressed microRNAs (199). In accordance with this result, in our validation study, in which the classification was also performed using IHC, we did not find differences in miR-155-5p expression between GCB and non-GCB subtypes either, which could be pointing to relevant differences between classifications that may affect the expression of this microRNA. On the other hand, miR-221-3p was found upregulated in the non-GCB subgroup in four of the six studies in which it was analyzed. However, in our validation study, we did not find significant differences in its expression, which in addition to previous controversy, casts doubt on its usefulness as a biomarker for subtype classification.

Focusing on microRNAs as predictive biomarkers of response to R-CHOP treatment, five studies were identified with no agreement in the microRNAs considered (148,175,176,179,190). Among them, upregulation of miR-27-3p (148), miR-34a-5p (176) and miR-224-5p (175) were associated with chemosensitivity, and miR-155-5p and miR-146-5p (179) were associated with chemoresistance (Annex Table 3). While further studies are needed to confirm these preliminary

results, our validation study does not support the value of these microRNAs as biomarkers of response to R-CHOP treatment.

Finally, the implications of microRNAs in prognosis in DLBCL was analyzed in 20 studies finding 50 significant microRNAs (147,148,150–156,167,172,173,175,177–180,184,186,187,190,193–196). Among them, the expression of miR-222-3p (151–154) and miR-155-5p (167,179,187,195) were found associated with prognosis in more than two studies with concordant results. In addition, mir-155-5p was also associated with worse outcome in the non-GCB subgroup (154). However, these microRNAs were analyzed in an equal or higher number of additional studies, including our validation study, without finding any association with prognosis, which means that none of the analyzed microRNAs were established as a reliable marker of prognosis. It is noteworthy that most studies failed to report the specific treatment regimens, a parameter of great relevance to find prognostic biomarkers, since prognosis is dependent on the specific treatment regimen.

Several limitations were faced while performing this systematic review. On one hand, the included studies presented great heterogeneity in sample sources, types of controls used or methodology for expression analysis. This methodological variability could be a source of differences in results among studies. Since the effect of such differences is difficult to determine in the context of a review, it would be of great relevance to reach a consensus and standardize the methodology of study used for future works in order to facilitate reproducibility and comparisons among studies.

In addition, there was variability in the cut-off value for statistical significance among studies, which we considered to be a potential source of heterogeneity. Moreover, there is a tendency to only publish statistically significant results, which leads to bias. All of these limitations in the published literature may be contributing to the lack of consistency in many of the results, which makes it difficult to draw final conclusions about the role of some of the microRNAs analyzed as biomarkers in DLBCL.

Finally, the studies performed usually considered a limited set of selected microRNAs, which limits the number of comparable results and centers the discussion on those microRNAs that are better known, leaving other microRNAs aside. Therefore, we considered that it was necessary to perform large-scale studies with a wider array of microRNAs using techniques such as next-generation sequencing that allow a deeper and unbiased identification of microRNAs with the potential to be used as biomarkers in DLBCL.

MICRORNA EXPRESSION SIGNATURE IN DLBCL BASED ON SMALL RNA SEQUENCING

Considering the limitations identified thanks to the systematic literature review, we decided to carry out a study to define a new microRNA signature in DLBCL through comprehensive small RNA sequencing. This provides expression profiles of microRNAs previously described but also novel candidates, that are not usually included in other studies, in which the most frequently used technologies are microarray or reverse transcription quantitative PCR (RT-qPCR).

MicroRNA expression signature for diagnosis of DLBCL

Overall, the highest differences in microRNA expression among groups were observed when we compared their expression in DLBCL and control samples. A principal component analysis including the expression of all the identified microRNAs shows a clear separation between the control group and DLBCL samples (Figure 9). This would suggest that microRNA profiles of DLBCL and normal samples vary greatly. Besides the fact that PCA reveals large differences in microRNA profiles between the groups, we also detected several significantly deregulated microRNAs when individual comparisons were carried out.

A total of 146 microRNAs showed a differential expression when we compared microRNA expression profiles between DLBCL samples at diagnosis and control samples. Of these microRNAs, 112 were more expressed in DLBCL patients. Notably, deregulation of most of these microRNAs had not been previously reported in DLBCL or in other malignancies. The most significantly upregulated microRNAs in DLBCL samples were miR-210-3p and miR-994, and the most downregulated microRNAs were miR-215-5p and miR-150, with a higher expression in healthy individuals.

MiR-210 has been demonstrated to be overexpressed in lymphomas and suggested to play an important role acting as a key regulator of cell cycle (205). In accordance with our findings, overexpression of miR-210-3p has been previously reported in DLBCL patients in comparison with normal B-cells in two studies (146,147) although no statistically significant association was found in other two studies (150,165). Interestingly, a number of microRNA profiling studies have shown elevation of miR-210 in a wide array of cancers such as prostate (206), gastrointestinal (207), breast (208) or lung cancer (209). Therefore, the evidence accumulated to date by other authors and the present study indicates that miR-210-3p could also act as an oncogene in DLBCL. New evidence indicates that miR-210 upregulation can decrease the efficacy of anti-tumor immune response and improve the ability of tumors to avoid immunosurveillance by attenuating the efficacy of the cell-mediated cytotoxicity (cytotoxic T lymphocytes (CTL) and Natural killer (NK) cells) (210), a mechanism involving downstream targets such as *PTPN1 ,* as shown with our interaction network analysis.

Upregulation of miR-944 has not been previously described in either DLBCL or other hematopoietic malignancies. Nevertheless, altered expression of miR-944 has been shown in many tumor types, with tumor suppressive or oncogenic roles depending on the malignancy. On the one hand, in breast cancer (211), cervical cancer (212) and endometrial cancer (213), the expression of miR-944 was significantly upregulated, suggesting that miR-944 promotes tumor progression by promoting cell proliferation, migration, and invasion. On the other hand, other studies provided evidence that miR-944 acts as a tumor suppressor being significantly

downregulated in colorectal cancer tissues (214,215). According to the results obtained in our study, it seems that, in DLBCL, it follows the same trend as in breast, cervical and endometrial cancer, as acting as an oncogene. However, the biological functions and mechanism of action of miR-944 in DLBCL are not yet known. Through our interaction network analysis, we identified *HECW2, S100PBP, PTP4A1* and *SIAH1* as targets of miR-944 of relevance in DLBCL. *HECW2* encodes a poorly characterized HECT domain ubiquitin ligase, which has been shown to stabilize tumor suppressor p73 and enhance its transcriptional activity (216). It is tempting to speculate that miR-944 may suppress *HECW2* expression leading to a downregulation of p73 and, thus, promoting tumorigenesis. However, further studies are warranted to determine the functional role of *HECW2* in DLBCL and p73-miR-944 regulatory network. Another miR-944 target, *S100PBP*, has been shown to suppress cell invasion and adhesion in pancreatic cancer cell lines (217). Given that *S100PBP* is downregulated in DLBCL, it would also be interesting to determine whether it is also involved in DLBCL cell invasion. Finally, downregulation of *SIAH1* has previously been described to promote cell proliferation, colony formation, migration and invasion, and the entry of cells into S phase and inhibit apoptosis in human breast cancer (218). Therefore, if it followed the same scheme in DLCBL, it would be another plausible mechanism of action for the involvement of miR-944 in the development of DLBCL.

Regarding downregulated microRNAs, miR-215 was the most significantly downregulated in our population. Accordingly, the expression of miR-215 in DLBCL had been previously studied by Wu *et al*., (219), also finding a significant downregulation in DLBCL patients. Regarding other hematological malignancies, Wang *et al*., 2010 (220) also reported down-regulation of miR-215 expression as a common event in in acute myeloid leukemia (AML). Remarkably, miR-215 is one of the most extensively studied microRNAs in different types of cancer and there are accumulating reports that it may function as either tumor suppressor or oncogene depending on the tumor type. In fact, it was first identified as a tumor suppressor in colorectal cancer (221,222) non-small cell lung cancer (223), and breast cancer (224), tumors in which its expression is downregulated, whereas it may act as an oncogene in gastric cancer, in which it is upregulated (225) and correlated with the progression of tumor invasion and stage of the disease (226,227). These data confirm the complexity of cancer regulation and may reflect the ambivalent role of miR-215 in tumor progression and suppression, depending on the particular tissue of origin of the tumor. In this case, our results deliver new insights into the suppressor potential of miR-215 in DLBCL.

Interestingly, miR-215 is located, together with miR-194-5p, which was de fourth most downregulated microRNA in our study, in the *miR-215-194-1* cluster at *1q41.1,* within the common fragile site FRA1H (1q41–q42.1) that is deleted in many types of cancers (228). The co-downregulation of these two microRNAs was also reported in other cancers, such as renal cell carcinoma (229), and multiple myeloma (230). Regarding their mechanism of action, Pichiorri *et al.,* (230) observed that, in multiple myeloma treated cells with enforced expression of these microRNAs, *MDM2* was dramatically down-regulated at protein and mRNA levels and this downregulation was inversely associated with higher p53 expression and p21 activation, which could be a plausible explanation for their role as tumor suppressors. To further explore the mechanisms by which miR-215 and miR-194 could affect cellular processes in DLBCL, we performed our interaction network analysis and identified a number of targets that are known to be involved in tumor progression. Among them, the activin receptor type 2B *(ACVR2B)* has

been previously described to be targeted by this two microRNAs in renal childhood neoplasms (231). This receptor plays a crucial role in the activation of activin, which is an important part of the transforming growth factor (TGF)-β signaling pathway and might influence tumorigenesis. These findings would support a tumor-suppressive function for the *miR-215-194-1* cluster and possible mechanisms of action, which makes them interesting targets for further research.

Among the microRNAs with a lower expression in DLBCL, we also found miR-150-5p, which had been previously analyzed in DLBCL in several studies. Downregulation of miR-150 has been previously described in leukemias and lymphomas, including DLBCL, and this microRNA might have a highly specific role in the development of different lymphatic neoplasias, including DLBCL, as we have described in the previous section.

Combined use of these five microRNAs (miR-210-3p, miR-944/miR-215-5p, miR-150-5p and miR194-5p) might further enhance the diagnostic accuracy over any single microRNA marker. Nevertheless, future studies are necessary to clarify whether the expression profile for these five microRNAs is capable of discriminating DLBCL from other types of tumors.

In brief, besides the confirmation of several deregulated microRNAs previously described, here we report the discovery of additional microRNAs that are deregulated in DLBCL and that had not been previously studied in this malignancy. Indeed, regarding the 20 most upregulated microRNAs (Table 12), only miR-146a-5p, miR-129-5p and miR-205 had been previously studied in DLBCL; and taking into account the 20 most downregulated microRNAs (Table 13), only miR-224-5p, miR-145-5p, miR-497-5p, miR-151b and miR-451 had been previously studied in DLBCL.

The present results further show that microRNA profiling may represent a novel reliable and standardizable diagnostic tool in the characterization of DLBCL. To further improve the analytical properties of the DLBCL diagnostic, it may be helpful to use these novel microRNA signatures together with the conventional biomarkers.

MicroRNA expression signature for the classification of DLBCL subtypes

DLBCL GCB and Non-GCB subgroups, have shown distinct oncogenic activation mechanisms, genomic abnormalities and clinical outcome (10,69). When treated with a regimen containing R-CHOP or R-CHOP like regimens, patients with GCB DLBCL have been reported to have a better survival independent of the IPI (31,232). Nevertheless, the prognostic value of classification remains unclear, especially considering that different methodologies are used for classification with uneven results. In this section, we analyzed whether microRNA expression varied between both subtypes and could contribute to ease the classification in the clinical setting.

A total of eight microRNAs showed a differential expression, when we compared microRNA expression profiles between GCB and Non-GCB DLBCL samples at diagnosis. Five of them were upregulated in GCB DLBCL patients (miR-129-2-3p, miR-4464, miR-3150b-3p, miR-138-5p and miR-129-5p) and three microRNAs were upregulated in Non-GCB subtype (miR-511-5p, miR-205-5p, and miR-3652).

Regarding the microRNAs upregulated in GCB DLBCL patients, higher levels of miR-129-5p and miR-138-5p in the GCB subtype were previously reported (147,150,155,187). Furthermore, microRNAs upregulated in GCB subtype appear to target transcripts that are known to be

deregulated in the formation of germinal center lymphomas (233), specially miR-129-3p/miR-129-5p, and miR-138-5p. The target genes of these microRNAs were predicted using miRTarBase online tool. These included genes related with the cell cycle (*CDKN1A*), MAPK and NFkB signaling *(MAPK1).* A high expression level of these microRNAs (miR-129-3p/miR-129-5p, and miR-138-5p) could negatively regulate the expression of cyclin-dependent kinase inhibitor, *CDKN1A* (also known as p21), allowing cells to overcome the G1 cell cycle checkpoint. Hence, we suggest that these microRNAs are essential for centroblasts to progress from G1 to S phase of the cell cycle, by downregulating *CDKN1A* (234). Interestingly, we also found *TP53* as a target gene of miR-129-3p/miR-129-5p, and miR-138-5p. *TP53* gene is a crucial tumor suppressor that mediates cell-cycle arrest, DNA repair, apoptosis, senescence, and autophagy (235,236). *TP53* dysfunction is implicated in lymphomagenesis and disease progression, and normal function of p53 is crucial for tumor suppression. While the prognostic significance of *TP53* mutations has been inconsistent in several cancers, recent studies have showed that the *TP53* mutations do stratify GCB-DLBCL, but not Non-GCB-DLBCL, into distinct subsets with a different OS (237), which could suggest that GCB-DLBCL is *TP53*-dependent and Non-GCB DLBCL is *TP53*-independent, and thus, which could explain the differences in expression of the aforementioned microRNAs among subtypes. By contrast, miR-4464 and miR-3150b-3p, also found upregulated in the current study, have not been previously described in the pathogenesis of DLBCL. Finally, miR-511-5p, miR-205-5p and miR-3652, the three microRNAs most upregulated in Non-GCB, they were not previously studied in the pathogenesis of DLBCL.

Therefore, the differential expression of these microRNAs might reveal different regulatory pathways and they could be useful in the classification of DLBCL patients. As we have seen before, the germinal center B cell is the normal counterpart of GCB-DLBCL and Non-GCB subtype of DLBCL resembles post–germinal center plasmablasts (10,74). A hallmark of non-GCB DLBCL is constitutive activation of the NF- κB pathway. In contrast, germinal center B-cell-like (GCB) DLBCLs have been shown to be addicted to the oncogenic activation of the PI3K/AKT pathway (233). However, the mechanism underlying the activation of PI3K/AKT pathway and its oncogenic role in DLBCL remains unclear.

Even if we have found eight microRNAs differentially expressed between subtypes, we must point out that, as mentioned above, a limitation we face in this study is that the Han's IHC algorithm was used to classify the patients. Even though studies have shown clearly that the IHC-based classification provided very similar outcome prediction compared with the GEP-based classification, which is the gold standard (25), variable reproducibility of IHC stains and interpretations because of subjective and technical factors affecting immunostaining, may be the reason for inconsistent results (238). In fact, in the systematic review, we have observed some differences in the microRNAs identified between studies that used GEP and those that used IHC. Therefore, further studies are required to clarify these differences, and, on the meantime, it might be of greater interest to identify microRNAs that are associated with treatment response or prognosis rather that those differentially expressed between subtypes.

Predictive microRNA signature for therapy response

Around 10–20% of patients with DLBCL will be refractory to first-line chemotherapy, and up to 30–40% will relapse after obtaining a complete remission. It would be of great relevance to

identify these patients from the beginning for a better assignment of first-line therapy because the outcome of DLBCL patients after failure to first-line induction treatment is dismal (239). Therefore, the identification of new indicators for the effective assessment of therapeutic efficacy is an urgent priority. However, the underlying mechanisms of the acquisition of resistance to chemotherapeutic agents are still poorly understood. As regulators of biological functions, microRNAs also influence drug resistance. For that reason, we sought to identify novel microRNAs associated with response to treatment in DLBCL patients to use them as biomarkers to stratify these patients and personalize the therapy; specially bearing in mind that the relationship between microRNA expression and therapeutic response in DLBCL has been poorly studied to date.

In the present study, using a small RNA sequencing approach, high expression of ten microRNAs (miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p and miR-3928-3p) was associated with good response to treatment. By contrast, high expression of miR-192-5p was associated with chemoresistance to R-CHOP therapy. Notably, deregulation profiling of most of these microRNAs in DLBCL has not been previously reported; but some of them have been studied in the context of other types of cancer.

We found that miR-12136 was the most significantly upregulated microRNA in patients with complete response and exhibited greater than a 25-fold increase in expression. Remarkably, miR-12136 is a microRNA that has been recently discovered and, therefore, the knowledge about this microRNA is very limited. However, the result obtained points to this microRNA as an interesting candidate for additional studies. Furthermore, we observed that miR-3681-5p and miR-3928-3p, which are other microRNAs that have not been studied until now, were both associated with good response and with good prognosis. We also observed that miR-129-5p was associated with good response to treatment (Table 15). Remarkably, in agreement with our results, low levels of miR-129-5p have been previously correlated with breast cancer chemoresistance, which supports the idea that high levels of this microRNA could be of relevance for treatment response under some therapeutic regimens (240). In addition, miR-127, which was associated with good response to treatment in our population, was previously described to have a role in apoptosis activation via p53 in breast cancer (241), which could be a plausible mechanism of action for its association with good response in DLBCL. Of note, previous studies linked miR-192 and chemoresistance in lung cancer (242) and in larynx and hypopharynx (243). Hence, those previous results further support a role of this microRNA in chemoresistance in DLBCL.

We propose miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p, miR-3928-3p, and miR-192-5p as microRNA signature associated with treatment response in DLBCL patients treated with R-CHOP, which could help identify high risk refractory patients and provide them a chance to optimize the first line treatment. In addition, in the future, microRNA-based treatments, in combination with traditional chemotherapy, may be a new strategy for the clinical management of DLBCL refractory patients. In this line, increasing the expression of miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p, and miR-3928-3p could be a good choice for those patients with a low expression of these microRNAs, due to their correlation with good response. This could be attained with the use of pre-

microRNAs, microRNA genes, or microRNA mimics (244–246). On the other hand, the inactivation of miR-192-5p through the use of antisense microRNA oligonucleotides, microRNA sponges, or microRNA inhibitors (245,246) could be a new therapeutic strategy for patients with a high expression of this microRNA. In this context, while microRNA-based therapy is a promising strategy in cancer, the major issue that it must face is its challenging delivery. Fortunately, major advances are being made in this sense.

Consequently, this information will be of benefit in further improving the therapeutic efficacy and development of personalized treatment in which microRNA expression could assist in the choice of the best treatment for each patient.

MicroRNA expression signature associated with outcome in DLBCL

In addition to treatment response, the ability to accurately predict survival may be crucial for initial treatment planning in patients with DLBCL. Nowadays, the IPI remains the gold-standard for prognosis prediction in DLBCL. However, this system does not reflect the heterogeneities of cell morphology, immune phenotype and molecular biology in DLBCL patients. Moreover, it has failed to predict prognosis in a considerable proportion of patients with B cell lymphoma in the Rituximab era (247). Previous studies have presented a variety of conclusions regarding which clinical factors truly affect DLBCL patient outcomes, with various contradictions among them. Therefore, new biomarkers would be needed to combine with clinical features. Accumulating evidence has revealed that microRNA expression may be a key biomarker to predict patient outcome. Besides serving as a better risk stratification tool than microRNAs associated with subtype (GCB vs. non-GCB), microRNAs associated with prognosis may also be useful for the development of specific therapeutic agents.

We identified seven microRNAs (miR-4444, miR-449c-5p, miR-3681-5p, miR-3928-3p, miR-449b-5p, miR-370-3p, miR-4424) significantly upregulated in patients with long term remission and four microRNAs (miR-133a-3p, miR-133a-3p, miR-208b-3p, miR-205-5p) upregulated in relapsed patients.

Among them, miR-3681-5p and miR-3928-3p had not been previously studied in DLBCL. In fact, miR-3681-5p and miR-3928-3p have been recently characterized and for that reason they have not been included in other studies. Our survival analysis reveals that miR-4444 is significantly associated with better OS and PFS. Until now, the expression pattern of miR-4444 in DLBCL and its prognostic significance have not been investigated systematically, but it was previously reported as part of a model based on six microRNAs associated with a poor prognosis in colon adenocarcinoma (248). One possible explanation of these contradictory result is that miR-4444 could have different targets and could participate in different pathways, playing different roles depending on the cell type, the tumor pattern of gene expression and the type of therapy, which makes the results non comparable. Further studies are granted to understand its role in DLBCL.

Interestingly, miR-205 was also significantly associated with worse OS in our group of DLBCL patients uniformly treated with immunochemotherapy. Furthermore, multivariate analysis revealed that miR-205-5p was associated with worse OS and PFS independently of IPI and subtype. Interestingly, subtype was not associated with survival, probably due to the fact that these patients had been treated with rituximab which has been found to overcome the negative

prognosis associated with Non-GCB DLBCL (249). Anyway, the combination of microRNA expression patterns with IPI status and DLBCL subtype could improve the stratification of patients with different prognosis, leading to a better personalized treatment. In the present study, we identified that the expression of miR-205-5p appears to be very useful in DLBCL because it is not only associated with bad prognosis and survival, independent of IPI and subtype, but it was also upregulated in DLBCL patients and in Non-GCB patients.

In order to deepen into the mechanisms of action of this microRNA in DLBCL, as the miR-205 target genes that are deregulated in DLBCL have been studied as part of the microRNA-mRNA interaction network analysis strategy, a protein-protein interaction (PPI) network was constructed (Figure 15). *ERBB3* possessed the highest number of interaction nodes. Somatic mutations of *ERBB3*, which encodes a member of the epidermal growth factor receptor family, are common in colorectal and gastric cancers, but rare in hematological malignancies. The function of this gene remains elusive. Among these 36 target proteins, *PTEN* was also key node protein. *PTEN* is the major negative regulator of the PI3K/AKT signaling pathway which is constitutively activated in 25-50% of DLBCL according to recent studies (250). *PTEN* loss has been associated with poorer prognosis in many solid tumors including DLBCL and is significantly related to advanced disease, chemotherapy resistance, and poor survival (251–254). Therefore, this could be a plausible explanation for its role in DLBCL.

Collectively, our data indicate that miR-4444 and miR-205 are associated with the pathogenesis of DLBCL and are implicated with patient prognosis. To the best of our knowledge, the present study is the first to demonstrate the prognosis predictive value of miR-4444 and miR-205 for patients with DLBCL, and provide a novel direction for treatment. Thus, we infer that earlier adoption of a more intensive therapeutic program may benefit the patients with miR-4444 low expression and miR-205 high expression. Our results point to the development of personalized treatment in which microRNA expression can assist in the choice of the best treatment option for each patient. However, their specific mechanism and prognostic value require further exploration and verification.

Interestingly, most previous studies of microRNAs associated with DLBCL prognosis have been performed with DLBCL treated in pre-Rituximab era. Thus, this study provides a very interesting starting point in this context. We consider that our results should be validated in independent studies with adecuate cohort size to validate robust prognosis biomarkers.

DECIPHERING THE MECHANISM OF ACTION OF MICRORNAS IN DIFFUSE LARGE B CELL LIMPHOMA THROUGH A microRNA-mRNA INTERACTION NETWROK ANALYSIS

To gain insight in the global mechanism of deregulated microRNAs, we also performed a microRNA-mRNA interaction network, since deregulation of microRNAs may result in the aberrant expression of microRNA target genes, resulting in the acceleration of lymphomagenesis. Integration analysis determined that up to 214 genes could be affected in DLBCL as a consequence of microRNA deregulation, since they showed an expression pattern in accordance with microRNA mechanism. In this line, there were a total of 138 genes lowly expressed in DLBCL whose downregulation could be caused by the increased expression

observed for the microRNAs found in the current study. In the same line, 76 genes showed a high expression that could be explained by the downregulation of microRNAs. By function enrichment analysis, an over representation of binding and catalytic proteins such us transcriptional factors and kinases among these genes was observed. *In silico* analysis determined that these genes are over-represented in pathways of cancer, mainly PI3K-Akt and MAPK signaling pathways. Interestingly, several genes in these pathways are targeted by more than one microRNA deregulated in the current study, and several microRNAs targeted more than one gene in these pathways, what makes them strong candidates contributing to the pathogenesis of DLBCL. We explore the mechanisms by which deregulated microRNAs contribute to DLBCL focusing in those target genes and microRNAs showing the strongest implication.

Among the most interesting downregulated genes targeted by more than one microRNA, we found *FOXO1* and *PTEN,* involved in PI3K-Akt pathway (255)*,* predicted to be targeted by 5 and 4 microRNAs, respectively. *FOXO1* is a transcription factor for p27, p21, FasL, and Bim, which function as tumor suppressors by blocking the G_1/S transition and inducing apoptosis and DNA repair. *FOXO1* plays a critical role in establishing and maintaining the B cell specific differentiation program, but it is also responsible for cell death due to an inappropriate BCR signaling (256,257). Loss of FOXO1 function leads to promote tumorigenesis by favoring resistance to stress, proliferation and increased cellular survival (258). Abnormal cells that would normally undergo apoptosis may instead survive in the absence of *FOXO1,* which results in tumor expansion. Hence, loss of FoxO function leads to a decreased ability to induce this cell cycle arrest which leads to tumor development (259). Regarding the lymphoid malignancies, *FOXO1* was shown to be a tumor suppressor in Hodgkin lymphoma (260) and dysregulation of *FOXO1* has been previously reported in DLBCLs (261). It has been previously described that *FOXO1* represses the protooncogene *MYC* and inhibitor of apoptosis *BCL2L1* in primary mediastinal B cell lymphoma (262). In addition, *FOXO1* phosphorylation and inactivation is inversely correlated with length of survival in patients with AML (263). In the current study, up to 5 microRNAs, miR-182-5p, miR-183-5p, miR-135a-5p, miR-9-5p and miR-9-3p, directly targeted and suppressed *FOXO1* expression, and therefore, they could contribute to DLBCL pathogenesis silencing this gene. In the same line, *PTEN,* previously mentioned, is a tumor suppressor that acts upstream *FOXO1* negatively regulating *AKT.* Without PTEN regulation, activated *AKT* phosphorylates *FOXO1*, which is subsequently exported from the nucleus into the cytoplasm and degraded by proteasomes (264). MiR-155, miR-320a, miR-205 and miR-182-5p targeted *PTEN* in our study, and then, they could reduce its expression and disrupt the negative regulation of *AKT*. Interestingly, miR-182-5p targeted both genes and could be a crucial microRNA modulating the PI3K/AKT pathway by targeting *PTEN* upstream and *FOXO1* downstream. Supporting our findings, a relation between miR-182 and miR-183 and *FOXO1* suppression had been previously described in classical Hodgkin lymphoma (260).

Another interesting result is the downregulation of *GSK3β*, a gene predicted to be targeted by miR-183-5p, miR-182-5p, miR-9-5p, miR-129-1-3p and miR-1246. *GSK3β* is a critical protein kinase that phosphorylates numerous proteins in cells and thereby impacts multiple pathways such as Wnt signaling pathway. This pathway plays a pivotal role in tumorigenesis in various cancers including DLBCL (265,266). In the absence of Wnt, a β-catenin destruction complex, consisting of Axis inhibition protein (*AXIN*), adenomatous polyposis coli (*APC*), and glycogen

synthase kinase 3β (*GSK-3β*), phosphorylates β-catenin, leading to its proteolytic degradation (267). Wnt signaling inactivates *GSK3β* and prevents it from phosphorylating β-catenin, thus stabilizing β-catenin in the cytoplasm. As β-catenin accumulates, it translocates into the nucleus where it binds to TCF/LEF and dramatically increases their transcriptional activity. Genes up-regulated by TCF/LEF include proto-oncogenes, such as *c-myc* and *cyclin-D1,* which promotes proliferation and evade apoptosis (268). Thus, downregulation of *GSK3β* by miR-183-5p, miR-182-5p, miR-9-5p, miR-129-1-3p and miR-1246 could enhance accumulation of β-catenin and activation of proto-oncogenes. The suppression of *GSK-3β* also enhanced drug resistance in cancer cells through its ability to regulate Wnt/β-catenin signaling (269). Moreover, the overexpression of those microRNAs could enhance DLBCL cell proliferation, invasion, and apoptosis suppression through its regulation of *GSK-3β*.

These data pointed to miR-182-5p, miR-183-3p and miR-9-5p cooperation to contribute to the pathogenesis of DLBCL playing an oncogenic role in DLBCL by comprehensively regulating the PI3K/AKT/FOXO1/Wnt pathway.

We also observed miR-612, miR-663a and miR-155-3p as direct negative regulators of *TP53*. The tumor suppressor p53 plays a crucial role in maintaining genomic stability and tumor suppression. As a transcription factor, p53 controls the initiation of cell cycle and cell division (270). Extensive mutation search demonstrated that the loss of function mutations in *TP53* gene is present in over 50% of malignant tumors (271). Moreover, it has been demonstrated that p53 is associated with a variety of tumors and regulated by some certain microRNAs (272,273). Of the three microRNAs, miR-612 has previously identified as associated with esophageal squamous cell carcinoma development and metastasis through an inhibitory action on mRNA and protein expression of *TP53* (274). These microRNAs might bind to 3´-UTR of the *TP53* mRNA, downregulating *p53* level and function, negatively regulating p53-mediated apoptosis and cell cycle arrest, being important components in the p53 network.

Finally, it is worth to mention the role of miR-146a-5p in this analysis, the candidate microRNA more abundantly upregulated as a diagnosis biomarker. This microRNA showed interactions with up to 11 genes downregulated in DLBCL, including BRCA2*, RHOA, SOS1, STAT1, CCND2, CXCR4, NOTCH1, RAC1, ROCK1, SMAD4,* and *TGFB1.* miR-146a-5p was previously described as one of the key microRNA involved in breast cancer pathogenesis associated with *BRCA1* (275). In this regard, the *BRCA1* gene have the crucial role in breast cancer tumor suppression as the implication in DNA damage repair (276). In addition, even though miR-146a-5p and *BRCA1* feed-back loop has not been previously described in lymphoma, we proposed that miR-146a-5p may inhibit *BRCA1* translation in DLBCL. In addition, Luo *et al*., found that the loss of *SMAD4* expression may be related with the proliferation of DLBCL cells by enhancing transforming growth factor β (TGF-β) pathway signaling pathway (277). *NOTCH1* is a tumor suppressor gene which activity is significantly reduced in skin cancer (278). In fact, an attractive possibility could be that downregulation of NOTCH1 expression, is due, at least in part, to compromised p53 function (279).

Regarding the upregulated genes in DLBCL as a consequence of microRNAs downregulation, the most significant result is *BCL2*. This gene was supposed to be regulated by 135a-5p, miR-234-5p, miR-139-5p, miR-451a and miR-497-5p, but the loss of expression of the mentioned microRNAs

could contribute to its overexpression. Evidence revealed that elevated expression of anti-apoptotic members such as *BCL2* is one of the major contributing factors to B cell lymphomagenesis (280). Since then, many studies have determined that *BCL2* is one of the most important oncogenes through its inhibition of apoptosis and that it causes lymphoma development, in particular in backgrounds with c-MYC overexpression (281). *BCL2* protein mostly localizes in mitochondria, and it inhibits apoptosis by preventing the release of Cytochrome *C* from the mitochondria to the cytoplasm. *BCL2* is upregulated by translocation or other mechanismsin approximately 50% of DLBCL (282). The loss of microRNA-mediated silencing of *BCL2* could also contribute to this overexpression. In fact, previous studies have confirmed that deletion or downregulation or microRNAs, such as miR-15 and miR-16, are involved in the overexpression of *BCL2* (283).Thus, downregulation of miR-135a-5p, miR-234-5p, miR-139-5p, miR-451a and miR-497-5p, could contribute to *BCL2* overexpression in DLBCL, reducing apoptosis of lymphoma cells. All these microRNAs, except miR-234-5p, were among the top 20 microRNAs downregulated in DLBCL in our population, so it would be interesting to study these associations in other populations.

The downregulation of miR-145-5p, candidate microRNA as a diagnosis biomarker, could contribute to the overexpression of genes such as *STAT1, MMP1, JAG1, VEGFA, EPAS1* and*, FZD7.* In accordance with our results, *STAT1* is consistently downregulated in other lymphomas (284) and other cancers (285). *STAT1* typically promotes apoptosis by inducing the expression of members of the cell surface death receptor family and their ligands (286). However, there are some different views on the role of *STAT1* in DLBCL, since other studies proposed that *STAT1* acts as an oncogene in DLBCL (287). Therefore, further experimental data are still needed to verify the function of *STAT1* in DLBCL. The upregulated expression of *MMP1* is of particular interest because it has been previously reported to be upregulated in other cancers, such as esophageal cancer (288). The matrix metalloproteinases (MMPs), mediate many of the changes in the microenvironment during tumor progression. Among them, *MMP1* degrades various components of the extracellular matrix and enable cancer cells to invade and metastasize (289). Regarding *JAG1*, several independent studies report the overexpression of NOTCH1 and the ligand *Jagged1* in multiple myeloma during disease progression (290). *EGFR* may also be found at high levels on some types of cancer cells, which causes these cells to grow and divide (291). *EGFR* is known to be overexpressed in ~30% of gastric cancer cases, and may serve as an effective biomarker for predicting the clinical benefit of anti-EGFR therapy (292). However, the mechanism underlying the regulation of EGFR expression in DLBCL remains unknown.

There is a rapidly increasing understanding of knowledge about microRNAs and their intimate involvement in tumorigenesis and tumor progression. Through this mRNA-microRNA interaction analysis we proposed a mechanism by which deregulated microRNAs could contribute to DLBCL pathogenesis, highlighting the importance of PI3K/AKT/FOXO1/Wnt pathway and BCL2 involvement. Some microRNAs could act in a cooperative manner, such as miR-182-5p, miR-183-3p and miR-9-5p, and others, like miR-146a-5p, seem to be master regulators of genes involved in DLBCL. With this method, we identified candidate biomarkers from a large number of gene sets rather than a small number of differentially expressed gene sets. Identified co-expression microRNA-mRNA pairs in the current study can be applied as targets for clinical co-detection of DLBCL.

LIMITATIONS AND STRENGTHS

During the development of this study, several strengths and limitations were found.

In our present study, we analyzed microRNA expression in FFPE lymph node specimens from DLBCL patients. Firstly, the availability of FFPE lymph node biopsies enabled us to have a long term follow up close to 20 years for the study population, diagnosed with DLBCL between 1999 and 2018. Although we consider 5 years overal survival and event free survival as a robust end point for disease related outcome, we consider an advantage the disponibility of this follow up long period. In addition, the study includes patients of the three major hospitals at the Basque Country, which has allowed us to collect a relatively large patient population. However, the quality of RNA extracted from FFPE blocks is poor and it is usually degraded into fragments of fewer than 300 bases in length (293,294) and chemically modified by methylol groups during formalin fixation (295). Although the RNA extracted from FFPE blocks is often compromised, compared to mRNA, microRNAs are relatively resistant to RNase degradation probably due to their small size and secondary structure (296). In fact, previous studies concluded that the expression of microRNAs from archived FFPE tissue samples was in good correlation with fresh frozen samples and that formalin fixation did not significantly alter the stability of the microRNAs (297). Undeniably, the RNA would be better preserved in Fresh Frozen samples, however, this type of material is rarely available. In these cases, FFPE is usable for microRNA analyses and allows for retrospective large studies where the results can be correlated with clinical parameters of patients.

In this study, a comprehensive analysis of microRNA profiles was performed by next-generation sequencing technology. At present, quantitative measurement of microRNA expression is most commonly accomplished by real-time PCR or microarrays. To our knowledge, few reports exist in which microRNA sequencing were used to identify microRNA signatures in cancer, and only one which analyzed microRNAs with NGS in DLBCL (150). NGS methods are becoming increasingly prevalent technologies, because they allow the identification of all the described microRNAs, which is not possible with other techniques and, as shown by our study, in which many of the associated microRNAs had not been previously studied, it is of great relevance.

On the other hand, patients were diagnosed in three different hospitals with different follow up routines, which could be a source of heterogeneity, although the treatment protocols were similar. Finally, considering that many of the microRNAs that we have identified as possible biomarkers in DLBCL have not been previously studied, it would be necessary to validate their role in other populations. Prospective studies in larger cohorts of DLBCL samples are warranted to further validate our findings and explore new therapeutic options based on the microRNA profile of each patient.

MicroRNA-mRNA network analysis are based on large sets of experimentally validated data. However, a limitation of the microRNA network is that it was built upon deregulated microRNAs and genes of different patients. Another downside of this method is that the microRNA networks can lead to wrong causal inferences which shown contradictory results in gene expression. Ideally, microRNA regulatory networks should be predicted microRNA and gene expression of the same patients. However, this limitation must be assumed due to the design of the study. The greatest challenge remains to validate these interactions at the bench.

CONCLUSIONS

In diffuse large B cell lymphoma:

1. Four microRNAs (miR-150-5p, miR-146a-5p, miR-155-5p and miR-21-5p) were consistently deregulated in DLBCL according to the literature and further validated in our cohort of patients. However, the limited number of microRNAs analyzed in previous studies and the inconsistency in results did not allow us to identify reliable signatures for classification, treatment response, or prognosis.
2. There are major differences in microRNA expression among DLBCL samples and controls identified by the RNAseq analysis of our cohort of patients. The most significantly upregulated microRNAs were miR-210-3p and miR-944 and the most downregulated were miR-215-5p and miR-150-5p.
3. Regarding differences between subtypes, miR-129-2-3p was the most upregulated microRNA in GCB DLBCL and miR-511-5p was the most upregulated in Non-GCB DLBCL. The role of the upregulation of miR-129-2-3p in GCB subtype could be mediated by its targeting of transcripts that are known to be deregulated in the formation of germinal center lymphomas.
4. A microRNA signature comprising miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p, miR-3928-3p, and miR-192-5p could identify high risk refractory patients. Moreover, they could be considered as targets for microRNA-based therapy in the future.
5. MiR-4444 and miR-205-5p expression could be biomarkers of good and poor prognosis in DLBCL, respectively. The effect of miR-205-5p could be mediated by PTEN regulation.
6. The overexpression of specific microRNAs could contribute to the pathogenesis of DLBCL through the regulation of the PI3K/AKT/FOXO1/Wnt pathways, since genes of these pathways are targeted by multiple microRNAs identified as deregulated in DLBCL in the present study.
7. MiR-9-5p, miR-146a-5p and miR-182-5p could act as master regulators in DLBCL, targeting multiple genes deregulated in DLBCL.
8. NGS technology is of great value, since it allows detecting associations even with poorly studied microRNAs.

In summary, our study remarks that microRNAs could play an important role as biomarkers in diagnosis, classification, treatment response and prognosis in DLBCL, as well as in the pathogenesis of the disease.

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ANNEX

Annex table 1: Differentially expressed microRNAs in DLBCL patients compared with healthy control individuals.

Significant microRNAs	Result	n DLBCL	n Control	Sample Source	Method	Nº microRNAs	Reference
miR-21	up	45	23	Tissue	qRT-PCR	1	Chen 2020
miR-214	down	15	15	FF	qRT-PCR	1	Sun J-R 2019
miR-222-3p	up	74	26	FF	qRT-PCR	1	Sun S 2019
miR-101	down	30	30 (NLN)	FF	qRT-PCR	1	Huang Y 2019
miR-195	down	36	NA	FF	qRT-PCR	1	Wang 2019
miR-101	down	72	30	FF	qRT-PCR	1	Huang 2019
miR-27a	up	409	477	Tissue	qRT-PCR	1	Tang 2019
miR-let-7a	down	13	11	FF	qRT-PCR	1	Malpeli 2018
miR-23a	up	70	30	FFPE	qRT-PCR	1	Xu 2018
miR-155	up	82	-	FFPE	qRT-PCR	2550 array	Wu 2018
miR-195	down	20	-	Tissue	qRT-PCR	1	He 2018
miR-155	up	29	32 (RLH)	Tissue	qRT-PCR	1	Li 2017
miR-4532	up						
miR-1915-3p	up						
miR-187-3p	up						
miR-4485	up						
miR-4284	up						
miR-4508	up						
miR-1973	up						
miR-663a	up						
miR-877-5p	up						
miR-3195	up	5	4 (RLH)	Tissue	nanostring	800	Jia 2017
miR-596	up						
miR-4516	up						
miR-4488	up						
miR-636	up						
miR-27b-3p	down						
miR-150-5p	down						
miR-200b-3p	down						
miR-205-5p	down						
miR-342-3p	down						
miR-203	down						
miR-21	up	55	20 (NLN)	FF and FFPE	qRT-PCR	1	Liu 2017
miR-21	up	26	10 (NLN)	FFPE	qRT-PCR	1	Song 2017
miR-10a	down	9	9 (RLH)	FF	qRT-PCR	1	Fan 2016
miR-4284	up						
miR-21-5p	up						
miR-142-3p	up						
miR-155-5p	up						
miR-3182	up						
miR-16-5p	up						
miR-142-5p	up						
miR-451a	up						
miR-29a-3p	up						
miR-342-3p	up						
miR-17-5p	up						
miR-20a-5p	up						
miR-let-7g-5p	up						
miR-23b-3p	up						
miR-19b-3p	up						
miR-15a-5p	up						
miR-491-3p	up						
miR-4484	down						Tamaddon
miR-143	down	24	14 (NLN)	FFPE	array	3100 probes	2016

Annex table 1: Differentially expressed microRNAs in DLBCL patients compared with healthy control individuals (Continuation).

Significant microRNAs	Result	N DLBCL	n Control	Sample Source	Method	Nº microRNAs	Reference
miR-125b-5p	down						
miR-30c-1-3p	down						
miR-4534	down						
miR-7491	down						
miR-711	down						
miR-5571	down						
miR-630-5p	down						
miR-483-5p	down						
miR-4741	down						
miR-4778-5p	down						
miR-3158-5p	down						
miR-320a	down						
miR-101	down						
miR-224	down	258	40 (NLN)	FFPE	qRT-PCR	1	Ni 2015
miR-155	up	22	6 (NLN)	Biopsy	qRT-PCR	1	Huskova 2015
miR-16-1	up						
miR-16-2	up						
miR-27a	up						
miR-103	up	63	5 GCB cells	FF	qRT-PCR	11	Troppan 2015
miR-185	up						
miR-199	up						
miR-497	up						
miR-21	up						
miR-17-92	up	200	11 (NT)	FFPE	qRT-PCR	3	Go 2015
miR-155	up						
miR-126	up						
miR-10b	up						
miR-145	up						
miR-126	up						
miR-424	up						
miR-134	up						
miR-199a-2	up						
miR-127	up						
miR-379	up						
miR-127	up						
miR-199b	up						
miR-143	up						
miR-144	up						
miR-199b	up						
miR-139	up						
miR-199a-2	up						
miR-130a	up						
miR-542	up						
miR-125a	up						
miR-218-2	up						
miR-99b	up						
miR-125b-2	up						
miR-10a	up						
miR-145	up						
miR-455	up						
miR-214	up						
miR-628	up						
miR-146b	up						
miR-100	up						
miR-497	up						
miR-1301	up	92	15	FF	sequencing	miRNAome	Lim 2015

Annex table 1: Differentially expressed microRNAs in DLBCL patients compared with healthy control individuals (Continuation).

Significant microRNAs	Result	n DLBCL	n Control	Sample Source	Method	Nº microRNAs	Reference
miR-451a	up						
miR-1247	up						
miR-574	up						
miR-195	up						
miR-340	up						
miR-326	up						
miR-196a-2	up						
miR-146b	up						
miR-338	up						
miR-675	up						
miR-337	up						
miR-511-2	up						
miR-10393-3p	up						
miR-99a	up						
miR-22	up						
miR-let-7c	up						
miR-217	up						
miR-654	up						
miR-452	up						
miR-503	up						
miR-455	up						
miR-let-7b	up						
miR-let-7e	up						
miR-450a-2	up						
miR-136	up						
miR-let-7a-2	up						
miR-196b	up						
miR-362	up						
miR-224	up						
miR-203a	down						
miR-205	down						
miR-20b	down						
NOVELM00290	down						
miR-4491	down						
miR-3150b	down						
miR-28	down						
miR-20b	down						
miR-129-2	down						
miR-3917	down						
miR-10392-5p	down						
miR-363	down						
miR-486	down						
miR-3934	down						
miR-138-1	down						
miR-3681	down						
NOVELM00288	down						
miR-27a	down						
miR-23a	down						
miR-589	down						
miR-10397-5p	down						
miR-4746	down						
miR-151a	down						
NOVELM00113	down						
miR-331	down						
miR-17	down						
miR-942	down						
miR-629	down						

Annex table 1: Differentially expressed microRNAs in DLBCL patients compared with healthy control individuals (Continuation).

Significant microRNAs	Result	n DLBCL	n Control	Sample Source	Method	Nº microRNAs	Reference
miR-128-2	down						
miR-15b	down						
miR-3615	down						
miR-200b	down						
miR-181a-1	down						
miR-130b	down						
miR-582	down						
miR-616	down						
miR-185	down						
miR-146-a	up	56	28 (RLH)	Tissue	qRT-PCR	1	Zhuang 2014
miR-23a	up	104	28	FFPE	qRT-PCR	1	Wang 2014
miR-146b	down						
miR-320d	down	106	30 (RLH)	FFPE	qRT-PCR	939	Wu 2014
miR-200c	NS	61	13 (NLN)	Tissue	qRT-PCR	1	Berglund 2013
miR-150	down						
miR-29b	down						
miR-29a	down						
miR-142-3p	down						
miR-142-5p	down						
miR-145	down	45 (DC); 75 (VC)	10 (DC); 6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
miR-143	down						
miR-195	down						
miR-497	down						
miR-494	up						
miR-638	up						
miR-21	up						
miR-155	up						
miR-16	down	12	7	FFPE	qRT-PCR	4	Handal 2013
miR-150	up						
miR-155	up	90	31 (RLN)	FFPE	qRT-PCR	2	Zhong 2012
miR-146a	up						
miR-18b	up						
miR-19b	up						
miR-20a	up						
miR-92	up	36	5 (NLN)	Tissue	qRT-PCR	8	Fassina 2012
miR-93	up						
miR-106a	up						
miR-150	down						
miR-210	up	58	7 (NLN)	FFPE	qRT-PCR	157	Roehle 2008
miR-155	up						
miR-106a	up						
miR-17-5p	up						
miR-150	down						
miR-145	down						
miR-328	down						
miR-139	down						
miR-99a	down						
miR-10a	down						
miR-95	down						
miR-149	down						
miR-let-7e	down						
miR-320	down						
miR-151	down						
miR-21	up	48	6 (NBC)	FF and FFPE	qRT-PCR	3	Lawrie 2007
miR-155	up						

Annex table 1: Differentially expressed microRNAs in DLBCL patients compared with healthy control individuals (Continuation).

Significant microRNAs	Result	n DLBCL	n Control	Sample Source	Method	Nº microRNAs	Reference
miR-221	up						
miR-155	up	23	2	FF	Semi RT-PCR	1	Eis 2005

Abbreviations: RLH: Reactive lymphoid hyperplasia; NLN: normal lymph node tissues; NT: normal tonsil; FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; DC: discovery cohort; VC: validation cohort; NBC: normal B cell samples.

Annex table 2: Differentially expressed microRNAs in DLBCL subtypes.

Significant microRNAs	Result	n GCB	n non-GCB	Sample source	Method	Nº microRNAs	Reference
miR-155	Down GCB	248	242	FFPE	qRT-PCR	1	Due 2019
miR-197	Up GCB	14	37	FFPE	qRT-PCR	array	Yang 2018
miR-21	Down GCB	19	36	FF and FFPE	qRT-PCR	1	Liu 2017
0	-	29	29	FF	qRT-PCR	1	Marques 2016
0	-	6	15	Biopsy	qRT-PCR	1	Huskova 2015
miR-155	Down GCB	53	95	FFPE	qRT-PCR	8	Go 2015
miR-28-3p	Up GCB	32	27	FFPE	qRT-PCR/array	377	Iqbal 2015
miR-28-5p	Up GCB						
miR-331-5p	Up GCB						
miR-589	Up GCB						
miR-129-3p	Up GCB						
miR-597	Up GCB						
miR-542-3p	Down GCB						
miR-155	Down GCB						
miR-1270	Up GCB	41	30	FF	sequencing	miRNAome	Lim 2015
miR-129-1-3p	Up GCB						
miR-129-2-3p	Up GCB						
miR-129-5p	Up GCB						
miR-138-1-3p	Up GCB						
miR-138-5p	Up GCB						
miR-151a-3p	Up GCB						
miR-151b	Up GCB						
miR-181a-5p	Up GCB						
miR-196b-5p	Up GCB						
miR-210-3p	Up GCB						
miR-28-3p	Up GCB						
miR-28-5p	Up GCB						
miR-301a-5p	Up GCB						
miR-3074-5p	Up GCB						
miR-30e-3p	Up GCB						
miR-3150b-3p	Up GCB						
miR-331-3p	Up GCB						
miR-339-3p	Up GCB						
miR-3681-5p	Up GCB						
miR-3934-3p	Up GCB						
miR-423-3p	Up GCB						
miR-4746-5p	Up GCB						
miR-582-3p	Up GCB						
miR-582-5p	Up GCB						
miR-5989-3p	Up GCB						

Annex table 2: Differentially expressed microRNAs in DLBCL subtypes (Continuation).

Significant microRNAs	Result	n GCB	n non-GCB	Sample source	Method	Nº microRNAs	Reference
miR-664a-3p	Up GCB						
miR-744-5p	Up GCB						
miR-10397-5p	Down GCB						
miR-106a-5p	Down GCB						
miR-10b-5p	Down GCB						
miR-148a-5p	Down GCB						
miR-155-5p	Down GCB						
miR-17-5p	Down GCB						
miR-20a-5p	Down GCB						
miR-21-3p	Down GCB						
miR-221-3p	Down GCB						
miR-222-3p	Down GCB						
miR-222-5p	Down GCB						
miR-29b-1-5p	Down GCB						
miR-30b-3p	Down GCB						
miR-30d-3p	Down GCB						
miR-320a	Down GCB						
miR-363-3p	Down GCB						
miR-424-5p	Down GCB						
miR-503-5p	Down GCB						
miR-625-3p	Down GCB						
miR-625-5p	Down GCB						
miR-92a-a-5p	Down GCB						
NOVELM00288	Down GCB						
0	-	140	118	FFPE	qRT-PCR	1	Ni 2015
miR-199a **miR-497**	Up GCB Up GCB	36	17	FF	qRT-PCR	11	Troppan 2015
miR-320d	Down GCB	47	59	FFPE	qRT-PCR	2	Wu 2014
miR-155 **miR-146a**	Down GCB Down GCB	20	34	FF and FFPE	qRT-PCR/array	177	Caramuta 2013
miR-155	Down GCB	36	31	FF	qRT-PCR	1	Huang 2012
0	-	10	8	FF	qRT-PCR	2	Kim 2012

Annex table 2: Differentially expressed microRNAs in DLBCL subtypes (Continuation).

Significant microRNAs	Result	n GCB	n non-GCB	Sample source	Method	Nº microRNAs	Reference
miR-155	Down GCB	21	69	FFPE	qRT-PCR	2	Zhong 2012
miR-146a	Down GCB						
miR-331	Up GCB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno 2011
miR-151	Up GCB						
miR-28	Up GCB						
miR-454-3p	Up GCB						
miR-222	Down GCB						
miR-144	Down GCB						
miR-451	Down GCB						
miR-221	Down GCB						
0	-	8	17	FFPE	qRT-PCR	3	Nie 2010
miR-129	Up GCB	32	28	FFPE	Array	464	Lawrie 2009
miR-138	Up GCB						
miR-199b	Up GCB						
miR-421	Up GCB						
miR-520h	Up GCB						
miR-569	Up GCB						
miR-616	Up GCB						
miR-620	Up GCB						
miR-653	Up GCB						
miR-132	Down GCB						
miR-146b	Down GCB						
miR-155	Down GCB						
miR-186	Down GCB						
miR-190	Down GCB						
miR-194	Down GCB						
miR-21	Down GCB						
miR-213	Down GCB						
miR-221	Down GCB						
miR-222	Down GCB						
miR-301a-5p	Down GCB						
miR-30d	Down GCB						
miR-340	Down GCB						
miR-363	Down GCB						
miR-422b	Down GCB						
miR-518a	Down GCB						

Annex table 2: Differentially expressed microRNAs in DLBCL subtypes (Continuation).

Significant microRNAs	Result	n GCB	n non-GCB	Sample source	Method	Nº microRNAs	Reference
miR-660	Down GCB						
miR-106b	NA						
miR-140-3p	NA						
miR-142-3p	NA						
miR-142-5p	NA						
miR-151-5p	NA						
miR-16	NA						
miR-184	NA						
miR-191	NA						
miR-19a	NA	20	20	Tissue	Array	113	Zhang 2009
miR-19b	NA						
miR-20a	NA						
miR-28-5p	NA						
miR-299-5p	NA						
miR-30c	NA						
miR-30e	NA						
miR-32	NA						
miR-526b	NA						
miR-583	NA						
miR-129	NA						
miR-133a	NA						
miR-133b	NA						
miR-138	NA	25	25	FFPE	qRT-PCR	157	Roehle 2008
miR-151	NA						
miR-155	NA						
miR-199b	NA						
miR-27b	NA						
miR-155	Down GCB						
miR-21	Down GCB	16	18	FF and FFPE	qRT-PCR	3	Lawrie 2007
miR-221	Down GCB						
miR-155	Down GCB	4	19	FF	Semi. RT-PCR	1	Eis 2005

Abbreviations: GCB: Germinal center B-cell like; FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; NA: not available.

Annex Table 3: Differentially expressed microRNAs as prediction to response to R-CHOP therapy.

Significant microRNAs	Result	n DLBCL	Sample source	Method	Nº microRNAs	Reference
miR-27	FR	201	Tissue	nanostring	800	Jia 2017
miR-34a	FR	62	FF	qRT-PCR	1	Marques 2016
0	-	22	Biopsy	qRT-PCR	1	Huskova 2015
miR-224 **miR-146**	FR UFR	258	FFPE	qRT-PCR	1	Ni 2015

Abbreviations: UFR: unfavorable response; FR: favorable response; FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded.

Annex Table 4: Differentially expressed microRNAs as prognosis biomarkers.

Significant microRNAs	Result	n DLBCL	Sample source	Method	Nº microRNAs	Reference
miR-222-3p	Up: ↓ OS (Non GCB)	74 Non GCB	Tissue	qRT-PCR	1	Shanshan 2019
miR-155	Down: ↓ OS and PFS (GCB)	73	TIssue	qRT-PCR	1	Due 2019
miR-101	Down: lower survival	72	FF	qRT-PCR	1	Huang 2019
miR-197	Down: ↓ PFS	51	FFPE	qRT-PCR	array	Yang 2018
miR-155	Up: ↓ PFS	82	FFPE	qRT-PCR	array	Wu 2018
miR-101-3p	NS	100	FFPE	qRT-PCR	1	Cui 2017
miR-27b	Down: ↓ OS	202	Tissue	qRT-PCR	1	Jia 2017
miR-34a	Up: ↑ OS	62	FF	qRT-PCR	1	Marques 2016
miR-155	Up: lower survival	118	FF	qRT-PCR	1	Zhu 2016
miR-23a	Up: ↓ OS	104	FFPE	qRT-PCR	1	Wang 2014
miR-155 **miR-16** **miR-363** **miR-24**	Up: ↓ OS Up: ↓ OS Up: ↓ OS Up: ↑ OS	79	FFPE	qRT-PCR	8	Iqbal 2015
miR-214-5p **miR-28-5p** **miR-324-5p** **miR-339-3p** **miR-5586-5p** **NOVELM00203M**	Up: ↑ OS and EFS Up: ↑ OS and EFS Up: ↓ OS and PFS Up: ↑ OS and EFS Up: ↑ OS and EFS Up: ↓ OS and PFS	92	FF	sequencing	miRNAome	Lim 2015
miR-224	Up: ↑ OS and PFS	258	FFPE	qRT-PCR	1	Ni 2015
miR-17-5p **miR-19-3p** **miR-20a-5p** **miR-106a-5p** **miR-150-5p** **miR-342-3p** **miR-181a-5p** **miR-140-3p**	Up: poor prognosis Up: poor prognosis Up: poor prognosis Up: poor prognosis Down: poor prognosis Down: poor prognosis Down: poor prognosis Down: poor prognosis	83	FFPE	qRT-PCR/array	±900	Shepshelovich 2015
miR-199a **miR-497**	Up: ↑ OS and DFS Up: ↑ OS and DFS	58	FF	qRT-PCR	11	Troppan 2015
miR-17-92 **miR-21**	Up: ↓ OS and PFS Up: ↓ OS and PFS	200	FFPE	qRT-PCR	3	Go 2015
miR-146b-5p **miR-320d**	Down: ↓ PFS Down: ↓ PFS and OS	12 (DC); 106 (VC)	FFPE	qRT-PCR	939	Wu 2014

Annex Table 4: Differentially expressed microRNAs as prognosis biomarkers (Continuation).

Significant microRNAs	Result	n DLBCL	Sample source	Method	Nº microRNAs	Reference
miR-200c	Up: ↓ OS	61	Tissue	qRT-PCR	1	Berglund 2013
miR-146a	Down: ↑ PFS	90	FFPE	qRT-PCR	2	Zhong 2012
miR-155	Up: ↑ PFS					
miR-181	Down: ↑ PFS					
miR-18a	Up: ↓ OS	176	FFPE	qRT-PCR	11	Alencar 2011
miR-222	Up: ↓ OS					
miR-221	Up: ↑ OS and PFS					
miR-331	Up: ↑ OS and PFS					
miR-222	Up: ↓ OS and PFS					
miR-93	Up: ↓ OS and PFS	36/240	FFPE	qRT-PCR/array	470/9	Montes-Moreno 2011
miR-148a	NA					
miR-151	NA					
miR-28-5p	NA					
miR-451	NA					
miR-491	NA					
miR-100	Up: ↑ EFS					
miR-199a	Up: ↑ EFS					
miR-199b	Up: ↑ EFS					
miR-23a	Up: ↑ EFS					
miR-24	Up: ↑ EFS					
miR-27a	Up: ↑ EFS					
miR-30e	Up: ↑ EFS	64	FFPE	Array	464	Lawrie 2009
miR-330	Up: ↑ EFS					
miR-425	Up: ↑ EFS					
miR-302	Up: ↓ OS and PFS					
miR-608	Up: ↓ EFS					
miR-637	Up: ↓ EFS					
miR-222	Up: ↓ OS and PFS	106	FFPE	qRT-PCR	3	Malumbres 2009
miR-let-7g	Down: ↑ EFS					
miR-195	Down: ↑ EFS					
miR-19a	Down: ↑ OS					
miR-21	Down: ↑ OS					
miR-23a	Down: ↑ OS	58	Biopsy	qRT-PCR	157	Roehle 2008
miR-27a	Down: ↑ OS					
miR-34a	Down: ↑ OS					
miR-127	Up: ↓ OS and PFS					
miR-21	Up: RFS	35	FF and FFPE	qRT-PCR	3	Lawrie 2007

Abbreviations: FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; OS: overall survival, PFS: progression-free survival; EFS: event free survival; RFS: relapse free survival.

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0).

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-210-3p	MIMAT0000267	843.0	3.51	$2.33x10^{-29}$	$1.45x10^{-26}$
hsa-miR-944	MIMAT0004987	60.9	4.10	$2.19x10^{-23}$	$6.80x10^{-21}$
hsa-miR-12136	MIMAT0049032	76.1	26.94	$3.64x10^{-20}$	$6.47x10^{-18}$
hsa-miR-3681-5p	MIMAT0018108	75.3	5.16	$3.33x10^{-20}$	$6.47x10^{-18}$
hsa-miR-425-3p	MIMAT0001343	152.2	1.60	$1.04x10^{-17}$	$1.62x10^{-15}$
hsa-miR-378i	MIMAT0019074	25.0	3.01	$3.01x10^{-17}$	$4.16x10^{-15}$
hsa-miR-106b-3p	MIMAT0004672	1394.7	1.32	$3.80x10^{-17}$	$4.73x10^{-15}$
hsa-miR-4454	MIMAT0018976	183.5	2.35	$1.01x10^{-16}$	$1.04x10^{-14}$
hsa-miR-1291	MIMAT0005881	354.8	4.02	$1.84x10^{-16}$	$1.76x10^{-14}$
hsa-miR-7974	MIMAT0031177	111.8	3.46	$1.87x10^{-15}$	$1.45x10^{-13}$
hsa-miR-183-5p	MIMAT0000261	891.2	3.40	$5.77x10^{-15}$	$3.59x10^{-13}$
hsa-miR-146a-5p	MIMAT0000449	33085.6	2.11	$2.05x10^{-14}$	$1.16x10^{-12}$
hsa-miR-2467-5p	MIMAT0019952	25.6	2.33	$7.35x10^{-14}$	$3.81x10^{-12}$
hsa-miR-4420	MIMAT0018933	8.1	4.63	$2.02x10^{-13}$	$1.01x10^{-11}$
hsa-miR-19a-3p	MIMAT0000073	1660.1	1.77	$7.91x10^{-13}$	$3.79x10^{-11}$
hsa-miR-1248	MIMAT0005900	202.5	2.69	$1.03x10^{-12}$	$4.73x10^{-11}$
hsa-miR-18a-3p	MIMAT0002891	54.1	2.05	$1.21x10^{-12}$	$5.36x10^{-11}$
hsa-miR-21-3p	MIMAT0004494	6544.0	1.86	$1.90x10^{-12}$	$7.88x10^{-11}$
hsa-miR-129a-5p	MIMAT0000242_1	25.1	4.62	$3.03x10^{-12}$	$1.22x10^{-10}$
hsa-miR-147b-3p	MIMAT0004928	92.6	3.41	$3.78x10^{-12}$	$1.47x10^{-10}$
hsa-miR-3691-5p	MIMAT0018120	6.6	2.59	$1.09x10^{-11}$	$3.88x10^{-10}$
hsa-miR-128-1-5p	MIMAT0026477	16.5	1.38	$1.90x10^{-11}$	$6.55x10^{-10}$
hsa-miR-1246	MIMAT0005898	4.6	4.47	$2.64x10^{-11}$	$8.86x10^{-10}$
hsa-miR-205-5p	MIMAT0000266	67.2	6.11	$3.81x10^{-11}$	$1.22x10^{-09}$
hsa-miR-769-3p	MIMAT0003887	21.6	2.53	$4.00x10^{-11}$	$1.25x10^{-09}$
hsa-miR-10395-5p	MIMAT0041621	120.1	2.24	$4.74x10^{-11}$	$1.44x10^{-09}$
hsa-miR-3917	MIMAT0018191	7.5	2.67	$1.05x10^{-10}$	$3.10x10^{-09}$
hsa-miR-4424	MIMAT0018939	110.8	4.39	$1.29x10^{-10}$	$3.72x10^{-09}$
hsa-miR-7706	MIMAT0030021	100.7	2.22	$2.11x10^{-10}$	$5.97x10^{-09}$
hsa-miR-4449	MIMAT0018968	77.2	1.99	$2.52x10^{-10}$	$6.96x10^{-09}$
hsa-miR-210-5p	MIMAT0026475	9.4	2.51	$5.01x10^{-10}$	$1.30x10^{-08}$
hsa-miR-19a-5p	MIMAT0004490	20.6	2.30	$6.23x10^{-10}$	$1.55x10^{-08}$
hsa-miR-7-1-5p	MIMAT0000252_2	87.2	1.92	$6.17x10^{-10}$	$1.55x10^{-08}$
hsa-miR-182-5p	MIMAT0000259	4036.2	2.63	$6.65x10^{-10}$	$1.60x10^{-08}$
hsa-miR-320a-5p	MIMAT0037311	16.4	3.77	$6.97x10^{-10}$	$1.64x10^{-08}$
hsa-miR-155-5p	MIMAT0000646	20773.1	1.99	$7.26x10^{-10}$	$1.67x10^{-08}$
hsa-miR-1307-3p	MIMAT0005951	443.6	1.61	$1.56x10^{-09}$	$3.34x10^{-08}$

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) (Continuation)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-1843	MIMAT0039764	78.3	1.49	$2.03x10^{-09}$	$4.22x10^{-08}$
hsa-miR-34a-5p	MIMAT0000255	759.4	1.11	$2.32x10^{-09}$	$4.72x10^{-08}$
hsa-miR-191-3p	MIMAT0001618	22.5	2.26	$2.57x10^{-09}$	$5.16x10^{-08}$
hsa-miR-185-3p	MIMAT0004611	41.1	2.21	$2.94x10^{-09}$	$5.78x10^{-08}$
hsa-miR-4518	MIMAT0019055	11.7	2.25	$3.33x10^{-09}$	$6.37x10^{-08}$
hsa-miR-3609	MIMAT0017986	56.4	2.31	$4.58x10^{-09}$	$8.32x10^{-08}$
hsa-miR-629-5p	MIMAT0004810	91.4	1.58	$4.62x10^{-09}$	$8.32x10^{-08}$
hsa-miR-3922-3p	MIMAT0018197	17.2	2.73	$7.13x10^{-09}$	$1.27x10^{-07}$
hsa-miR-9-1-5p	MIMAT0000441	128.0	3.32	$9.08x10^{-09}$	$1.59x10^{-07}$
hsa-miR-141-5p	MIMAT0004598	20.2	3.65	$1.60x10^{-08}$	$2.65x10^{-07}$
hsa-miR-19b-1-5p	MIMAT0004491	12.6	2.08	$1.60x10^{-08}$	$2.65x10^{-07}$
hsa-miR-3651	MIMAT0018071	63.2	1.88	$1.81x10^{-08}$	$2.96x10^{-07}$
hsa-miR-3176	MIMAT0015053	14.2	1.85	$1.90x10^{-08}$	$3.07x10^{-07}$
hsa-miR-130b-3p	MIMAT0000691	381.5	1.37	$4.01x10^{-08}$	$6.23x10^{-07}$
hsa-miR-3620-5p	MIMAT0022967	4.8	3.65	$5.74x10^{-08}$	$8.81x10^{-07}$
hsa-miR-92a-1-5p	MIMAT0004507	43.9	1.75	$6.64x10^{-08}$	$1.01x10^{-06}$
hsa-miR-1307-5p	MIMAT0022727	4232.8	2.31	$7.57x10^{-08}$	$1.13x10^{-06}$
hsa-miR-330-5p	MIMAT0004693	126.3	1.52	$7.71x10^{-08}$	$1.14x10^{-06}$
hsa-miR-5100	MIMAT0022259	153.9	2.32	$1.20x10^{-07}$	$1.74x10^{-06}$
hsa-miR-16-1-3p	MIMAT0004489	13.0	1.29	$1.22x10^{-07}$	$1.74x10^{-06}$
hsa-miR-10392-5p	MIMAT0041615	28.4	2.00	$1.51x10^{-07}$	$2.14x10^{-06}$
hsa-miR-3614-5p	MIMAT0017992	14.7	2.28	$1.73x10^{-07}$	$2.42x10^{-06}$
hsa-miR-3150b-3p	MIMAT0018194	50.7	2.38	$1.84x10^{-07}$	$2.52x10^{-06}$
hsa-miR-3677-3p	MIMAT0018101	13.6	2.27	$1.83x10^{-07}$	$2.52x10^{-06}$
hsa-miR-296-3p	MIMAT0004679	26.7	2.39	$2.02x10^{-07}$	$2.73x10^{-06}$
hsa-miR-1304-3p	MIMAT0022720	36.2	1.41	$2.05x10^{-07}$	$2.74x10^{-06}$
hsa-miR-208b-3p	MIMAT0004960	8.0	4.29	$2.66x10^{-07}$	$3.45x10^{-06}$
hsa-miR-769-5p	MIMAT0003886	2112.0	1.64	$2.80x10^{-07}$	$3.59x10^{-06}$
hsa-miR-9-1-3p	MIMAT0000442	8.1	3.14	$3.05x10^{-07}$	$3.87x10^{-06}$
hsa-miR-146a-3p	MIMAT0004608	27.7	2.13	$3.12x10^{-07}$	$3.92x10^{-06}$
hsa-miR-130b-5p	MIMAT0004680	91.9	1.28	$4.43x10^{-07}$	$5.46x10^{-06}$
hsa-miR-103a-2-5p	MIMAT0009196	8.7	1.08	$4.80x10^{-07}$	$5.86x10^{-06}$
hsa-miR-548k	MIMAT0005882	79.1	1.24	$5.14x10^{-07}$	$6.15x10^{-06}$
hsa-miR-3610	MIMAT0017987	26.0	2.95	$5.37x10^{-07}$	$6.19x10^{-06}$
hsa-miR-10394-3p	MIMAT0041620	13.2	2.92	$5.37x10^{-07}$	$6.19x10^{-06}$
hsa-miR-4775	MIMAT0019931	7.2	1.41	$5.24x10^{-07}$	$6.19x10^{-06}$
hsa-miR-17-3p	MIMAT0000071	334.1	1.14	$5.34x10^{-07}$	$6.19x10^{-06}$

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) (Continuation)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-181a-3p	MIMAT0000270	1153.4	1.35	$6.56x10^{-07}$	$7.42x10^{-06}$
hsa-miR-4521	MIMAT0019058	106.9	1.85	$7.20x10^{-07}$	$8.07x10^{-06}$
hsa-miR-3913-2-5p	MIMAT0018187_1	13.4	1.97	$7.97x10^{-07}$	$8.77x10^{-06}$
hsa-miR-6753-3p	MIMAT0027407	1.3	3.50	$8.61x10^{-07}$	$9.39x10^{-06}$
hsa-miR-3689f	MIMAT0019010	5.6	5.38	$1.10x10^{-06}$	$1.19x10^{-05}$
hsa-miR-378a-3p	MIMAT0000732	7208.5	1.42	$1.13x10^{-06}$	$1.21x10^{-05}$
hsa-miR-15a-3p	MIMAT0004488	4.9	1.52	$1.39x10^{-06}$	$1.48x10^{-05}$
hsa-miR-4487	MIMAT0019021	2.3	2.26	$1.60x10^{-06}$	$1.64x10^{-05}$
hsa-miR-4741	MIMAT0019871	3.9	3.00	$1.62x10^{-06}$	$1.65x10^{-05}$
hsa-miR-766-5p	MIMAT0022714	9.0	2.78	$1.67x10^{-06}$	$1.68x10^{-05}$
hsa-miR-4517	MIMAT0019054	3.5	2.28	$2.00x10^{-06}$	$1.99x10^{-05}$
hsa-miR-25-5p	MIMAT0004498	25.2	1.37	$2.08x10^{-06}$	$2.05x10^{-05}$
hsa-miR-4746-5p	MIMAT0019880	3.9	1.88	$2.14x10^{-06}$	$2.09x10^{-05}$
hsa-miR-10527-5p	MIMAT0041997	13.1	1.73	$2.32x10^{-06}$	$2.25x10^{-05}$
hsa-miR-30b-3p	MIMAT0004589	6.6	1.50	$2.38x10^{-06}$	$2.30x10^{-05}$
hsa-miR-3944-3p	MIMAT0018360	3.2	3.56	$2.61x10^{-06}$	$2.49x10^{-05}$
hsa-miR-4485-3p	MIMAT0019019	2.3	2.78	$3.14x10^{-06}$	$2.98x10^{-05}$
hsa-miR-576-3p	MIMAT0004796	18.8	1.98	$3.18x10^{-06}$	$3.00x10^{-05}$
hsa-miR-1303	MIMAT0005891	10.2	1.93	$4.24x10^{-06}$	$3.94x10^{-05}$
hsa-miR-21-5p	MIMAT0000076	140750.7	1.11	$4.37x10^{-06}$	$4.03x10^{-05}$
hsa-miR-15b-3p	MIMAT0004586	100.6	1.44	$4.61x10^{-06}$	$4.19x10^{-05}$
hsa-miR-9-2-5p	MIMAT0000441_2	118.5	3.01	$6.18x10^{-06}$	$5.49x10^{-05}$
hsa-miR-4524a-5p	MIMAT0019062	4.1	2.34	$6.15x10^{-06}$	$5.49x10^{-05}$
hsa-miR-942-5p	MIMAT0004985	25.3	1.15	$8.36x10^{-06}$	$7.27x10^{-05}$
hsa-miR-3652	MIMAT0018072	8.9	3.05	$9.54x10^{-06}$	$8.19x10^{-05}$
hsa-miR-454-5p	MIMAT0003884	12.7	0.93	$9.83x10^{-06}$	$8.37x10^{-05}$
hsa-miR-155-3p	MIMAT0004658	5.5	2.19	$1.09x10^{-05}$	$9.20x10^{-05}$
hsa-miR-939-5p	MIMAT0004982	4.1	2.27	$1.23x10^{-05}$	0.00010
hsa-miR-4690-5p	MIMAT0019779	2.1	3.85	$1.31x10^{-05}$	0.00011
hsa-miR-3913-1-5p	MIMAT0018187	12.1	1.79	$1.34x10^{-05}$	0.00011
hsa-miR-96-5p	MIMAT0000095	90.2	1.86	$1.40x10^{-05}$	0.00011
hsa-miR-873-3p	MIMAT0022717	7.5	3.79	$1.58x10^{-05}$	0.00013
hsa-miR-19b-1-3p	MIMAT0000074	4447.5	1.03	$1.59x10^{-05}$	0.00013
hsa-miR-1296-3p	MIMAT0026637	3.6	2.35	$1.79x10^{-05}$	0.00014
hsa-miR-3681-3p	MIMAT0018109	1.3	3.63	$2.06x10^{-05}$	0.00016
hsa-miR-18a-5p	MIMAT0000072	359.9	1.55	$2.56x10^{-05}$	0.00019
hsa-miR-151a-3p	MIMAT0000757	4394.7	0.82	$2.61x10^{-05}$	0.00019

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) (Continuation)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-503-5p	MIMAT0002874	5.9	1.43	$2.63x10^{-05}$	0.00019
hsa-miR-10393-3p	MIMAT0041618	12.9	1.96	$3.60x10^{-05}$	0.00026
hsa-miR-9-2-3p	MIMAT0000442_2	6.9	2.59	$3.73x10^{-05}$	0.00026
hsa-miR-573	MIMAT0003238	3.2	3.34	$3.88x10^{-05}$	0.00027
hsa-miR-4651	MIMAT0019715	1.5	2.64	$3.88x10^{-05}$	0.00027
hsa-miR-409-3p	MIMAT0001639	244.7	1.68	$4.14x10^{-05}$	0.00029
hsa-miR-7705	MIMAT0030020	13.5	1.03	$4.19x10^{-05}$	0.00029
hsa-miR-3615	MIMAT0017994	274.9	1.37	$4.43x10^{-05}$	0.00031
hsa-miR-589-5p	MIMAT0004799	168.8	1.03	$4.70x10^{-05}$	0.00032
hsa-miR-937-3p	MIMAT0004980	7.5	2.19	$5.14x10^{-05}$	0.00035
hsa-miR-188-3p	MIMAT0004613	3.6	1.20	$5.20x10^{-05}$	0.00035
hsa-miR-141-3p	MIMAT0000432	1540.8	1.80	$5.65x10^{-05}$	0.00038
hsa-miR-301b-3p	MIMAT0004958	99.9	1.43	$5.76x10^{-05}$	0.00038
hsa-miR-7702	MIMAT0030017	2.2	2.12	$6.19x10^{-05}$	0.00041
hsa-miR-33b-3p	MIMAT0004811	4.0	1.55	$6.22x10^{-05}$	0.00041
hsa-miR-30d-3p	MIMAT0004551	159.0	0.90	$6.53x10^{-05}$	0.00043
hsa-miR-183-3p	MIMAT0004560	3.0	2.02	$7.00x10^{-05}$	0.00045
hsa-miR-4491	MIMAT0019026	3.2	1.94	$7.00x10^{-05}$	0.00045
hsa-miR-671-5p	MIMAT0003880	50.7	1.71	$7.32x10^{-05}$	0.00047
hsa-miR-181b-2-5p	MIMAT0000257_1	3292.3	1.00	$7.48x10^{-05}$	0.00048
hsa-miR-3916	MIMAT0018190	4.2	1.62	$8.08x10^{-05}$	0.00051
hsa-miR-3136-5p	MIMAT0015003	4.1	1.04	$8.19x10^{-05}$	0.00051
hsa-miR-592	MIMAT0003260	5.4	1.46	$8.83x10^{-05}$	0.00055
hsa-miR-3195	MIMAT0015079	6.4	4.35	$9.65x10^{-05}$	0.00060
hsa-miR-9901	MIMAT0039321	21.8	5.88	0.00010	0.00064
hsa-miR-6852-5p	MIMAT0027604	2.5	1.30	0.00010	0.00064
hsa-miR-9-3-3p	MIMAT0000442_1	7.0	2.73	0.00011	0.00067
hsa-miR-424-3p	MIMAT0004749	27.9	1.13	0.00011	0.00068
hsa-miR-181d-5p	MIMAT0002821	233.5	0.77	0.00011	0.00068
hsa-miR-25-3p	MIMAT0000081	9225.6	0.80	0.00012	0.00074
hsa-miR-548e-5p	MIMAT0026736	17.8	0.83	0.00014	0.00080
hsa-miR-129b-2-5p	MIMAT0000242	19.0	2.62	0.00014	0.00084
hsa-let-7i-5p	MIMAT0000415	22108.6	0.56	0.00015	0.00087
hsa-miR-181b-3p	MIMAT0022692	11.9	1.19	0.00015	0.00090
hsa-miR-548j-5p	MIMAT0005875	5.3	1.22	0.00016	0.00092
hsa-miR-7977	MIMAT0031180	4065.0	1.39	0.00016	0.00094
hsa-miR-4677-3p	MIMAT0019761	28.0	0.60	0.00016	0.00094

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) (Continuation)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-598-5p	MIMAT0026620	1.2	3.21	0.00020	0.00113
hsa-miR-129-1-3p	MIMAT0004548	7.4	2.31	0.00021	0.00121
hsa-miR-2278	MIMAT0011778	1.3	1.88	0.00023	0.00127
hsa-miR-940	MIMAT0004983	9.9	0.94	0.00024	0.00136
hsa-miR-4523	MIMAT0019061	3.1	2.75	0.00029	0.00161
hsa-miR-9-3-5p	MIMAT0000441_1	91.3	2.57	0.00029	0.00162
hsa-miR-219a-1-3p	MIMAT0004567	4.0	1.58	0.00029	0.00162
hsa-miR-760	MIMAT0004957	5.8	1.43	0.00029	0.00162
hsa-miR-3150a-3p	MIMAT0015023	2.0	2.50	0.00030	0.00164
hsa-miR-6762-3p	MIMAT0027425	1.8	2.11	0.00031	0.00170
hsa-miR-3659	MIMAT0018080	1.2	3.77	0.00033	0.00178
hsa-miR-365a-5p	MIMAT0009199	4.3	1.43	0.00034	0.00182
hsa-miR-1976	MIMAT0009451	13.1	0.97	0.00034	0.00182
hsa-miR-4513	MIMAT0019050	2.7	3.20	0.00035	0.00183
hsa-miR-3174	MIMAT0015051	1.6	1.65	0.00035	0.00185
hsa-miR-423-3p	MIMAT0001340	2454.7	0.87	0.00037	0.00191
hsa-miR-4645-3p	MIMAT0019706	7.7	0.84	0.00037	0.00191
hsa-miR-4745-5p	MIMAT0019878	0.7	2.68	0.00038	0.00194
hsa-miR-30c-1-3p	MIMAT0004674	36.4	0.94	0.00039	0.00199
hsa-miR-6842-3p	MIMAT0027587	28.5	0.81	0.00041	0.00211
hsa-miR-612	MIMAT0003280	0.9	2.86	0.00043	0.00218
hsa-miR-127-3p	MIMAT0000446	3977.2	1.54	0.00045	0.00226
hsa-miR-1538	MIMAT0007400	7.3	1.72	0.00046	0.00231
hsa-miR-3144-3p	MIMAT0015015	2.8	3.18	0.00048	0.00240
hsa-miR-4677-5p	MIMAT0019760	2.0	2.06	0.00049	0.00246
hsa-miR-7703	MIMAT0030018	1.5	1.29	0.00050	0.00249
hsa-miR-6751-3p	MIMAT0027403	1.0	2.46	0.00050	0.00250
hsa-miR-873-5p	MIMAT0004953	33.4	2.07	0.00052	0.00259
hsa-miR-19b-2-3p	MIMAT0000074_1	1793.7	1.48	0.00054	0.00264
hsa-miR-548b-5p	MIMAT0004798	5.6	1.98	0.00056	0.00272
hsa-miR-6516-3p	MIMAT0030418	71.5	1.49	0.00059	0.00283
hsa-miR-10397-5p	MIMAT0041625	6.8	0.97	0.00059	0.00283
hsa-miR-3158-1-3p	MIMAT0015032	5.5	1.05	0.00061	0.00293
hsa-miR-4515	MIMAT0019052	0.7	2.36	0.00062	0.00293
hsa-miR-4802-5p	MIMAT0019981	0.9	2.28	0.00062	0.00293
hsa-miR-7-2-5p	MIMAT0000252	3.2	2.79	0.00063	0.00296
hsa-miR-4444-1	MIMAT0018962	10.3	1.85	0.00063	0.00296

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) (Continuation)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-3934-5p	MIMAT0018349	5.7	0.83	0.00065	0.00305
hsa-miR-3928-3p	MIMAT0018205	12.2	1.56	0.00070	0.00325
hsa-miR-7704	MIMAT0030019	266.5	3.73	0.00071	0.00329
hsa-miR-1910-5p	MIMAT0007884	1.6	2.76	0.00072	0.00334
hsa-miR-582-3p	MIMAT0004797	68.7	1.27	0.00073	0.00336
hsa-miR-4766-3p	MIMAT0019918	2.2	1.77	0.00074	0.00342
hsa-miR-30d-5p	MIMAT0000245	17897.8	0.67	0.00075	0.00342
hsa-miR-4767	MIMAT0019919	10.4	1.24	0.00078	0.00354
hsa-miR-3940-5p	MIMAT0019229	1.9	2.53	0.00084	0.00376
hsa-miR-6839-5p	MIMAT0027580	0.9	2.52	0.00084	0.00376
hsa-miR-148a-5p	MIMAT0004549	431.0	1.15	0.00085	0.00381
hsa-miR-664b-5p	MIMAT0022271	4.3	1.44	0.00093	0.00411
hsa-miR-548l	MIMAT0005889	2.1	1.26	0.00093	0.00412
hsa-miR-92a-3p	MIMAT0000092	52252.0	0.85	0.00100	0.00441
hsa-miR-4444-2	MIMAT0018962_1	11.4	1.88	0.00105	0.00459
hsa-miR-6850-3p	MIMAT0027601	0.7	2.76	0.00106	0.00462
hsa-miR-449a	MIMAT0001541	12.7	1.43	0.00107	0.00465
hsa-miR-146b-5p	MIMAT0002809	15631.5	0.96	0.00107	0.00465
hsa-miR-3678-5p	MIMAT0018102	0.9	1.85	0.00110	0.00474
hsa-miR-181b-1-5p	MIMAT0000257	495.1	1.00	0.00110	0.00474
hsa-miR-135b-5p	MIMAT0000758	8.6	1.38	0.00113	0.00484
hsa-miR-5009-5p	MIMAT0021041	0.5	2.34	0.00114	0.00489
hsa-miR-1270	MIMAT0005924	5.0	1.15	0.00117	0.00498
hsa-miR-378a-5p	MIMAT0000731	103.4	0.81	0.00121	0.00512
hsa-miR-6515-5p	MIMAT0025486	1.4	2.36	0.00123	0.00518
hsa-miR-3611	MIMAT0017988	8.3	1.68	0.00127	0.00535
hsa-miR-370-3p	MIMAT0000722	16.1	1.95	0.00129	0.00541
hsa-miR-17-5p	MIMAT0000070	1127.3	1.20	0.00130	0.00542
hsa-miR-324-3p	MIMAT0000762	74.5	0.60	0.00133	0.00551
hsa-miR-4668-5p	MIMAT0019745	4.4	2.47	0.00139	0.00573
hsa-miR-10394-5p	MIMAT0041619	2.6	2.54	0.00151	0.00615
hsa-miR-103a-1-5p	MIMAT0037306	0.9	1.74	0.00151	0.00615
hsa-miR-6818-3p	MIMAT0027537	2.1	1.93	0.00154	0.00628
hsa-miR-1226-5p	MIMAT0005576	0.5	2.43	0.00158	0.00640
hsa-miR-20a-5p	MIMAT0000075	1664.1	1.19	0.00159	0.00642
hsa-miR-1295a	MIMAT0005885	4.9	1.85	0.00188	0.00743
hsa-miR-4529-3p	MIMAT0019068	1.2	2.44	0.00195	0.00766

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) (Continuation)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-3064-5p	MIMAT0019864	2.2	1.47	0.00196	0.00770
hsa-miR-597-5p	MIMAT0003265	2.9	1.47	0.00218	0.00846
hsa-miR-219b-3p	MIMAT0019748	1.3	1.53	0.00225	0.00868
hsa-miR-7111-3p	MIMAT0028120	1.4	2.35	0.00232	0.00885
hsa-miR-632	MIMAT0003302	2.2	1.94	0.00251	0.00948
hsa-miR-4524a-3p	MIMAT0019063	1.1	1.83	0.00251	0.00948
hsa-miR-324-5p	MIMAT0000761	63.5	0.49	0.00281	0.01047
hsa-miR-6821-3p	MIMAT0027543	1.7	1.61	0.00328	0.01214
hsa-miR-542-3p	MIMAT0003389	36.7	0.73	0.00342	0.01258
hsa-miR-4687-3p	MIMAT0019775	0.7	2.10	0.00346	0.01266
hsa-miR-6840-5p	MIMAT0027582	1.0	2.15	0.00356	0.01300
hsa-miR-12135	MIMAT0049031	14.0	3.48	0.00372	0.01351
hsa-miR-5001-5p	MIMAT0021021	2.3	1.20	0.00371	0.01351
hsa-miR-219a-5p	MIMAT0000276	14.3	0.75	0.00400	0.01440
hsa-miR-138-1-3p	MIMAT0004607	18.2	1.63	0.00403	0.01445
hsa-miR-421	MIMAT0003339	186.1	0.73	0.00407	0.01453
hsa-miR-1286	MIMAT0005877	0.8	2.39	0.00410	0.01463
hsa-miR-193b-5p	MIMAT0004767	9.9	1.02	0.00412	0.01464
hsa-miR-182-3p	MIMAT0000260	1.0	2.16	0.00419	0.01481
hsa-miR-2861	MIMAT0013802	8.0	5.40	0.00428	0.01502
hsa-miR-100-3p	MIMAT0004512	9.8	0.95	0.00430	0.01506
hsa-miR-3181	MIMAT0015061	2.1	1.94	0.00433	0.01514
hsa-miR-425-5p	MIMAT0003393	1019.7	0.61	0.00438	0.01526
hsa-miR-663a	MIMAT0003326	42.3	3.20	0.00452	0.01570
hsa-miR-3661	MIMAT0018082	1.1	1.30	0.00470	0.01626
hsa-miR-3198	MIMAT0015083	1.3	3.43	0.00482	0.01657
hsa-miR-7848-3p	MIMAT0030423	0.5	2.30	0.00494	0.01681
hsa-miR-5094	MIMAT0021086	1.0	1.60	0.00495	0.01681
hsa-miR-4707-5p	MIMAT0019807	0.6	2.48	0.00549	0.01830
hsa-miR-3158-2-3p	MIMAT0015032_1	5.3	0.90	0.00548	0.01830
hsa-miR-191-5p	MIMAT0000440	31969.0	0.89	0.00549	0.01830
hsa-miR-135a-5p	MIMAT0000428	0.7	3.68	0.00583	0.01926
hsa-miR-3187-3p	MIMAT0015069	1.2	1.14	0.00594	0.01953
hsa-miR-331-5p	MIMAT0004700	18.7	0.74	0.00599	0.01961
hsa-miR-2355-3p	MIMAT0017950	6.3	1.15	0.00645	0.02094
hsa-miR-33a-5p	MIMAT0000091	277.1	0.77	0.00644	0.02094
hsa-miR-491-5p	MIMAT0002807	3.6	1.00	0.00716	0.02294

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) (Continuation)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-545-5p	MIMAT0004785	3.6	0.85	0.00730	0.02333
hsa-miR-4421	MIMAT0018934	1.3	2.28	0.00734	0.02343
hsa-miR-454-3p	MIMAT0003885	318.1	0.80	0.00767	0.02438
hsa-miR-6502-5p	MIMAT0025460	2.4	1.19	0.00792	0.02507
hsa-miR-625-3p	MIMAT0004808	41.4	0.71	0.00853	0.02694
hsa-miR-4539	MIMAT0019082	0.8	3.44	0.00866	0.02727
hsa-miR-1243	MIMAT0005894	0.4	2.20	0.00878	0.02759
hsa-miR-6854-5p	MIMAT0027608	0.5	1.97	0.00891	0.02786
hsa-miR-501-5p	MIMAT0002872	33.9	0.86	0.00908	0.02831
hsa-miR-6764-3p	MIMAT0027429	0.5	1.80	0.00959	0.02983
hsa-miR-431-5p	MIMAT0001625	5.3	1.21	0.00969	0.03005
hsa-miR-4703-3p	MIMAT0019802	1.2	1.96	0.00980	0.03034
hsa-miR-1306-3p	MIMAT0005950	2.2	1.27	0.01022	0.03153
hsa-miR-1229-3p	MIMAT0005584	1.1	1.13	0.01032	0.03176
hsa-miR-181a-5p	MIMAT0000256	45360.1	0.76	0.01043	0.03203
hsa-miR-2116-3p	MIMAT0011161	5.4	1.08	0.01123	0.03423
hsa-miR-147a	MIMAT0000251	0.6	3.15	0.01142	0.03474
hsa-miR-3155a	MIMAT0015029	1.4	1.13	0.01156	0.03506
hsa-miR-1181	MIMAT0005826	1.7	2.22	0.01164	0.03523
hsa-miR-5706	MIMAT0022500	0.9	1.29	0.01198	0.03617
hsa-miR-5582-3p	MIMAT0022280	0.6	1.48	0.01256	0.03766
hsa-miR-4479	MIMAT0019011	0.6	1.98	0.01282	0.03834
hsa-miR-6881-3p	MIMAT0027663	0.8	1.95	0.01302	0.03871
hsa-miR-1537-5p	MIMAT0026765	0.8	1.51	0.01301	0.03871
hsa-miR-3940-3p	MIMAT0018356	6.7	0.84	0.01304	0.03871
hsa-miR-222-5p	MIMAT0004569	7.3	1.22	0.01308	0.03875
hsa-miR-4647	MIMAT0019709	1.0	2.72	0.01370	0.04039
hsa-miR-127-5p	MIMAT0004604	30.8	1.08	0.01384	0.04071
hsa-miR-3654	MIMAT0018074	4.7	1.61	0.01397	0.04089
hsa-miR-651-5p	MIMAT0003321	23.8	0.62	0.01423	0.04155
hsa-miR-636	MIMAT0003306	2.0	0.88	0.01476	0.04279
hsa-miR-2277-5p	MIMAT0017352	9.5	0.81	0.01479	0.04279
hsa-miR-4496	MIMAT0019031	2.2	2.04	0.01504	0.04319
hsa-miR-4504	MIMAT0019040	0.4	1.87	0.01507	0.04319
hsa-miR-301a-5p	MIMAT0022696	1.7	0.93	0.01502	0.04319
hsa-miR-550a-5p	MIMAT0004800	2.1	0.97	0.01547	0.04413
hsa-miR-580-5p	MIMAT0026617	0.5	1.69	0.01566	0.04457

Annex table 5: All the results of upregulated microRNAs in DLBCL patients vs. controls (p<0.05 and log2foldchange >0) (Continuation)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-580-3p	MIMAT0003245	8.5	0.50	0.01624	0.04603

Annex table 6: All the results of downregulated microRNAs in DLBCL patients vs. controls (padj<0.05 and log2foldchange <0).

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-215-5p	MIMAT0000272	53.8	-4.42	6.74×10^{-35}	8.39×10^{-32}
hsa-miR-150-5p	MIMAT0000451	6310.7	-3.22	3.46×10^{-25}	1.44×10^{-22}
hsa-miR-224-5p	MIMAT0000281	47.7	-3.30	5.11×10^{-21}	1.27×10^{-18}
hsa-miR-194-1-5p	MIMAT0000460	37.8	-4.33	6.19×10^{-17}	7.00×10^{-15}
hsa-miR-452-3p	MIMAT0001636	7.2	-2.33	2.10×10^{-16}	1.87×10^{-14}
hsa-miR-335-5p	MIMAT0000765	127.6	-2.74	6.20×10^{-16}	5.14×10^{-14}
hsa-miR-145-5p	MIMAT0000437	2060.5	-2.16	2.65×10^{-15}	1.94×10^{-13}
hsa-miR-101-5p	MIMAT0004513	30.2	-1.69	5.46×10^{-15}	3.57×10^{-13}
hsa-miR-139-5p	MIMAT0000250	48.8	-2.26	5.40×10^{-15}	3.57×10^{-13}
hsa-miR-126-3p	MIMAT0000445	3182.5	-1.44	6.54×10^{-15}	3.88×10^{-13}
hsa-miR-497-5p	MIMAT0002820	332.5	-2.07	3.53×10^{-14}	1.91×10^{-12}
hsa-miR-212-5p	MIMAT0022695	14.4	-1.54	1.60×10^{-12}	6.88×10^{-11}
hsa-miR-10b-3p	MIMAT0004556	14.0	-1.60	5.28×10^{-12}	1.99×10^{-10}
hsa-miR-10a-3p	MIMAT0004555	10.2	-2.29	7.45×10^{-12}	2.73×10^{-10}
hsa-miR-95-3p	MIMAT0000094	18.1	-2.13	3.79×10^{-11}	1.22×10^{-09}
hsa-miR-125a-5p	MIMAT0000443	4941.1	-1.34	3.92×10^{-10}	1.04×10^{-08}
hsa-miR-125b-2-5p	MIMAT0000423_1	1975.9	-1.66	3.89×10^{-10}	1.04×10^{-08}
hsa-miR-151b	MIMAT0010214	24.8	-2.21	6.67×10^{-10}	1.60×10^{-08}
hsa-miR-874-5p	MIMAT0026718	10.1	-1.94	8.02×10^{-10}	1.81×10^{-08}
hsa-miR-10a-5p	MIMAT0000253	21228.4	-1.71	1.05×10^{-10}	2.33×10^{-08}
hsa-let-7c-5p	MIMAT0000064	764.1	-1.92	1.22×10^{-09}	2.66×10^{-08}
hsa-miR-504-5p	MIMAT0002875	9.1	-1.93	1.69×10^{-09}	3.56×10^{-08}
hsa-miR-551b-3p	MIMAT0003233	7.9	-2.25	2.97×10^{-09}	5.78×10^{-08}
hsa-miR-194-2-5p	MIMAT0000460_1	234.5	-2.52	4.25×10^{-09}	8.01×10^{-08}
hsa-miR-101-1-3p	MIMAT0000099	1463.8	-1.26	4.34×10^{-09}	8.07×10^{-08}
hsa-miR-642a-5p	MIMAT0003312	9.2	-1.50	1.02×10^{-08}	1.76×10^{-07}
hsa-miR-29a-3p	MIMAT0000086	7934.1	-1.14	1.38×10^{-08}	2.35×10^{-07}
hsa-miR-135a-1-5p	MIMAT0000428_1	4.3	-3.49	1.92×10^{-08}	3.07×10^{-07}
hsa-miR-99a-5p	MIMAT0000097	1513.3	-1.70	2.68×10^{-08}	4.23×10^{-07}
hsa-miR-339-3p	MIMAT0004702	159.2	-1.24	1.03×10^{-07}	1.51×10^{-06}
hsa-miR-549a-3p	MIMAT0003333	1.6	-2.39	2.28×10^{-07}	3.02×10^{-06}
hsa-miR-99a-3p	MIMAT0004511	15.8	-1.67	2.48×10^{-07}	3.25×10^{-06}
hsa-miR-335-3p	MIMAT0004703	253.9	-1.81	4.39×10^{-07}	5.46×10^{-06}
hsa-miR-549a-5p	MIMAT0037328	3.4	-2.44	4.93×10^{-07}	5.96×10^{-06}
hsa-miR-224-3p	MIMAT0009198	4.9	-1.60	5.92×10^{-07}	6.76×10^{-06}

Annex table 6: All the results of downregulated microRNAs in DLBCL patients vs. controls (padj<0.05 and log2foldchange <0) (Continuation).

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-452-5p	MIMAT0001635	67.8	-1.44	$7.92x10^{-07}$	$8.77x10^{-06}$
hsa-miR-140-3p	MIMAT0004597	2321.3	-1.01	$1.42x10^{-06}$	$1.50x10^{-05}$
hsa-miR-195-5p	MIMAT0000461	747.6	-1.72	$1.47x10^{-06}$	$1.53x10^{-05}$
hsa-let-7b-5p	MIMAT0000063	2879.4	-1.12	$1.55x10^{-06}$	$1.61x10^{-05}$
hsa-let-7g-5p	MIMAT0000414	8351.5	-1.24	$1.63x10^{-06}$	$1.65x10^{-05}$
hsa-miR-483-3p	MIMAT0002173	4.0	-2.00	$3.40x10^{-06}$	$3.18x10^{-05}$
hsa-miR-451a	MIMAT0001631	6085.0	-2.07	$4.55x10^{-06}$	$4.16x10^{-05}$
hsa-miR-125b-2-3p	MIMAT0004603	68.8	-1.39	$4.73x10^{-06}$	$4.26x10^{-05}$
hsa-miR-342-3p	MIMAT0000753	4801.5	-1.03	$6.23x10^{-06}$	$5.49x10^{-05}$
hsa-miR-204-5p	MIMAT0000265	78.2	-1.92	$6.34x10^{-06}$	$5.55x10^{-05}$
hsa-miR-670-3p	MIMAT0026640	0.9	-2.72	$8.61x10^{-06}$	$7.44x10^{-05}$
hsa-miR-1271-5p	MIMAT0005796	53.8	-1.61	$1.07x10^{-05}$	$9.06x10^{-05}$
hsa-miR-505-3p	MIMAT0002876	105.1	-0.53	$1.17x10^{-05}$	$9.77x10^{-05}$
hsa-miR-193a-3p	MIMAT0000459	115.1	-1.17	$1.50x10^{-05}$	0.00012
hsa-miR-197-3p	MIMAT0000227	316.1	-0.94	$1.60x10^{-05}$	0.00013
hsa-miR-6511b-3p	MIMAT0025848	1.3	-1.18	$1.68x10^{-05}$	0.00013
hsa-miR-29c-3p	MIMAT0000681	3703.5	-0.93	$1.72x10^{-05}$	0.00013
hsa-miR-4636	MIMAT0019693	1.1	-1.88	$1.95x10^{-05}$	0.00015
hsa-miR-190a-5p	MIMAT0000458	20.4	-1.20	$2.08x10^{-05}$	0.00016
hsa-miR-99b-5p	MIMAT0000689	7335.7	-1.01	$2.44x10^{-05}$	0.00018
hsa-miR-95-5p	MIMAT0026473	2.6	-1.28	$2.43x10^{-05}$	0.00018
hsa-miR-6803-3p	MIMAT0027507	2.7	-1.19	$2.69x10^{-05}$	0.00020
hsa-miR-135a-3p	MIMAT0004595	0.6	-2.46	$2.89x10^{-05}$	0.00021
hsa-miR-502-3p	MIMAT0004775	26.3	-0.84	$3.04x10^{-05}$	0.00022
hsa-miR-199a-1-3p	MIMAT0000232_1	72.7	-1.85	$3.07x10^{-05}$	0.00022
hsa-miR-585-3p	MIMAT0003250	2.3	-1.66	$3.24x10^{-05}$	0.00023
hsa-miR-216a-5p	MIMAT0000273	3.0	-2.19	$5.48x10^{-05}$	0.00037
hsa-miR-144-5p	MIMAT0004600	157.2	-1.91	$6.25x10^{-05}$	0.00041
hsa-miR-26a1-5p	MIMAT0000082_1	20323.2	-1.92	$6.66x10^{-05}$	0.00043
hsa-miR-101-2-3p	MIMAT0000099_1	8966.6	-1.11	$8.04x10^{-05}$	0.00051
hsa-miR-217-5p	MIMAT0000274	1.1	-2.15	$9.96x10^{-05}$	0.00062
hsa-miR-4662a-5p	MIMAT0019731	2.9	-1.37	0.00013	0.00077
hsa-let-7b-3p	MIMAT0004482	51.0	-0.79	0.00014	0.00080
hsa-miR-497-3p	MIMAT0004768	4.3	-1.13	0.00016	0.00093
hsa-miR-338-5p	MIMAT0004701	33.2	-1.44	0.00016	0.00093
hsa-miR-331-3p	MIMAT0000760	139.0	-0.73	0.00030	0.00164
hsa-miR-484	MIMAT0002174	480.5	-0.69	0.00031	0.00166
hsa-miR-676-3p	MIMAT0018204	1.1	-1.46	0.00034	0.00180
hsa-miR-143-5p	MIMAT0004599	81.1	-1.02	0.00034	0.00182
hsa-miR-23b-3p	MIMAT0000418	547.8	-1.11	0.00037	0.00193
hsa-miR-28-5p	MIMAT0000085	525.6	-1.00	0.00041	0.00211
hsa-let-7e-5p	MIMAT0000066	470.3	-0.93	0.00054	0.00264

Annex table 6: All the results of downregulated microRNAs in DLBCL patients vs. controls (padj<0.05 and log2foldchange <0) (Continuation).

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-320b-2	MIMAT0005792_1	6.5	-1.22	0.00057	0.00279
hsa-miR-181b-2-3p	MIMAT0031893	0.7	-1.45	0.00058	0.00279
hsa-miR-2114-5p	MIMAT0011156	1.7	-1.15	0.00062	0.00293
hsa-miR-1260a	MIMAT0005911	72.7	-1.75	0.00080	0.00361
hsa-miR-143-3p	MIMAT0000435	92690.9	-0.78	0.00083	0.00376
hsa-miR-190a-3p	MIMAT0026482	1.1	-1.45	0.00087	0.00390
hsa-miR-30c-2-5p	MIMAT0000244_1	1209.7	-1.14	0.00098	0.00433
hsa-miR-3622a-5p	MIMAT0018003	0.9	-1.63	0.00115	0.00489
hsa-miR-181a-2-5p	MIMAT0000256_1	8231.0	-0.94	0.00130	0.00542
hsa-miR-126-5p	MIMAT0000444	12938.0	-0.93	0.00145	0.00597
hsa-miR-376c-3p	MIMAT0000720	25.7	-1.13	0.00146	0.00598
hsa-miR-5571-3p	MIMAT0022258	3.7	-1.70	0.00160	0.00645
hsa-miR-551a	MIMAT0003214	1.2	-1.15	0.00162	0.00649
hsa-miR-628-5p	MIMAT0004809	14.3	-0.53	0.00167	0.00667
hsa-miR-150-3p	MIMAT0004610	62.6	-1.04	0.00167	0.00667
hsa-miR-144-3p	MIMAT0000436	576.9	-1.44	0.00171	0.00679
hsa-miR-500a-3p	MIMAT0002871	388.1	-0.56	0.00181	0.00717
hsa-miR-338-3p	MIMAT0000763	179.8	-1.10	0.00203	0.00794
hsa-miR-130a-3p	MIMAT0000425	677.2	-0.91	0.00208	0.00812
hsa-miR-409-5p	MIMAT0001638	11.0	-1.60	0.00219	0.00847
hsa-miR-561-5p	MIMAT0022706	4.9	-1.05	0.00222	0.00859
hsa-miR-31-5p	MIMAT0000089	94.3	-1.38	0.00232	0.00885
hsa-miR-5683	MIMAT0022472	24.0	-1.69	0.00231	0.00885
hsa-miR-142-5p	MIMAT0000433	61555.7	-0.81	0.00233	0.00888
hsa-miR-5193	MIMAT0021124	0.7	-1.21	0.00243	0.00921
hsa-miR-6737-3p	MIMAT0027376	1.6	-1.10	0.00256	0.00963
hsa-miR-26a-2-3p	MIMAT0004681	14.1	-0.75	0.00263	0.00985
hsa-miR-365b-3p	MIMAT0022834	35.7	-2.01	0.00274	0.01025
hsa-let-7a-3-5p	MIMAT0000062_1	4442.2	-1.05	0.00300	0.01115
hsa-miR-26b-5p	MIMAT0000083	10400.2	-0.86	0.00329	0.01214
hsa-miR-192-3p	MIMAT0004543	3.9	-1.79	0.00346	0.01266
hsa-let-7a-2-5p	MIMAT0000062	3740.8	-1.41	0.00380	0.01375
hsa-miR-628-3p	MIMAT0003297	8.5	-0.71	0.00387	0.01396
hsa-miR-675-3p	MIMAT0006790	1.8	-1.37	0.00420	0.01481
hsa-miR-514a-2-3p	MIMAT0002883_1	0.3	-1.89	0.00419	0.01481
hsa-miR-342-5p	MIMAT0004694	133.8	-0.68	0.00456	0.01580
hsa-miR-100-5p	MIMAT0000098	10399.0	-0.77	0.00480	0.01655
hsa-miR-652-5p	MIMAT0022709	14.9	-0.56	0.00487	0.01671
hsa-miR-32-5p	MIMAT0000090	219.0	-0.80	0.00490	0.01676
hsa-miR-202-5p	MIMAT0002810	0.6	-1.86	0.00499	0.01692
hsa-miR-5690	MIMAT0022482	3.2	-0.99	0.00507	0.01713
hsa-miR-212-3p	MIMAT0000269	17.4	-0.75	0.00525	0.01770

Annex table 6: All the results of downregulated microRNAs in DLBCL patients vs. controls (padj<0.05 and log2foldchange <0) (Continuation).

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-4526	MIMAT0019065	0.4	-1.49	0.00539	0.01811
hsa-miR-152-3p	MIMAT0000438	257.5	-0.77	0.00565	0.01880
hsa-miR-3912-3p	MIMAT0018186	12.7	-0.69	0.00584	0.01926
hsa-miR-509-3-3p	MIMAT0002881_1	1.3	-2.18	0.00584	0.01926
hsa-miR-145-3p	MIMAT0004601	403.6	-0.98	0.00595	0.01953
hsa-miR-29b-2-3p	MIMAT0000100_1	189.5	-1.09	0.00634	0.02070
hsa-miR-29b-1-3p	MIMAT0000100	811.1	-0.82	0.00668	0.02155
hsa-miR-199a-2-3p	MIMAT0000232	271.3	-1.07	0.00668	0.02155
hsa-let-7c-3p	MIMAT0026472	0.8	-1.45	0.00669	0.02155
hsa-let-7e-3p	MIMAT0004485	9.5	-0.68	0.00715	0.02294
hsa-miR-153-2-3p	MIMAT0000439_1	12.2	-1.46	0.00768	0.02438
hsa-miR-584-5p	MIMAT0003249	16.6	-0.67	0.00882	0.02764
hsa-miR-24-1-5p	MIMAT0000079	3.1	-0.74	0.01056	0.03236
hsa-miR-151a-5p	MIMAT0004697	2051.2	-0.83	0.01074	0.03284
hsa-miR-3613-3p	MIMAT0017991	16.1	-0.48	0.01220	0.03676
hsa-miR-195-3p	MIMAT0004615	25.2	-0.50	0.01256	0.03766
hsa-let-7a-3p	MIMAT0004481	39.1	-0.53	0.01334	0.03942
hsa-let-7d-5p	MIMAT0000065	613.2	-0.66	0.01390	0.04078
hsa-miR-31-3p	MIMAT0004504	4.1	-1.28	0.01453	0.04233
hsa-miR-26a-2-5p	MIMAT0000082	29705.3	-0.76	0.01475	0.04279
hsa-miR-376a-5p	MIMAT0003386	5.9	-1.03	0.01488	0.04296
hsa-miR-6513-3p	MIMAT0025483	0.7	-0.94	0.01543	0.04413
hsa-miR-488-3p	MIMAT0004763	4.8	-1.49	0.01605	0.04560
hsa-miR-574-3p	MIMAT0003239	266.7	-0.80	0.01707	0.04826
hsa-miR-361-5p	MIMAT0000703	364.4	-0.66	0.01749	0.04935
hsa-miR-215-5p	MIMAT0000272	53.8	-4.42	6.74x10-35	8.39x10-32

Annex table 7: All the results of upregulated microRNAs in Non-GCB DLBCL patients vs. GCB DLBCL patients (p<0.05 and log2foldchange <0).

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-625-3p	MIMAT0004808	39.4	-1.34	$1.19x10^{-07}$	0.00006
hsa-miR-625-5p	MIMAT0003294	19.3	-1.13	$5.40x10^{-06}$	0.00135
hsa-miR-511-5p	MIMAT0002808	7.6	-2.01	$9.92x10^{-06}$	0.00142
hsa-miR-185-3p	MIMAT0004611	40.4	-1.80	$7.70x10^{-06}$	0.00142
hsa-miR-205-5p	MIMAT0000266	20.3	-3.30	$4.10x10^{-05}$	0.00342
hsa-miR-1307-3p	MIMAT0005951	422.0	-1.18	$8.68x10^{-05}$	0.00620
hsa-miR-296-3p	MIMAT0004679	31.6	-1.94	0.00020	0.01033
hsa-miR-4453	MIMAT0018975	1.2	-1.93	0.00036	0.01707
hsa-miR-451a	MIMAT0001631	5979.3	-1.74	0.00038	0.01707
hsa-miR-144-3p	MIMAT0000436	572.9	-1.70	0.00043	0.01809
hsa-miR-6821-3p	MIMAT0027543	1.6	-1.97	0.00065	0.02583
hsa-miR-320a-3p	MIMAT0000510	2407.6	-1.35	0.00078	0.02904
hsa-miR-3652	MIMAT0018072	8.9	-2.44	0.00088	0.03137
hsa-miR-7706	MIMAT0030021	81.1	-1.17	0.00102	0.03414
hsa-miR-144-5p	MIMAT0004600	154.1	-1.66	0.00136	0.03996
hsa-miR-27a-5p	MIMAT0004501	37.7	-0.85	0.00135	0.03996
hsa-miR-3610	MIMAT0017987	20.3	-1.89	0.00157	0.04483

Annex table 8: All the results of upregulated microRNAs in GCB DLBCL patients vs. Non-GCB DLBCL patients (p<0.05 and log2foldchange >0).

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-28-3p	MIMAT0004502	3415.2	1.45	$3.36x10^{-11}$	$3.36x10^{-08}$
hsa-miR-129-2-3p	MIMAT0004605	4.9	4.12	$1.50x10^{-06}$	0.00050
hsa-miR-181a-2-5p	MIMAT0000256_1	6688.9	1.33	8.83x10-^{06}	0.00142
hsa-miR-4464	MIMAT0018988	2.9	3.91	$1.21x10^{-05}$	0.00151
hsa-miR-3150b-3p	MIMAT0018194	49.0	2.00	$1.90x10^{-05}$	0.00211
hsa-miR-181a-5p	MIMAT0000256	39940.6	1.23	$2.40x10^{-05}$	0.00240
hsa-miR-28-5p	MIMAT0000085	475.7	1.24	$3.85x10^{-05}$	0.00342
hsa-miR-181b-2-5p	MIMAT0000257_1	3046.9	1.06	$4.75x10^{-05}$	0.00366
hsa-miR-138-1-5p	MIMAT0000430_1	568.1	2.00	$9.92x10^{-05}$	0.00661
hsa-miR-486-2-3p	MIMAT0004762_1	27.6	1.75	0.00015	0.00891
hsa-miR-129-5p	MIMAT0000242	18.2	2.90	0.00014	0.00891
hsa-miR-181a-2-3p	MIMAT0004558	166.9	1.02	0.00017	0.00951
hsa-miR-181d-5p	MIMAT0002821	213.7	0.71	0.00024	0.01176
hsa-miR-151b	MIMAT0010214	22.0	1.56	0.00040	0.01761
hsa-miR-181a-3p	MIMAT0000270	1066.6	0.97	0.00067	0.02583
hsa-miR-4677-3p	MIMAT0019761	25.5	0.57	0.00093	0.03219
hsa-miR-582-3p	MIMAT0004797	65.7	1.28	0.00114	0.03663
hsa-miR-200b-3p	MIMAT0000318	15.7	1.56	0.00128	0.03996
hsa-miR-151a-5p	MIMAT0004697	1783.9	1.09	0.00166	0.04618

Annex table 9: All the results of microRNAs associated with good response to treatment (p<0.05)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-12136	MIMAT0049032	120.0	25.73	$1.29x10^{-13}$	$9.70x10^{-11}$
hsa-miR-129a-5p	MIMAT0000242_1	42.7	5.09	$2.86x10^{-09}$	$1.08x10^{-06}$
hsa-miR-129-1-3p	MIMAT0004548	7.4	4.08	$1.86x10^{-06}$	0.00035
hsa-miR-3150b-3p	MIMAT0018194	50.7	2.33	$1.60x10^{-05}$	0.00241
hsa-miR-409-3p	MIMAT0001639	244.7	1.91	$4.36x10^{-05}$	0.00547
hsa-miR-127-3p	MIMAT0000446	3977.2	2.01	$6.14x10^{-05}$	0.00661
hsa-miR-3681-5p	MIMAT0018108	75.3	2.34	0.00016	0.01507
hsa-miR-28-3p	MIMAT0004502	3740.9	1.02	0.00021	0.01729
hsa-miR-136-5p	MIMAT0000448	60.2	1.34	0.00033	0.02251
hsa-miR-127-5p	MIMAT0004604	30.8	1.92	0.00036	0.02279
hsa-miR-370-3p	MIMAT0000722	16.1	2.59	0.00041	0.02380
hsa-miR-145-5p	MIMAT0000437	2060.5	1.07	0.00074	0.03719
hsa-miR-4464	MIMAT0018988	3.3	3.55	0.00117	0.04641
hsa-miR-129b-5p	MIMAT0000242	27.5	2.88	0.00102	0.04641
hsa-miR-3928-3p	MIMAT0018205	16.5	2.03	0.00113	0.04641

Annex table 10: All the results of microRNAs associated with bad response to treatment (p<0.05)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-192-5p	MIMAT0000222	10375.5	-2.41	$1.60x10^{-07}$	$4.01x10^{-05}$
hsa-miR-222-3p	MIMAT0000279	1984.9	-1.10	0.00027	0.02010
hsa-miR-221-3p	MIMAT0000278	5082.8	-1.14	0.00061	0.03302
hsa-miR-4454	MIMAT0018976	183.5	-1.05	0.00107	0.04641

Annex table 11: All the results of microRNAs associated with good prognosis (p<0.05)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-4444	MIMAT0018962	10.3	2.26	$6.20x10^{-05}$	0.00897
hsa-miR-449c-5p	MIMAT0010251	9.8	2.22	$4.75x10^{-05}$	0.00897
hsa-miR-3615	MIMAT0017994	322.3	1.55	$5.32x10^{-05}$	0.00897
hsa-miR-3681-5p	MIMAT0018108	75.3	2.03	0.00022	0.01427
hsa-miR-3928-3p	MIMAT0018205	16.5	2.03	0.00018	0.01427
hsa-miR-449b-5p	MIMAT0003327	7.2	2.22	0.00037	0.02168
hsa-miR-1287-5p	MIMAT0005878	19.7	1.75	0.00039	0.02168
hsa-miR-146a-3p	MIMAT0004608	27.7	1.49	0.00053	0.02495
hsa-miR-423-3p	MIMAT0001340	3193.5	1.15	0.00049	0.02495
hsa-miR-370-3p	MIMAT0000722	16.1	2.12	0.00071	0.02954
hsa-miR-184	MIMAT0000454	14.6	1.98	0.00071	0.02954
hsa-miR-4424	MIMAT0018939	110.8	2.32	0.00107	0.04174

Annex table 12: All the results of microRNAs associated with bad prognosis (p<0.05)

MicroRNA	Ids	Base Mean	Log2 Fold Change	p-value	p adj
hsa-miR-133a-2-3p	MIMAT0000427_1	470,9	-3,81	$1,53x10^{-06}$	0,00102
hsa-miR-133a-1-3p	MIMAT0000427	69,3	-2,53	$6,78x10^{-05}$	0,00897
hsa-miR-338-3p	MIMAT0000763	179,8	-1,39	0,00011	0,01186
hsa-miR-208b-3p	MIMAT0004960	12,9	-3,09	0,00014	0,01329
hsa-miR-205-5p	MIMAT0000266	62,5	-3,37	0,00021	0,01427

Annex Table 13: 138 Downregulated target genes which interact with upregulated microRNAs

CXCR4	*SOX4*	*CYLD*	*ELAVL1*	*ATF1*	*CUL4A*	*TUG1*
REST	*EZR*	*CCND2*	*DICER1*	*TP53BP1*	*RUNX2*	*PRKAR1A*
SNCA	*MCL1*	*MAPK1*	*PDCD2*	*FBXW7*	*SUZ12*	*FANCM*
RAD52	*MNT*	*WASF2*	*PTBP3*	*CNOT6L*	*BCLAF1*	*FNDC3A*
KRAS	*BTRC*	*RECK*	*MALAT1*	*DRD2*	*CPM*	*CTNNB1*
IRAK2	*JUND*	*STAT1*	*ALDH5A1*	*SIKE1*	*BAX*	*SPAG9*
POU2F2	*TSC22D3*	*SRC*	*FOXN3*	*PRKCE*	*KLF8*	*TIA1*
ETS1	*NOTCH1*	*ILF3*	*INPP5A*	*CUL5*	*PTP4A1*	*TRIM8*
FOXO3	*TP53*	*KMT5A*	*PSMA3*	*SIAH1*	*REL*	*HES1*
IRAK1	*IL24*	*ARPP19*	*PTPN2*	*HOXD10*	*MTDH*	*NFAT5*
ROCK1	*SMAD4*	*FLOT1*	*ZFAND4*	*NFATC3*	*YWHAZ*	*WNK1*
FOXO1	*CREB1*	*PTEN*	*ITGB3*	*PIK3CG*	*RHOA*	*FGFR1*
RARG	*FGFR2*	*BMPR2*	*ACSL4*	*PPP2CB*	*PDPK1*	*ATM*
BRCA2	*CASP8*	*YY1*	*RAC1*	*TGFBR2*	*CACNB2*	*QKI*
APC	*MTSS1*	*ERN1*	*FOXP3*	*MTHFD1*	*IGF2BP3*	*MSH3*
PTPN1	*EGR1*	*SKP2*	*HIF3A*	*BCL2L11*	*DMD*	*RBAK*
XIST	*AP3B1*	*AKT2*	*FOXP1*	*STAT3*	*DLG1*	*CCNJ*
FAF1	*EGLN2*	*CDK6*	*TP53INP1*	*SOX2*	*CAMKK2*	*RAP2A*
E2F5	*TGFB1*	*DYRK1A*	*XIAP*	*SOS1*	*PRKAA1*	
ITGB1	*BCL2*	*GSK3B*	*SP1*	*LMNA*	*STAT5B*	

Annex Table 14: 76 Upregulated target genes which interact with upregulated microRNAs.

KLF4	*SOX4*	*EDNRA*	*HOXA10*	*ZEB2*	*ITSN1*	*CEMIP*
MYO6	*AP2M1*	*SRGAP1*	*JAG1*	*BMPR2*	*FAM162A*	*MTDH*
ROBO1	*EGFR*	*CDH2*	*BRCA1*	*EPAS1*	*RHOT1*	*NID1*
PTEN	*NR4A2*	*HBEGF*	*ID4*	*RREB1*	*CD28*	*DLC1*
STAT1	*SMAD4*	*PTPN12*	*SERINC5*	*IGF1*	*IL10*	*PTPN14*
YES1	*VEGFA*	*ITGA9*	*MEST*	*DPYSL2*	*YAP1*	*TCEAL9*
TNC	*WNK1*	*SOCS2*	*MMP1*	*MTSS1*	*ACTC1*	*PRKCA*
PARP8	*FZD7*	*CD44*	*MMP12*	*CELF2*	*ADGRL4*	*CHD1*
MIF	*MMP2*	*SERPINE1*	*APH1A*	*SRCIN1*	*SPTLC1*	*PLAUR*
APC	*MMP9*	*ITGB8*	*ABHD17C*	*CHEK1*	*CEBPD*	*Reck*
MERTK	*BCL2*	*KRAS*	*MYO5A*	*TRIB1*	*MYOCD*	

Annex table 15: Upregulated microRNAs and downregulated target genes signaling pathways in cancer.

microRNA	*Entrez-gene name*
hsa-miR-612 **hsa-miR-2861**	*AKT2*
hsa-miR-135a-5p **hsa-miR-663a** **hsa-miR-129-5p**	*APC*
hsa-miR-7-5p **hsa-miR-182-5p** **hsa-miR-205-5p** **hsa-miR-135a-5p** **hsa-miR-9-5p**	*BCL2*
hsa-miR-182-5p **hsa-miR-146a-5p**	*CCND2*
hsa-miR-129-5p **hsa-miR-320a**	*CDK6*
hsa-miR-146a-5p **hsa-miR-146a-3p** **hsa-miR-9-5p** **hsa-miR-663a**	*CXCR4*
hsa-miR-182-5p **hsa-miR-183-5p** **hsa-miR-9-5p** **hsa-miR-9-3p** **hsa-miR-135a-5p**	*FOXO1*
hsa-miR-183-5p **hsa-miR-182-5p** **hsa-miR-9-5p** **hsa-miR-129-1-3p** **hsa-miR-1246**	*GSK3B*
hsa-miR-9-3p **hsa-miR-9-5p**	*HES1*
hsa-miR-183-5p **hsa-miR-9-3p**	*ITGB1*
hsa-miR-320a **hsa-miR-9-3p** **hsa-miR-129-5p**	*MAPK1*
hsa-miR-129-5p **hsa-miR-9-5p** **hsa-miR-146a-5p**	*NOTCH1*

microRNA	*Entrez-gene name*
hsa-miR-182-5p **hsa-miR-155-3p** **hsa-miR-205-5p** **hsa-miR-320a**	*PTEN*
hsa-miR-320a **hsa-miR-146a-5p**	*RAC1*
hsa-miR-146a-5p **hsa-miR-135a-5p**	*ROCK1*
hsa-miR-146a-5p **hsa-miR-182-5p** **hsa-miR-205-5p** **hsa-miR-183-5p**	*SMAD4*
hsa-miR-129-5p **hsa-miR-612**	*SP1*
hsa-miR-663a **hsa-miR-146a-5p**	*TGFB1*
hsa-miR-612 **hsa-miR-663a** **hsa-miR-155-3p**	*TP53*
hsa-miR-7-5p	*BAX*
hsa-miR-9-5p	*BCL2L11*
hsa-miR-146a-5p	*BRCA2*
hsa-miR-19b-1-5p	*CASP8*
hsa-miR-320a	*CTNNB1*
hsa-miR-205-5p	*EGLN2*
hsa-miR-9-5p	*ETS1*
hsa-miR-573	*FGFR1*
hsa-miR-19b-1-5p	*FGFR2*
hsa-miR-18a-3p	*KRAS*
hsa-miR-7-5p	*MSH3*
hsa-miR-146a-5p	*RHOA*
hsa-miR-7-5p	*SKP2*
hsa-miR-146a-5p	*SOS1*
hsa-miR-146a-5p	*STAT1*
hsa-miR-1181	*STAT3*
hsa-miR-9-5p	*TGFBR2*
hsa-miR-182-3p	*STAT5B*
hsa-miR-7-5p	*XIAP*

Annex table 16: Downregulated microRNAs and upregulated target genes signaling pathways in cancer.

microRNA	*Entrez-gene name*
hsa-miR-451a	*BCL2*
hsa-miR-497-5p	*BCL2*
hsa-miR-135a-5p	*BCL2*
hsa-miR-224-5p	*BCL2*
hsa-miR-139-5p	*BCL2*
hsa-miR-145-5p	*EGFR*
hsa-miR-135a-5p	*EGFR*
hsa-miR-217	*KRAS*
hsa-miR-224-5p	*KRAS*
hsa-miR-217	*PTEN*
hsa-miR-216a-5p	*PTEN*
hsa-miR-483-3p	*SMAD4*
hsa-miR-224-5p	*SMAD4*
hsa-miR-145-5p	*STAT1*
hsa-miR-150-5p	*STAT1*
hsa-miR-150-5p	*VEGFA*
hsa-miR-145-5p	*VEGFA*
hsa-miR-135a-5p	*APC*
hsa-miR-224-5p	*EDNRA*
hsa-miR-145-5p	*EPAS1*
hsa-miR-145-5p	*FZD7*
hsa-miR-483-3p	*IGF1*
hsa-miR-145-5p	*JAG1*
hsa-miR-145-5p	*MMP1*
hsa-miR-451a	*MMP2*
hsa-miR-451a	*MMP9*
hsa-miR-150-5p	*PRKCA*

Annex Table 17: Genes of cancer signaling pathways targeted by downregulated target genes.

Entrez-gene ID	*Entrez-gene name*
2113	*ETS1:* ETS proto-oncogene 1, transcription factor
208	*AKT2:* AKT serine/threonine kinase 2
2260	*FGFR1:* fibroblast growth factor receptor 1
2263	*FGFR2:* fibroblast growth factor receptor 2
2308	*FOXO1:* forkhead box O1
324	*APC:* APC, WNT signaling pathway regulator
331	*XIAP:* X-linked inhibitor of apoptosis
4437	*MSH3:* mutS homolog 3
6502	*SKP2:* S-phase kinase associated protein 2
387	*RHOA:* ras homolog family member A
6654	*SOS1:* SOS Ras/Rac guanine nucleotide exchange factor 1
6667	*SP1:* Sp1 transcription factor
581	*BAX:* BCL2 associated X, apoptosis regulator
596	*BCL2:* BCL2, apoptosis regulator
6772	*STAT1:* signal transducer and activator of transcription 1
6774	*STAT3:* signal transducer and activator of transcription 3
6777	*STAT5B:* signal transducer and activator of transcription 5B
675	*BRCA2:* BRCA2, DNA repair associated
4851	*NOTCH1:* notch 1
1499	*CTNNB1:* catenin beta 1
841	*CASP8:* caspase 8
2932	*GSK3B:* glycogen synthase kinase 3 beta
894	*CCND2:* cyclin D2
7048	*TGFBR2:* transforming growth factor beta receptor 2
7157	*TP53:* tumor protein p53
1021	*CDK6:* cyclin dependent kinase 6
3280	*HES1:* hes family bHLH transcription factor 1
7040	*TGFB1:* transforming growth factor beta 1
5594	*MAPK1:* mitogen-activated protein kinase 1
5728	*PTEN:* phosphatase and tensin homolog
3688	*ITGB1:* integrin subunit beta 1
7852	*CXCR4:* C-X-C motif chemokine receptor 4
5879	*RAC1:* Rac family small GTPase 1
3845	*KRAS:* KRAS proto-oncogene, GTPase
112398	*EGLN2:* egl-9 family hypoxia inducible factor 2
10018	*BCL2L11:* BCL2 like 11
6093	*ROCK1:* Rho associated coiled-coil containing protein kinase 1
4089	*SMAD4:* SMAD family member 4

Annex Table 18: Genes of cancer signaling pathways targeted by upregulated target genes.

Entrez-gene ID	*Entrez-gene name*
8324	FZD7: frizzled class receptor 7
182	JAG1: jagged 1
4312	MMP1: matrix metallopeptidase 1
4313	MMP2: matrix metallopeptidase 2
4318	MMP9: matrix metallopeptidase 9
324	APC: APC, WNT signaling pathway regulator
596	BCL2: BCL2, apoptosis regulator
6772	STAT1: signal transducer and activator of transcription 1
7422	VEGFA: vascular endothelial growth factor A
3479	IGF1: insulin like growth factor 1
5578	PRKCA: protein kinase C alpha
5728	PTEN: phosphatase and tensin homolog
3845	KRAS: KRAS proto-oncogene, GTPase
1909	EDNRA: endothelin receptor type A
1956	EGFR: epidermal growth factor receptor
2034	EPAS1: endothelial PAS domain protein 1
4089	SMAD4: SMAD family member 4

cancers

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Review

Systematic Review of the Potential of MicroRNAs in Diffuse Large B Cell Lymphoma

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Received: 28 December 2018; Accepted: 22 January 2019; Published: 26 January 2019

Abstract: Diffuse large B cell lymphoma (DLBCL) is the most common subtype of invasive non-Hodgkin's lymphoma (NHL). DLBCL presents with variable backgrounds, which results in heterogeneous outcomes among patients. Although new tools have been developed for the classification and management of patients, 40% of them still have primary refractory disease or relapse. In addition, multiple factors regarding the pathogenesis of this disease remain unclear and identification of novel biomarkers is needed. In this context, recent investigations point to microRNAs as useful biomarkers in cancer. The aim of this systematic review was to provide new insight into the role of miRNAs in the diagnosis, classification, treatment response and prognosis of DLBCL patients. We used the following terms in PubMed" ((‘Non-coding RNA’) OR (‘microRNA’ OR ‘miRNA’ OR ‘miR’) OR (‘exosome’) OR (‘extracellular vesicle’) OR (‘secretome’)) AND (‘Diffuse large B cell lymphoma’ OR ‘DLBCL’)" to search for studies evaluating miRNAs as a diagnosis, subtype, treatment response or prognosis biomarkers in primary DLBCL in human patient populations. As a result, the analysis was restricted to the role of miRNAs in tumor tissue and we did not consider circulating miRNAs. A total of thirty-six studies met the inclusion criteria. Among them, twenty-one were classified in the diagnosis category, twenty in classification, five in treatment response and nineteen in prognosis. In this review, we have identified miR-155-5p and miR-21-5p as miRNAs of potential utility for diagnosis, while miR-155-5p and miR-221-3p could be useful for classification. Further studies are needed to exploit the potential of this field.

Keywords: lymphoma; microRNA; diagnosis; classification; treatment response; prognosis

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults, accounting for 30–40% of all non-Hodgkin lymphoma cases [1]. DLBCL presents a very diverse clinical and genetic background, which leads to highly heterogeneous outcomes among patients [2].

The first line therapy for this aggressive malignancy consists of combined chemotherapy including rituximab, prednisone, doxorubicin, vincristine and cyclophosphamide (R-CHOP). Around 75–80% of patients achieve complete remission with R-CHOP therapy. Nevertheless, approximately up to 40 % of

patients have primary refractory disease or relapse [3]. Worryingly, those patients tend to respond poorly to additional chemotherapy lines, which remains a major cause of morbidity and mortality [4].

In order to identify DLBCL patients with a higher risk of a poor response to therapy, different tools have been developed. The International Prognostic Index (IPI) predicts overall and progression free survival based on five risk factors: age, tumor stage, serum lactate dehydrogenase (LDH) concentration, performance status and number of extra nodal disease sites [5]. Using those factors, the IPI distinguishes four risk groups with different 5-year overall survival, ranging from 26 to 73% [6]. Nevertheless, some patients present an unfavorable course of disease despite having a good prognostic index. Another profiling tool uses gene expression profiling (GEP) or immunohistochemical analysis to define two molecular subtypes with different clinical outcomes independent of IPI stratification: the germinal center B-cell-like (GCB) DLBCL, and the activated B-cell-like (ABC) DLBCL. The 5-year survival rates are 60% for GCB and 40% for ABC subtype [7,8]. However, it is not possible to identify all the patients that will not respond to therapy with these profiling tools. Therefore, new biomarkers are needed for a better patient stratification.

In this sense, important knowledge is emerging regarding novel molecular and biological candidates with diagnostic, predictive and prognostic potential in DLBCL, including microRNAs (miRNAs) [9]. MiRNAs are small, non-coding RNAs with a role in gene expression regulation at the post-transcriptional level. They bind the 3′ untranslated region (UTR) of a target mRNA, which leads to their repression or degradation [10]. Through this mechanism, miRNAs regulate more than 50% of known human genes [11], including genes of the 10 main routes involved in cancer [12].

Accordingly, recent research has shown the potential role of miRNAs as diagnostic, classification and prognostic predictors in cancer [13]. For instance, miR-21 has been intensively studied as a diagnosis tool, being found upregulated in many types of cancer including non-small cell lung cancer (NSCLC) [14]. Other examples include miR-10b, which has been described as a useful tool for the classification of papillary renal cell carcinoma type 2 [15], and miR-183, high expression levels of which have been associated with poor prognosis in different cancer types such as colorectal cancer, pancreatic cancer, lung cancer, gastric cancer, and breast cancer [16].

Abnormal expression of miRNAs is also common in B cell neoplasms, including B cell lymphoma. However, there is inconsistency in the data reported. Consequently, the aim of this systematic review was to clarify the role of deregulated miRNAs in DLBCL tumor samples as more systematically-defined diagnostic, subtype, prediction of treatment response and prognostic biomarkers.

2. Results

The detailed search results are included in Figure 1. In brief, the search strategy provided a total of 508 records in the PubMed database. Once the duplicated articles were removed, 338 remained. Of these 338, 239 were excluded after reading the abstract because they did not meet the inclusion criteria. Then, the full texts of the remaining 99 studies, which focused on miRNAs in DLBCL, were read carefully. Additionally, another 63 articles were excluded because there were other coexisting pathologies, miRNAs were not analyzed in the tumor sample, DLBCL was not primarily considered, they did not assess the role of miRNAs in diagnosis, subtype, prediction of treatment response or prognosis, or miRNA expression changes were not considered. A total of 36 studies investigating the role of miRNA expression changes as biomarkers in DLBCL tumor samples were included. Twenty-one of them considered miRNAs as putative DLBCL diagnosis biomarkers, twenty in subtype classification, five in treatment response and nineteen of the studies searched for markers for their role in prognosis.

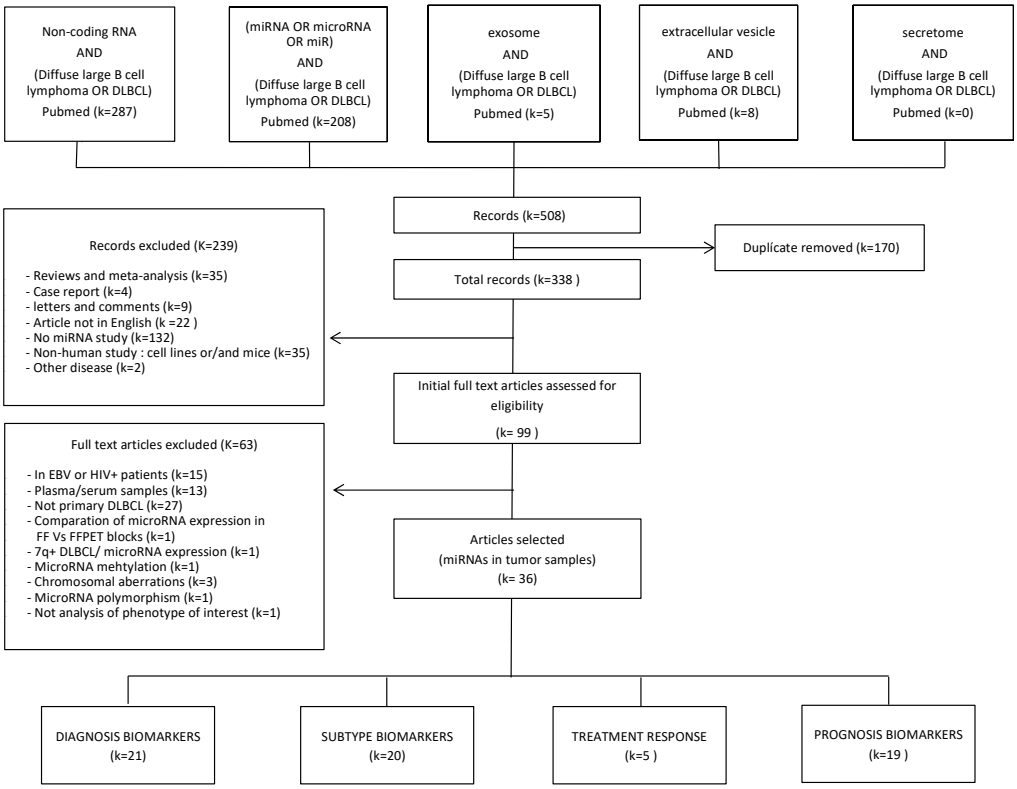

Figure 1. Flow-chart diagram of study selection. k = number of records.

2.1. Tumor Tissue miRNAs as Biomarkers for Diagnosis in DLBCL

Twenty-one studies analyzed the expression of miRNAs by comparing DLBCL cases vs healthy controls [17–37]. These 21 studies provided a total of 140 differentially expressed microRNAs in DLBCL patients compared with healthy control individuals as shown in Table S1.

Regarding the miRNAs that were concordantly deregulated in more than two studies, we identified two miRNAs that were repeatedly reported to be up-regulated in DLBCL patients (miR-155-5p [17,22,24,26,28,30,32–34], miR-21-5p [19,22,26,28,33,36], although some studies did not find a significant association (miR-155-5p [27,29], miR-21-5p [27,32]).We also identified two miRNAs with contradictory results. On the one hand, miR-150-5p was found to be down-regulated in DLBCL patients in four studies [18,28,31,32] and contradictorily up-regulated in DLBCL patients in another study [29], while no significant association was reported in the remaining study [27]. On the other hand, miR-146a/b-5p was found upregulated in three studies [22,27,30] while it was shown to be discordantly downregulated in another study [21], and not significantly associated with DLBCL in two studies [27,28] (Table 1).

2.2. Tumor Tissue miRNAs as Biomarkers for DLBCL Subtype Classification

Twenty studies analyzed the role of tumor tissue miRNAs to distinguish between GCB and ABC DLBCL subtypes and their characteristics are provided in Table S2 [19,21,23–28,30,32–34,38–45]. These studies found 93 miRNAs differentially expressed between GCB and ABC DLBCL samples. Among these 93 differentially expressed miRNAs, five miRNAs were concordantly reported in more than two studies. Four of them were reported as down-regulated (miR-155-5p [26–28,30,33,34,38,41,44], miR-221-3p [27,33,41,45], miR-222-3p [27,41,45], and miR-146a/b-5p [28,30,41]) or unchanged (miR-221-3p [28,43], miR-222-3p [28,32,38,43] and miR-146a/b-5p [21,27,28,38,43]) in GCB samples, whereas miR-28-5p was found to be up-regulated [28,41] or unchanged [28,41] in the same subtype (Table 2).

2.3. Tumor Tissue miRNAs as Biomarkers for Prediction of Treatment Response in DLBCL

Five studies were focused in the role of miRNAs in DLBCL tissue as predictive biomarkers of response to R-CHOP treatment [18,23,24,30,42]. The characteristics of each study are shown in Table S3. A total of five miRNAs were differentially expressed between good and poor responders. Three microRNAs were found to be associated with a favorable response to therapy (miR-27-3p [18], miR-34a-5p [24] and miR-224-5p [23]), whereas miR-155-5p [30] and miR-146-5p [30] were found to be associated with chemoresistance. However, each miRNA was analyzed in only one study, without any of the results being replicated.

2.4. Tumor Tissue miRNAs as Biomarkers for DLBCL Prognosis

The relevance of tumor tissue miRNAs for prognosis in DLBCL patients was analyzed in nineteen studies [18,20,21,23,25–27,30,32,33,37,38,41,42,45–49]. A total of 50 miRNAs with significant reported associations with patient survival were found (Table S4).

Considering the miRNAs with concordant significant results in more than two studies, miR-222-3p, and miR-155-5p were identified. Up-regulation of miR-222-3p [45,48,49] and miR-155-5p [30,38,46] was associated with a worse outcome in three different studies in each case. However, four and eight studies, respectively, did not find any association with prognosis for miR-222-3p [27,32,41,47] and miR-155-5p [26,27,32,33,41,47–49] (Table 3).

2.5. Pathway Enrichment Analysis

Predicted target genes for the miRNA that presented the highest evidence of being involved in DLBCL diagnosis (miR-155-5p and miR-21-5p) and in subtype classification (miR-155-5p, miR-221-3p) were identified in silico. Using these lists of genes, we searched for over-represented pathways that could be linked to DLBCL.

MAPK signaling pathway—Homo sapiens (KEGG) was significantly enriched among the predicted target genes of the two miRNAs associated with DLBCL diagnosis (Table S5). Regarding the targets of the two miRNAs associated with DLBCL subtype, signaling by receptors tyrosine kinases (Reactome) was significantly enriched (Table S6). The predicted target genes covered more than 20% of the genes included in these two pathways (Table 4).

Table 1. miRNAs significantly associated with DLBCL diagnosis in more than two studies.

Significant miRNAs	Result	*n* DLBCL	*n* Control	Sample Source	Method	n miRNAs	Reference
miR-155-5p	up	29	32 (RLH)	Tissue	qRT-PCR	1	Li et al. 2017 [17]
		22	6 (NLN)	Biopsie	qRT-PCR	1	Huskova et al. 2015 [24]
		200	11 (NT)	FFPE	qRT-PCR	3	Go et al. 2015 [26]
		45 (DC);75 (VC)	10 (DC);6 (VC) (NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
		90	31 (RLN)	FFPE	qRT-PCR	2	Zhong et al. 2012 [30]
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle et al. 2008 [32]
		48	6 (NBC)	FF and FFPE	qRT-PCR	3	Lawrie et al. 2007 [33]
		23	2	FF	Semi RT-PCR	1	Eis et al. 2005 [34]
		24	14 (NLN)	FFPE	array	3100 probes	Tamaddon et al. 2016 [22]
	NS	92	15	FF	sequencing	miRNAome	Lim 2015 et al. [27]
		12	7	FFPE	qRT-PCR	4	Handal et al. 2013 [29]
miR-21-5p	up	55	20 (NLN)	FF and FFPE	qRT-PCR	1	Liu et al. 2017 [19]
		26	10 (NLN)	FFPE	qRT-PCR	1	Song et al. 2017 [36]
		200	11 (NT)	FFPE	qRT-PCR	3	Go et al. 2015 [26]
		45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
		48	6 (NBC)	FF and FFPE	qRT-PCR	3	Lawrie et al. 2007 [33]
		24	14 (NLN)	FFPE	array	3100 probes	Tamaddon et al. 2016 [22]
	NS	92	15	FF	sequencing	miRNAome	Lim et al. 2015 [27]
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle et al. 2008 [32]
	up	12	7	FFPE	qRT-PCR	4	Handal et al. 2013 [29]
	down	45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
		36	5 (NLN)	Tissue	qRT-PCR	8	Fassina et al. 2012 [31]
		58	7 (NLN)	FFPE	qRT-PCR	157	Roehle et al. 2008 [32]
		5	4 (RLH)	Tissue	nanostring	800	Jia et al. 2017 [18]
	NS	92	15	FF	sequencing	miRNAome	Lim et al. 2015 [27]
miR-146b-5p	down	106	30 (RLH)	FFPE	qRT-PCR	939	Wu et al. 2014 [21]
	NS	45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
	up	92	15	FF	sequencing	miRNAome	Lim et al. 2015 [27]
		24	14 (NLN)	FFPE	array	3100 probes	Tamaddon et al. 2016 [22]
miR-146a-5p	up	90	31 (RLN)	FFPE	qRT-PCR	2	Zhong et al. 2012 [30]
		24	14 (NLN)	FFPE	array	3100 probes	Tamaddon et al. 2016 [22]
	NS	45 (DC);75 (VC)	10 (DC);6 (VC)(NLN)	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
		92	15	FF	sequencing	miRNAome	Lim et al. 2015 [27]

RLH: Reactive lymphoid hyperplasia; NLN: normal lymph node tissues; NT: normal tonsil; FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; DC: discovery cohort; VC: validation cohort; NBC: normal B cell samples; Up: statistically significantly upregulated in DLBCL patients; NS: no significant difference between patients and controls; Down: significantly downregulated in DLBCL patients.

Table 2. miRNAs significantly associated with DLBCL subtype in more than two studies.

Significant miRNAs	Result	*n* GCB	*n* ABC	Sample Source	Method	n miRNAs	Reference
miR-155-5p	Down GCB	53	95	FFPE	qRT-PCR	8	Go et al. 2015 [26]
		32	27	FFPE	qRT-PCR/array	377	Iqbal et al. 2015 [38]
		20	34	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
		36	31	FF	qRT-PCR	1	Huang et al. 2012 [44]
		21	69	FFPE	qRT-PCR	2	Zhong et al. 2012 [30]
		32	28	FFPE	Array	464	Lawrie et al. 2009 [41]
		16	18	FF and FFPE	qRT-PCR	3	Lawrie et al. 2007 [33]
		4	19	FF	Semiq. RT-PCR	1	Eis et al. 2005 [34]
		41	30	FF	sequencing	miRNAome	Lim et al. 2015 [27]
	NA	25	25	FFPE	qRT-PCR	157	Roehle et al. 2008 [32]
miR-221-3p	Down GCB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno et al. 2011 [45]
		32	28	FFPE	Array	464	Lawrie et al. 2009 [41]
		16	18	FF and FFPE	qRT-PCR	3	Lawrie et al. 2007 [33]
		41	30	FF	sequencing	miRNAome	Lim et al. 2015 [27]
	NS	20	20	Tissue	Array	113	Zhang et al. 2009 [43]
		20	34	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
miR-222-3p	Down GCB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno et al. 2011 [45]
		32	28	FFPE	Array	464	Lawrie et al. 2009 [41]
		41	30	FF	sequencing	miRNAome	Lim et al. 2015 [27]
	NS	25	25	FFPE	qRT-PCR	157	Roehle et al. 2008 [32]
		20	20	Tissue	Array	113	Zhang et al. 2009 [43]
		32	27	FFPE	qRT-PCR/array	377	Iqbal et al. 2015 [38]
		20	34	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
miR-146a-5p	Down GCB	20	34	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
		21	69	FFPE	qRT-PCR	2	Zhong et al. 2012 [30]
	NS	41	30	FF	sequencing	miRNAome	Lim et al. 2015 [27]
		20	20	Tissue	Array	113	Zhang et al. 2009 [43]
miR-146b-5p	Down GCB	32	28	FFPE	Array	464	Lawrie et al. 2009 [41]
	NS	32	27	FFPE	qRT-PCR/array	377	Iqbal et al. 2015 [38]
		41	30	FF	sequencing	miRNAome	Lim et al. 2015 [27]
		20	20	Tissue	Array	113	Zhang et al. 2009 [43]
		47	59	FFPE	qRT-PCR	2	Wu et al. 2014 [21]
		20	34	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
miR-28-5p	Up GCB	11	18	FFPE	qRT-PCR/array	470	Montes-Moreno et al. 2011 [45]
		32	27	FFPE	qRT-PCR/array	377	Iqbal et al. 2015 [38]
		41	30	FF	sequencing	miRNAome	Lim et al. 2015 [27]
		20	20	Tissue	Array	113	Zhang et al. 2009 [43]
	NS	20	34	FF and FFPE	qRT-PCR/array	177	Caramuta et al. 2013 [28]
		32	28	FFPE	Array	464	Lawrie et al. 2009 [41]

GCB: Germinal center B-cell like; FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; NA: not available; Up: statistically significantly upregulated in the subtype of DLBCL patients; NS: no significant difference between patient subtypes; Down: significantly downregulated in the subtype of DLBCL patients.

Table 3. miRNAs significantly associated with DLBCL prognosis in more than two studies.

Significant miRNAs	Result	*n* DLBCL	Sample Source	Method	n miRNAs	Reference
miR-222-3p	Up: ↓OS	176	FFPE	qRT-PCR	11	Alencar et al. 2011 [48]
	Up: ↓PFS and OS	36/240	FFPE	qRT-PCR/array	470/9	Montes-Moreno et al. 2011 [45]
	Up: ↓OS and PFS	106	FFPE	qRT-PCR	3	Malumbres et al. 2009 [49]
	NS	64	FFPE	Array	464	Lawrie et al. 2009 [41]
		92	FF	sequencing	miRNAome	Lim et al. 2015 [27]
		58	Biopsie	qRT-PCR	157	Roehle et al. 2008 [32]
		83	FFPE	qRT-PCR/array	±900	Shepshelovich et al. 2015 [47]
miR-155-5p	Up: ↓survival	118	FF	qRT-PCR	1	Zhu et al. 2016 [46]
	Up: ↓OS	79	FFPE	qRT-PCR	8	Iqbal et al. 2015 [38]
	Down: ↑PFS	90	FFPE	qRT-PCR	2	Zhong et al. 2012 [30]
	NS	176	FFPE	qRT-PCR	11	Alencar et al. 2017 [48]
		200	FFPE	qRT-PCR	3	Go et al. 2015 [26]
		35	FF and FFPE	qRT-PCR	3	Lawrie et al. 2007 [33]
		64	FFPE	Array	464	Lawrie et al. 2009 [41]
		92	FF	sequencing	miRNAome	Lim et al. 2015 [27]
		106	FFPE	qRT-PCR	3	Malumbres et al. 2009 [49]
		58	Biopsie	qRT-PCR	157	Roehle et al. 2008 [32]
		83	FFPE	qRT-PCR/array	±900	Shepshelovich et al. 2015 [47]

FF: fresh frozen; FFPE: formalin-fixed paraffin-embedded; OS: overall survival; PFS: progression-free survival; EFS: event free survival; RFS: relapse free survival; Up: statistically significantly upregulated expression; NS: no significant association between expression and patient outcome; Down: significantly downregulated expression. ↓: decreased; ↑: increased.

Table 4. Coverage of DLBCL related pathway with miRNA predicted targets.

Phenotype	Pathway Name Num. of Genes Database	miRNA	*p*-value	FDR	n Target Genes	Coverage
DLBCL Diagnosis	MAPK signaling pathway (Homo sapiens) 295 genes (KEGG)	miR-155-5p miR-21-5p	3.42×10^{-07}	0.000293	73	24.7%
DLBCL Subtype	Signaling by Receptor Tyrosine Kinases 422 genes (Reactome)	miR-155-5p miR-221-3p	1.09×10^{-07}	6.25×10^{-05}	103	24.4%

p-value: absolute *p*-value; FDR: corrected *p*-value by "False Discovery Rate" method; pathway source: associated database.

3. Discussion

In this systematic review, we have performed an in depth analysis of the current literature in relation to the potential role of miRNA expression in tumor biopsies as biomarker for diagnosis, subtype characterization, treatment response and prognosis in patients with DLBCL.

Regarding the suitability of miRNAs as diagnostic biomarkers in DLBCL, twenty one articles were identified, in which a total of four miRNAs (miR-155-5p [17,22,24,26,28,30,32–34], miR-21-5p [19,22,26,28,33,36], miR-150-5p [18,28,29,31,32]) and miR-146a/b-5p [21,22,27,30] were found to be significantly deregulated in DLBCL patients in more than two studies with concordant results. Among them, miR-155-5p and miR-21-5p presented the most consistent results, being found upregulated in DLBCL patients in most studies.

MiR-155-5p, was the most widely studied miRNA and was found to be upregulated in DLBCL patients in nine of the studies in which it was analyzed [17,22,24,26,28,30,32–34] while no significant association was found in the other two studies [27,29]. Among the two studies that did not find a significant association between mir-155-5p and DLBCL, one presented the smallest sample size with nineteen patients [29], and the other study followed stricter criteria for statistically significant associations [27]. In agreement with these results, previous studies have suggested that miR-155 could represent an onco-miR as its expression is activated in many tumors, i.e., prostate cancer, breast cancer, and other tumors, particularly those of the lymphoid tissue [50–52]. A possible explanation for its implication in DLBCL is that the validated targets of this miRNA include known hallmarks of DLBCL, such as *SOSC* or *SHIP1* [53].

On the other hand, it is noteworthy that miR-21-5p, which was analyzed in eight independent studies, was significantly upregulated in DLBCL patients in six of them [19,22,26,28,33,36], while no statistically significant association was found in the other two studies [27,32]. In agreement with this observation, miR-21 has been reported to be deregulated in most cancers, such as colorectal cancer, acting as an oncogene [54]. High levels of miR-21 have also been observed in B-NHLs. Overall, miR-21 is considered to be an onco-miR that acts through the inhibition of the expression of different phosphatases, such as PDCD4 (Programmed Cell Death 4) and PTEN (Phosphatase And Tensin Homolog), which control the activity of signaling pathways like AKT and MAPK [55].

Given that miR-155-5p and miR-21-5p seem the best candidates as putative diagnostic tools in patients with DLBCL, their functional implication was inferred by in silico analysis. This analysis showed that MAPK signaling pathway is over-represented among the combined predicted target genes of miR-155-5p and miR-21-5p (Table 4). Interestingly, the genes predicted to be targeted by miR-155-5p and miR-21-5p are in the first steps of the signaling cascade (*CACN, RTK, IL1R* or *NIK*). Aberrant expression of this pathway is a major and highly prevalent oncogenic event in many human cancers [56], including NHL [57], which could explain the role of these miRNAs in DLBCL. In this regard, miR-21-5p is also one of the most frequently upregulated circulating microRNAs previously described as a non-invasive diagnosis biomarker [9].

The utility of microRNAs for DLBCL classification has been analyzed by twenty studies. A total of five miRNAs (miR-155-5p [26–28,30,33,34,38,41,44], miR-221-3p [27,33,41,45], miR-222-3p [27,41,45], miR-146a/b-5p [28,41,43], and miR-28-5p [27,38,43,45]) were found to be deregulated in more than two studies. However, miR-222-3p, miR-146a/b-5p, and miR-28-5p showed contradictory results since they were not found to be significantly related to DLBCL classification in four [28,32,38,43], five [21,27,28,38,43] and two studies [28,41], respectively. Some of the discrepancies might be due to the fact that subtype classification of the DLBCL patients was performed by GEP or IHC, which makes the studies less comparable due to the variable reproducibility of IHC stains and interpretations. The only miRNAs that showed more consistent results were miR-155-5p and miR-221-3p. MiR-155-5p was found to be upregulated in the ABC subgroup in nine out of ten studies and only found to be not associated in a study which used IHC for classification and a more stringent requirement for differentially expressed miRNAs [32]. Mir-221-3p was found to be upregulated in the ABC subgroup in four of the six studies in which it was analyzed.

Taking into account the two most consistent microRNAs related with DLBCL classification, miR-155-5p and miR-221-3p, in silico analysis showed that the Tyrosine Kinase pathway was over-represented among their predicted target genes (Table 4). Among the target genes of both microRNAs, we found *PIK3R1* (p85), which is a negative regulator of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway. Our data could indicate that overexpression of miR-155-5p and miR-221-3p in ABC subgroup repressed *PIK3R1* (p85), the PIK regulatory subunit, activating the PI3K-AKT signaling pathway in this subtype. However, it should be noted that it would be difficult to classify different DLBCL subtypes simply based on those two miRNAs. Thus, additional molecular biomarkers would be needed for clinical application.

Focusing on miRNAs as predictive biomarkers of response to R-CHOP treatment, five studies were identified with no agreement in the miRNAs considered [18,23,24,30,42]. Among them, upregulation of miR-27-3p [18], miR-34a-5p [42] and miR-224-5p [23] were associated with chemosensitivity and miR-155-5p and miR-146-5p [30] were associated with chemoresistance (Table S3). Further studies are needed to confirm these preliminary results.

Finally, the implications of microRNAs in prognosis in DLBCL has been analyzed in nineteen studies including 50 significant miRNAs [18,20,21,23,25–27,30,32,33,41,42,45–49]. Among them, the expression of miR-222-3p [45,48,49], and miR-155-5p [30,38,46] were found to be associated with prognosis in more than two studies with concordant results. However, these miRNAs were analyzed in an equal or higher number of additional studies without finding any association with prognosis, which means that none of the analyzed miRNAs were established as a reliable marker of prognosis. It is noteworthy that most studies failed to report the specific treatment regimens, which would be of relevance in order to find prognostic biomarkers since prognosis is dependent on the specific treatment regimen.

Several limitations were faced while performing this systematic review. On the one hand, the studies performed usually considered a limited set of selected miRNAs, which limits the number of comparable results and centers the discussion on those miRNAs that are better known, leaving other miRNAs aside. It is necessary to perform large-scale studies with a wider array of miRNAs using techniques such as next-generation sequencing that allow the identification of new miRNAs. On the other hand, most studies analyzed in this revision relied on tissue-based miRNA detection using qRT-PCR. As a result, it is difficult to know whether the differentially expressed miRNAs directly result from DLBCL or from the cancer-associated microenvironment. Single-cell RNA sequencing methods, developed in recent years, may provide a better approach to achieve this goal in future studies. Further, the included studies present great heterogeneity in sample sources, types of controls used or methodology for expression analysis. This methodological variability could be a source of differences in results among studies. Since the effect of such differences is difficult to determine in the context of a review, it would be of great relevance to reach a consensus and standardize the methodology of study used for future studies in order to facilitate reproducibility and comparisons among studies.

In addition, there is variability in the cut-off value for statistical significance among studies, which we considered to be a potential source of heterogeneity. Finally, there is a tendency to only publish statistically significant results, which leads to bias. All of these limitations in the published literature may be contributing to the lack of consistency in many of the results, which makes it difficult to draw final conclusions about the role of some of the miRNAs analyzed as biomarkers in DLBCL.

4. Materials and Methods

4.1. Systematic Review

4.1.1. Search Strategy

A systematic search with the terms "((‘Non-coding RNA’) OR (‘miRNA’ OR ‘microRNA’ OR ‘miR’) OR (‘exosome’) OR (‘extracellular vesicle’) OR (‘secretome’)) AND (‘Diffuse large B cell lymphoma’

OR 'DLBCL')", following the same strategy used in our previously published review on circulating miRNAs [9] was performed using the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/), including articles published until December 2017.

4.1.2. Inclusion and exclusion criteria

Independent original studies that evaluated the expression of miRNAs in DLBCL tumor tissue as diagnosis, subtype, prediction of treatment response or prognosis biomarkers in human patient populations were included. Exclusion criteria encompassed: articles not including original data (reviews, meta-analyses, letters, and comments), case reports, abstracts, articles not published in English, studies that did not include miRNA data on human populations, and studies on diseases other than DLBCL. After full text revision, articles that included other diseases, analyzed circulating miRNAs, were focused on non-primary DLBCL, did not assess the role of miRNAs in diagnosis, subtype, treatment response, or prognosis, or did not analyze miRNA expression, were excluded. References within the identified studies were reviewed to identify additional matches. Study selection was performed by two researchers independently (AL and BS) and disagreements were resolved by consensus.

4.1.3. Data Extraction

The following information was extracted from each study: publication year, type of tissue sample analyzed, characteristics of the study population, methodology, number of miRNAs studied, and the list of differentially expressed miRNAs provided. Only the miRNAs that were reported as statistically significant in more than two studies with consistent results were selected.

4.2. Data Analysis

4.2.1. Target Genes Selection

In order to predict the putative target genes for the miRNAs identified in the systematic search, miRWalk 2.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) [58] was used. Only those genes predicted by 6 or more of the 12 available prediction algorithms available at miRWalk 2.0 were taken into account.

4.2.2. Pathway Enrichment Analysis

In order to analyze pathways enrichment within the lists of predicted target genes, the over-representation analysis module of the ConsensusPathDB web tool (CPdB) (http://consensuspathdb.org/) was used [59]. Within this tool, KEGG (https://www.genome.jp/kegg/) [60], Reactome (https://reactome.org/PathwayBrowser/) [61] and BioCarta (http://cgap.nci.nih.gov/Pathways/BioCarta_Pathways) databases were interrogated, assuming a conservative *p*-value cutoff of 0.0001.

5. Conclusions

In this systematic review, we have identified that the expression of miR-155-5p and mir-21-5p shows the potential for utility in diagnosis, while mir-155-5p and mir-21-5p could be of use for DLBCL classification. Nevertheless, other associations between miRNA expression and DLBCL phenotypes showed contradictory results. We can conclude that this is a very promising field of study, which could also help to identify novel therapeutic targets and strategies to guide treatment choice. In order to exploit the potential of this field, it would be of particular interest to perform large-scale studies with large sample sizes and a wider array of miRNAs, including unknown miRNAs.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/2/144/s1, Table S1: Differentially expressed microRNAs in DLBCL patients compared with healthy control individuals, Table S2: Differentially expressed microRNAs in DLBCL subtypes, Table S3: Differentially expressed microRNAs as prediction to response to R-CHOP therapy, Table S4: Differentially expressed microRNAs as prognosis biomarkers, Table S5: Pathways significantly enriched among hsa-miR-155-5p and hsa-miR-21-5p (DLBCL diagnosis) target genes predicted by 6 or more databases, Table S6: Pathways significantly enriched among hsa-miR-155-5p, hsa-miR-221-3p (DLBCL subtype) target genes predicted by 6 or more databases.

Author Contributions: E.L.-L. and A.G.-O. contributed to the conception and design of the study. A.L.-E., B.S.-Z. and M.L.-S., acquired and analyzed the data. A.L.-E., B.S.-Z., E.L.-L. and A.G.-O. interpreted the results and drafted the article. E.L.-L and A.G.-O. acquired and administrated the funding. All authors have read, reviewed critically and approved the final manuscript.

Funding: This study was funded by the Basque Government (IT989-16), and Bioef-EiTB maratoia (BIO15/CA/022). The funding source did not have any role in the study design, collection, analysis and interpretation of data, writing of the report, or decision to submit the article for publication.

Conflicts of Interest: The authors declare no conflict of interest.

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Potential of microRNAs in the diagnosis, classification and prognosis of Diffuse Large B Cell Lymphoma though miRNA-Sequencing.

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Abstract

Diffuse Large B Cell Lymphoma (DLBCL) is the most common lymphoid malignancy in adults. Despite being considered a single disease, DLBCL presents with variable backgrounds in terms of morphology, genetics, and biological behavior, which results in heterogeneous outcomes among patients. Although new tools have been developed for the classification and management of patients, 40% of them still have primary refractory disease or relapse. In addition, multiple factors regarding the pathogenesis of this disease remain unclear and identification of novel biomarkers is needed. In this context, recent investigations point to microRNAs as useful biomarkers in cancer as well as important players in the development of the disease. However, regarding DLBCL, up to date, there is inconsistency in the data reported. Therefore, in this work, the main goals were to determine a microRNA set with utility as biomarkers for DLBCL diagnosis, classification, prognosis and treatment response, as well as to decipher the mechanism of action of deregulated microRNAs in the origin of the disease. To achieve these goals, we analyzed microRNA expression in a cohort of 78 DLBCL patients and 17 controls using small RNA sequencing, and performed a microRNA-mRNA interaction network

analysis. This way, we were able to define new microRNA expression signatures for diagnosis, classification, treatment response and prognosis, and we identified plausible mechanisms of action by which deregulated microRNAs could be involved in DLBCL pathogenesis. In summary, our study remarks that microRNAs could play an important role as biomarkers in diagnosis, classification, treatment response and prognosis in DLBCL, as well as in the pathogenesis of the disease.

Introduction

Diffuse Large B Cell Lymphoma (DLBCL) is an aggressive type of non-Hodgkin lymphoma that represents the most common lymphoid malignancy in adults (1). Despite the great heterogeneity observed among patients with DLBCL in terms of morphology, genetics, and biological behavior, most of them are treated with standard chemotherapy regimens, which allow complete remission in 75-80% of patients (2,3). Nevertheless, the remaining patients will be refractory to first-line chemotherapy, and 30–40% will relapse after obtaining a complete remission (4). Remarkably, it would be of great relevance to identify these patients from the beginning for a better assignment of therapy because the outcome of DLBCL patients after failure to first-line treatment is very poor. Although different prognostic predictors exist, such as the International Prognostic Index (IPI), they fail to predict prognosis in a considerable proportion of patients (5). Therefore, the identification of new biomarkers for a better management of DLBCL patients is an urgent priority.

In this context, microRNAs (miRNAs), as post-transcriptional regulators of gene expression and biological functions, can also influence drug resistance and have shown potential as diagnostic, classification and prognostic predictors in cancer. For instance, miR-150-5p was downregulated in DLBCL in four studies (6–9), while showed upregulated expression in another study (10) and

unchanged in another one (11). Similarly, miR-222-3p was associated with good prognosis in four studies (12–15) while no association was observed in other four studies (8,11,16,17). For that reason, in a recently published systematic review (18) we sought to identify a signature of miRNAs of relevance in DLBCL. However, we observed that the studies performed usually considered a limited set of selected microRNAs, which limits the number of comparable results and centers the discussion on those microRNAs that are better known, leaving other microRNAs aside. Therefore, we considered that it was necessary to perform large-scale studies with a wider array of microRNAs using techniques such as next-generation sequencing that allow a deeper and unbiased identification of microRNAs with the potential to be used as biomarkers in DLBCL.

Consequently, the main goals of the present study were to determine a microRNA set with utility in DLBCL diagnosis, classification, prognosis and treatment response through the analysis of all the existing microRNAs in DLBCL using small RNA sequencing, as well as to decipher the mechanism of action of deregulated microRNAs in the origin of the disease.

Materials and Methods

Population of study

Patient samples were obtained at diagnosis from formalin-fixed paraffin-embedded (FFPE) samples from a total of 78 DLBCL patients (50 patients with long-term complete remission, 12 patients with refractory disease, and 16 patients that relapsed within 10 years after diagnosis). Samples were collected from 1999 to 2018 at the Hematology Units of 3 Spanish reference hospitals (Cruces University Hospital, Donostia University Hospital, and Araba University Hospital). Control samples were obtained from non-tumoral ganglia from individuals without DLBCL, which were collected at Araba University Hospital. Demographic and clinical data were collected from patient's medical files by two independent clinical researchers. For each patient the collected data included: age, sex, clinical stage (I-IV), B symptoms (yes/no), IPI score (0-5), subtype (GCB or non-GCB), response to treatment (Complete response (CR), not complete response (not CR)), LDH (normal or high), β-2 microglobulin (normal or high), outcome, and 5-year progression free survival (PFS) and overall survival (OS) (Table 1). Demographic information was also obtained for the controls. The study was approved by the Clinical Research Ethical Committee of the Basque Country (P2016121). Signed informed consent was obtained from each participant and the study was carried out according to the Declaration of Helsinki.

Sample processing, Small RNA-seq library preparation and sequencing

RNA was isolated from FFPE samples with the miRNeasy FFPE Kit (Qiagen, Hilden, Germany). One µg of total RNA was used for library preparation with TruSeq small RNA Sample Prep Kit (Illumina), according to the manufacturer's protocol. Libraries were analyzed using Agilent DNA High Sensitivity chip and sequenced Single Read, 50nts (v4) on Illumina's HiSeq 2500 with a depth of approximately 10 million reads per sample. All the process was carried out at the Centre for Genomic Regulation (CRG).

Bioinformatic analysis and differential miRNA expression

Reads were trimmed for the presence of the small RNA adapter using Skewer and filtered removing every sequence shorter than 15 and longer than 40 bases. The remaining ones were then aligned to the reference genome (GRCh38) using ShortStack and the resulting alignments used by htseq-count for determining the number of tags per gene. We used the annotation v27 from Gencode consortium. Finally, the count matrix was reduced to only miRNA genes. Every small RNA with less than 10 reads considering the sum of the reads in every condition was excluded, and we used the remaining genes for differential expression analysis with DESeq2. P-values were adjusted using False discovery rate (p-adj). Differentially expressed miRNAs were

those with p-adj <0.05 and a $\log_2$ fold change (logFC) > 2 or <-2. Principal component analysis was performed using the prcomp package from R to analyze the transformed read counts of all miRNAs.

Survival analysis

Survival analyses were conducted to estimate the effect on the progression-free survival (PFS) and overall survival (OS) of those miRNAs differentially expressed between samples at diagnosis from patients with long-term remission and those that relapsed. The median value of the expression of those differentially expressed miRNAs was considered to categorize patients into low or high expression groups. Kaplan–Meier analysis was used to define the survival curves and the log-rank test was used to assess significance. For the multivariate analysis, we also considered the subtype and IPI status using the Cox proportional hazards (Cox PH) method. All calculations were performed using the Survival R package. Significant associations with survival were those with p-value <0.05.

miRNA-mRNA interaction network

Using our own data on miRNA expression (DLBCL patients vs controls) and mRNA expression data contained in the Gene Expression Omnibus (GEO) database (19), we constructed a miRNA-mRNA interaction network to elucidate the mechanism of DLBCL development. First, for those miRNA deregulated at diagnosis (DLBCL patients vs controls), target genes were identified. MirTarbase (20) (http://mirtarbase.cuhk.edu.cn/php/index.php) was used to search for experimentally validated miRNA-mRNA target interactions. For the current study, only strong miRNA-mRNA interactions were selected. Secondly, for a comprehensive search of databases in GEO, we used "DLBCL and expression profiling by array" terms to identify relevant research publications with an unlimited starting publication date until September 2020. The only microarray dataset (GSE56315) containing gene expression information of both DLBCL samples (n=55) and healthy controls (n=33) was downloaded from the GEO (http://www.ncbi.nlm.nih.gov/geo/) (21,22). Differentially expressed genes (DEG) were identified using GEO2R tool (https://www.ncbi.nlm.nih.gov/geo/) with default parameters. We established the following inclusion criteria for the DEGs: upregulated genes must have a logFC≥2 and p-adj<0.05, while downregulated genes must have a logFC≤–2 and p-adj<0.05. Finally, experimentally validated targets of differentially deregulated miRNAs were overlapped with mRNAs differentially expressed obtained from GEO databases as represented in Figure 1. The intersection of the upregulated and downregulated genes was mapped using the Venn package (http://bioinformatics.psb.ugent.be/webtools/Venn/). Gene Ontology (GO) functional

annotations, Protein ANalisis THroug Evolutionary Relationships (PANTHER) Classification System, and signaling pathway enrichment analysis (Consensus Pathway database http://cpdb.molgen.mpg.de/) were conducted with overlapping genes.

Results

MiRNAs deregulated in DLBCL

A total number of 1584 microRNAs were identified through the miRNA sequencing analysis. A principal component analysis (PCA) was performed including the expression of all the miRNAs to obtain an overview of the grouping of samples according to their variance. The samples seem to cluster with little difference between complete response, refractory and relapsed groups, whereas the control group samples cluster as a clearly separate group (Supplementary Figure 1). To obtain a comprehensive list of miRNAs deregulated in DLBCL, we compared the expression of each miRNA in 78 DLBCL samples at diagnosis with 17 non-tumoral ganglia from individuals without DLBCL. We noted that 146 miRNAs exhibited statistically significant differences in expression between samples of DLBCL patients at diagnosis and control samples. Of these miRNAs, 122 exhibited increased abundance in DLBCL, while 24 miRNAs exhibited decreased abundance. The 20 most discriminatory up- and down-regulated miRNAs are listed in Table 2.

MiRNAs differentially expressed among DLBCL subtypes

By analyzing expression profiling of 32 GCB and 21 Non-GCB DLBCL samples at diagnosis, eight miRNAs were found to be differentially expressed. Five of them were upregulated in GCB DLBCL patients (miR-129-2-3p, miR-4464, miR-3150b-3p, miR-138-5p and miR-129-5p) and three miRNAs were upregulated in Non-GCB subtype (miR-511-5p, miR-205-5p, miR-3652) (Table 3).

MiRNAs associated with DLBCL treatment response

Eleven miRNAs were differentially expressed between samples at diagnosis from patients with long-term complete remission (n=50) and refractory patients (n=12), of which, ten miRNAs were upregulated in patients with complete remission (miR-12136, miR-129-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129-5p and miR-3928-3p) and one miRNA was downregulated in the same group of patients (miR-192-5p) (Table 4).

MiRNAs as prognostic biomarkers in DLBCL and their impact on survival

MiRNA expression at diagnosis of 50 patients with long term remission and 16 patients that relapsed within 10 years after diagnosis was compared. The results revealed that seven miRNAs (miR-4444, miR-449c-5p, miR-3681-5p, miR-3928-3p, miR-449b-5p, miR-370-3p, and miR-4424)

were significantly upregulated in patients with long term remission and three miRNAs (miR-133a-3p, miR-208b-3p, and miR-205-5p) were significantly upregulated in patients that relapsed (Table 5).

Patient survival curves were generated to represent 5-year PFS and OS, dividing the patients into high-expression and low-expression groups according to the mean expression of each of the ten miRNAs significantly associated with prognosis. The long-rank test revealed that, among the miRNAs associated with good prognosis, high expression of miR-4444 was associated with better 5-year OS and PFS (OS p-value=0.0169, and PFS p-value=0.0162) (Figure 2). Additionally, considering the miRNAs associated with bad prognosis, high expression of miR-205-5p was associated with poorer 5-year OS (p=0.0444) (Figure 2). We also performed Cox PH multivariate analysis including two established indicators of DLBCL patient outcome (COO-Subtype and IPI) as covariates. The results of this analysis revealed that miR-205-5p was associated with worse 5-year OS (p=0.02) and PFS (p=0.02) independently of IPI and subtype.

miRNA-mRNA interaction network analysis

For the 122 upregulated miRNAs found in our study, we identified a total of 482 experimentally validated targets using MirTarbase. Regarding the 24 downregulated miRNAs from our study, a total of 375 experimentally validated target genes were identified.

The GSE56315 expression profile dataset identified through the search in the GEO database, contains gene expression information of 55 DLBCL samples and 33 human healthy samples analyzed with the Affymetrix Human Genome U133 Plus 2.0 Array. Using these data, a total of 1918 significantly upregulated genes and 4545 downregulated genes were found in DLBCL patients compared with healthy control individuals.

Integration of both datasets showed that downregulated miRNAs targeted a total of 138 genes among those highly expressed in DLBCL (Supplementary Table 1) and upregulated miRNAs a total of 76 targets among those genes downregulated in DLBCL (Supplementary Table 2). Some miRNAs showed interactions with several targets (Supplementary Table 3). For instance, miR-9-5p was shown to target 25 genes downregulated in DLBCL, and miR-146a-5p and miR-182-5p were shown to target 24 and 20 genes downregulated in DLBCL, respectively. Some target genes showed interactions with several miRNAs (Supplementary Table 4). For instance, *FOXO1*, *BCL2*, *GS3KB* and *PTEN*, downregulated in DLBCL, are targeted by 7, 5 ,5 and 4 miRNAs deregulated in DLBCL, respectively. In order to evaluate the pathways that could be affected by the deregulated miRNAs through their target genes, we performed a pathway enrichment analysis using the ConsensusPathDB web tool. Among the most significantly over-represented pathways, we

identified some involved in cancer, such as FoxO signaling pathway, signaling by Receptor Tyrosine Kinases, and PI3K-Akt signaling pathway (Figure 3).

Discussion

In this study, we identified new signatures of miRNAs of relevance in DLBCL with potential to improve diagnosis, subtype characterization and treatment response through small RNA sequencing. To our knowledge, few reports exist in which miRNA sequencing were used to identify miRNA signatures in cancer, and only one which analyzed miRNAs with NGS in DLBCL (11). Notably, deregulation of most of these miRNAs had not been previously reported in DLBCL.

Regarding miRNAs deregulated at diagnosis in DLBCL, a total of 146 miRNAs differentially expressed between DLBCL samples and controls were identified. Of note, all miRNAs we previously proposed as a consistent signature of deregulation in DLBCL in a systematic review (miR-150-5p, miR-146a-5p, miR-155-5p and miR-21-5p) (18) were identified in the current study following a similar expression pattern. Moreover, we also identified other miRNAs highly deregulated in DLBCL, miR-210-3p being the most significantly upregulated, and miR-215-5p the most significantly downregulated. Overexpression of miR-210-3p had been previously reported in DLBCL patients in comparison with normal B-cells in two studies (7,8), although no statistically significant association was found in other two studies (11,23). Of nothe, miR-210 is also overexpressed in other lymphomas and a wide array of solid tumors and could play an important role acting as a key regulator of cell cycle (24–28). Therefore, the evidence accumulated to date by other authors and the present study indicates that miR-210-3p could also act as an oncogene in DLBCL. On the other hand, miR-215 was the most significantly downregulated miRNA in our population, in line with the only previous study that analyzed this miRNA in DLBCL (29). Remarkably, miR-215 is one of the most extensively studied microRNAs in different types of cancer and there are accumulating reports that it may function as either tumor suppressor or oncogene depending on the tumor type (30–36).

Comparing miRNA expression in DLBCL GCB and Non-GCB subgroups, a total of eight miRNAs were identified with subtype classification potential. Five of them were upregulated in GCB DLBCL patients (miR-129-2-3p, miR-4464, miR-3150b-3p, miR-138-5p and miR-129-5p) and three miRNAs were upregulated in Non-GCB subtype (miR-511-5p, miR-205-5p, and miR-3652). Of note, miR-28-5p, which was proposed as a biomarker of GCB DLBCL in our previous systematic review pending further confirmation (18), was also differentially expressed among subtypes, although the logFC did not reach the threshold set for the present study. Interestingly, higher

levels of miR-129-5p and miR-138-5p in the GCB subtype had been previously reported (8,11,16,37). As part of the miRNA-mRNA interaction network analysis, we observed that they could target transcripts that are known to be deregulated in the formation of germinal center lymphomas (38), including genes related with the cell cycle (*CDKN1A*), MAPK and NFkB signaling *(MAPK1).* Interestingly, we also found *TP53* as a target gene of miR-129-3p/miR-129-5p, and miR-138-5p. *TP53* gene is a crucial tumor suppressor that mediates cell-cycle arrest, DNA repair, apoptosis, senescence, and autophagy (39,40). *TP53* dysfunction is implicated in lymphomagenesis and disease progression. While the prognostic significance of *TP53* mutations has been inconsistent in several cancers, recent studies have showed that the *TP53* mutations do stratify GCB-DLBCL, but not Non-GCB-DLBCL, into distinct subsets with a different OS (41), which could suggest that GCB-DLBCL is *TP53*-dependent and Non-GCB DLBCL is *TP53*-independent, and thus, could support the role of miR-129-3p/miR-129-5p, and miR-138-5p in GBC subtype.

We also identified a predictive miRNA signature for therapy response, which included the upregulation of ten miRNAs (miR-12136, miR-129a-5p, miR-129-1-3p, miR-3150b-3p, miR-127-3p, miR-3681-5p, miR-370-3p, miR-4464, miR-129b-5p and miR-3928-3p) in association with good response to R-CHOP DLBCL treatment, and the upregulation of miR-192-5p associated with chemoresistance to R-CHOP therapy. Notably, deregulation profiling of most of these miRNAs in DLBCL has not been previously reported; but some of them have been studied in the context of other types of cancer. MiR-12136 was the most significantly upregulated miRNA in patients with complete response and exhibited greater than a 25-fold increase in expression in comparison with those that relapse. Although miR-12136 has been recently discovered and, therefore, the knowledge about this miRNA is very limited, our result points to this miRNA as an interesting candidate for additional studies. On the other hand, previous studies had linked miR-192 and chemoresistance in lung cancer (42) and in larynx and hypopharynx (43). Hence, those previous results further support a role of this microRNA in chemoresistance in DLBCL. In addition to being putative biomarkers for R-CHOP treatment response prediction, these miRNAs could also be considered in the future as novel therapeutic targets for personalized miRNA-based therapy, which could be an alternative to be used as adjuvant therapy, although further studies are needed in this area of research before such therapies can be translated to the clinic.

In addition to treatment response, the ability to accurately predict survival may be crucial for initial treatment planning in patients with DLBCL. We identified seven miRNAs (miR-4444, miR-449c-5p, miR-3681-5p, miR-3928-3p, miR-449b-5p, miR-370-3p, miR-4424) significantly upregulated in patients with long term remission and four miRNAs (miR-133a-3p, miR-133a-3p,

miR-208b-3p, miR-205-5p) upregulated in relapsed patients. In addition, the survival analysis reveals that high expression of miR-4444 is significantly associated with better OS and PFS. Until now, the expression pattern of miR-4444 in DLBCL and its prognostic significance have not been investigated systematically, but it was previously reported as part of a model based on six miRNAs associated with a poor prognosis in colon adenocarcinoma (44). One possible explanation of these contradictory result is that miR-4444 could participate in several pathways, playing different roles depending on the cell type, the tumor pattern of gene expression and the type of therapy, which makes the results non comparable. Further studies are warranted to understand its role in DLBCL. Interestingly, miR-205 was also significantly associated with worse OS in our group of DLBCL patients uniformly treated with immunochemotherapy. Multivariate analysis revealed that miR-205-5p was associated with worse OS and PFS independently of IPI and subtype. Therefore, the combination of miRNA expression patterns with IPI status and DLBCL subtype could improve the stratification of patients with different prognosis, leading to a better personalized treatment.

To gain insight into the global mechanism of action of deregulated miRNAs, we also performed a miRNA-mRNA interaction network analysis. Integration analysis determined that up to 214 genes could be affected in DLBCL because of miRNA deregulation, as reflected by their expression pattern. In this line, there were a total of 138 genes with low expression in DLBCL, and this downregulation could be due, at least in part, to the increased expression of miRNAs identified in the current study. Moreover, 76 genes were highly expressed, and the decreased expression of some miRNAs here identified could contribute to their increased expression. Among the most interesting downregulated genes targeted by more than one miRNA deregulated in DLBCL, we identified *FOXO1* and *PTEN,* involved in PI3K-Akt pathway (45), predicted to be targeted by 5 and 4 deregulated miRNAs, respectively. *FOXO1* is a tumor suppressor and its downregulation leads to promoting tumorigenesis by favoring resistance to stress, proliferation, and increased cellular survival (46). Abnormal cells that would normally undergo apoptosis may instead survive in the absence of *FOXO1,* which results in tumor expansion (47). In fact, downregulation of *FOXO1* has been previously reported in DLBCLs (48). In the current study, miR-182-5p, miR-183-5p, miR-135a-5p, miR-9-5p, and miR-9-3p, were identified to directly target *FOXO1*. In the same line, *PTEN* is a tumor suppressor that acts upstream *FOXO1* negatively regulating *AKT.* Without PTEN regulation, activated AKT phosphorylates FOXO1, which is subsequently exported from the nucleus into the cytoplasm and degraded by the proteasome (49). Interestingly, *PTEN* is the major negative regulator of the PI3K/AKT signaling pathway, which is constitutively activated in 25-50% of DLBCL according to

recent studies (50). *PTEN* loss has been associated with poorer prognosis in many solid tumors, including DLBCL, and is significantly related to advanced disease, chemotherapy resistance, and poor survival (51–54). miR-155, miR-320a, miR-205 and miR-182-5p were shown to target *PTEN* in our study. Interestingly, miR-182-5p targeted both genes and could be a crucial miRNA modulating the PI3K/AKT pathway by targeting *PTEN,* upstream, and *FOXO1,* downstream. Further supporting our findings, a relation between miR-182 and miR-183 and *FOXO1* suppression have been previously described in classical Hodking lymphoma (55).

Regarding the upregulated genes in DLBCL which could be a consequence of miRNA downregulation, the most significant result is *BCL2*. Evidence revealed that elevated expression of anti-apoptotic members such as *Bcl-2* is one of the major contributing factors to B cell lymphomagenesis (56). Since then, many studies have determined that *BCL2* is one of the most important oncogenes in cancer and that it is linked with lymphoma development, particularly when c-MYC is overexpressed (57). This gene should be negatively regulated by miR-135a-5p, miR-234-5p, miR-139-5p, miR-451a, and miR-497-5p, but the low of expression of the mentioned miRNAs observed in DLBCL could contribute to BCL2 overexpression. In fact, previous studies have confirmed that deletion or downregulation or miRNAs, such as miR-15 and miR-16, are involved in the overexpression of *BCL2* (58). All the above mentioned miRNAs, except for miR-234-5p, were among the top 20 downregulated miRNAs in DLBCL in our population, so it would be interesting to study these associations in other populations.

Finally, this study has some limitations that need to be addressed. First, we analyzed miRNA expression from FFPE blocks, in which RNA quality is usually compromised. However, compared to mRNA, miRNAs are relatively resistant to RNase degradation (59), and previous studies demonstrated that miRNA expression from FFPE samples is in good correlation with fresh frozen samples (60). Secondly, patients were diagnosed in three different hospitals with different follow up routines, which could be a source of heterogeneity, although the treatment protocols were similar. Finally, we also have to mention that in this study the Han's IHC algorithm was used to classify the patients into GCB and Non-GCB subtypes. Even though studies have shown clearly that the IHC-based classification provided very similar outcome prediction compared with the GEP-based classification, which is the gold standard (61), variable reproducibility of IHC stains and interpretations, may be the reason for inconsistent results (62).

In conclusion, in the present study, a comprehensive analysis of miRNA profiles next-generation sequencing technology allowed us to identify signatures that could be of relevance in DLBCL. Prospective studies in larger cohorts of DLBCL samples are warranted to further validate our findings and explore new therapeutic options based on the miRNA profile of each patient.

Moreover, we explored the mechanisms by which deregulated miRNAs contributes to DLBCL pathogenesis, with miR-182-5p playing a key role in PI3K/AKT pathway.

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Table 1: Demographic and clinical characteristics of DLBCL patients at diagnosis (n=78)

Variables	Patients with DLBCL (n=78)	Controls (n=17)
Age		
Mean (range)	58.66 (21-81)	67.76 (31-86)
≥60	43	4
<60	34	13
NA	1	0
Sex		
Male	41	9
Female	37	8
Stage		
I	7	
II	11	
III	23	
IV	27	
NA*	10	
B symptoms		
Yes	31	
No	31	
NA*	18	
IPI scores		
Low risk (0-1)	15	
Low to intermediate risk (2)	13	
Intermediate to high risk (3)	15	
High risk (4-5)	20	
NA*	15	
Subtype IHC or molecular		
GCB	32	
Non-GCB	21	
NA*	25	
Therapy response		
CR	50	
not CR	28	
LDH		
Normal	23	
Augmented	40	
NA*	17	
β2-MG, β 2 microglobulin		
Normal	22	
Augmented	39	
NA*	17	
5 years PFS		
Median, months	39.68	
5 years OS		
Median (range), months	46.63	

Abbreviations: CR, complete response; DLBCL, diffuse large B-cell lymphoma; GCB, germinal center B cell; IHC, immunohistochemistry; IPI, International Prognostic Index; β2-MG, β 2 microglobulin; LDH, lactate dehydrogenase; PFS, progression free survival; OS, Overall survival; NA, not available; *Several variables contain missing values due to the lack of information in patient´s medical files

Table 2: The 15 most upregulated and downregulated microRNAs in DLBCL patients vs. controls.

MicroRNA	Base Mean	log2FoldChange	p-value	padj
hsa-miR-210-3p	842.9	3.51	2.33×10^{-29}	1.45×10^{-26}
hsa-miR-944	60.9	4.10	2.19×10^{-23}	6.80×10^{-21}
hsa-miR-12136	76.05	26.94	3.64×10^{-20}	6.47×10^{-18}
hsa-miR-3681-5p	75.3	5.16	3.33×10^{-20}	6.47×10^{-18}
hsa-miR-378i	24.9	3.01	3.01×10^{-17}	4.16×10^{-15}
hsa-miR-4454	183.4	2.35	1.01×10^{-16}	1.04×10^{-14}
hsa-miR-1291	354.7	4.02	1.84×10^{-16}	1.76×10^{-14}
hsa-miR-7974	111.8	3.46	1.87×10^{-15}	1.45×10^{-13}
hsa-miR-183-5p	891.1	3.40	5.77×10^{-15}	3.59×10^{-13}
hsa-miR-146a-5p	33085.5	2.11	2.05×10^{-14}	1.16×10^{-12}
hsa-miR-2467-5p	25.56	2.33	7.35×10^{-14}	3.81×10^{-12}
hsa-miR-4420	8.09	4.63	2.02×10^{-13}	1.01×10^{-11}
hsa-miR-1248	202.5	2.69	1.03×10^{-12}	4.73×10^{-11}
hsa-miR-18a-3p	54.06	2.05	1.21×10^{-12}	5.36×10^{-11}
hsa-miR-129-5p	25.1	4.62	3.03×10^{-12}	1.22×10^{-10}
hsa-miR-215-5p	53.8	-4.42	6.74×10^{-35}	8.39×10^{-32}
hsa-miR-150-5p	6310.7	-3.22	3.46×10^{-25}	1.44×10^{-22}
hsa-miR-224-5p	47.7	-3.30	5.11×10^{-21}	1.27×10^{-18}
hsa-miR-194-5p	37.8	-4.33	6.19×10^{-17}	7.00×10^{-15}
hsa-miR-452-3p	7.2	-2.33	2.10×10^{-16}	1.87×10^{-14}
hsa-miR-335-5p	127.6	-2.74	6.20×10^{-16}	5.14×10^{-14}
hsa-miR-145-5p	2060.5	-2.16	2.65×10^{-15}	1.94×10^{-13}
hsa-miR-139-5p	48.8	-2.26	5.40×10^{-15}	3.57×10^{-13}
hsa-miR-497-5p	332.5	-2.07	3.53×10^{-14}	1.91×10^{-12}
hsa-miR-10a-3p	10.2	-2.29	7.45×10^{-12}	2.73×10^{-10}
hsa-miR-95-3p	18.1	-2.13	3.79×10^{-11}	1.22×10^{-09}
hsa-miR-151b	24.8	-2.21	6.67×10^{-10}	1.60×10^{-08}
hsa-miR-551b-3p	7.9	-2.25	2.97×10^{-09}	5.78×10^{-08}
hsa-miR-194-5p	234.5	-2.52	4.25×10^{-09}	8.01×10^{-08}
hsa-miR-135a-5p	4.3	-3.49	1.92×10^{-08}	3.07×10^{-07}

Positive Log2 Fold change represented the upregulation of microRNAs in DLBCL patients compared with healthy control individuals; Negative Log2 Fold change represented the downregulation of microRNAs in DLBCL patients compared with healthy control individuals; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Table 3. MicroRNAs differentially expressed between GCB and Non-GCB DLBCL subtypes

MicroRNA	Base Mean	log2FoldChange	p-value	padj
hsa-miR-129-2-3p	4.9	4.12	$1.50x10^{-06}$	0.00050
hsa-miR-4464	2.9	3.91	$1.21x10^{-05}$	0.00151
hsa-miR-3150b-3p	49.0	2.00	$1.90x10^{-05}$	0.00211
hsa-miR-138-5p	568.1	2.00	$9.92x10^{-05}$	0.00661
hsa-miR-129-5p	18.2	2.90	0.00014	0.00891
hsa-miR-511-5p	7.6	-2.01	$9.92x10^{-06}$	0.00142
hsa-miR-205-5p	20.3	-3.30	$4.10x10^{-05}$	0.00342
hsa-miR-3652	8.9	-2.44	0.00088	0.03137

Positive Log2 Fold change represented the upregulation of microRNAs in GCB DLBCL patients compared with Non-GCB individuals; and negative, upregulation in Non-GCB DLBCL; Negative Log2 Fold change represented the upregulation of microRNAs in Non-GCB DLBCL patients compared with GCB individuals; and negative, upregulation in Non-GCB DLBCL; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Table 4. MicroRNAs significantly associated with good or poor response to treatment.

MicroRNA	Base Mean	log2FoldChange	pvalue	padj
hsa-miR-12136	120.0	25.73	$1.29x10^{-13}$	$9.70x10^{-11}$
hsa-miR-129a-5p	42.7	5.09	$2.86x10^{-09}$	$1.08x10^{-06}$
hsa-miR-129-1-3p	7.4	4.08	$1.86x10^{-06}$	0.00035
hsa-miR-3150b-3p	50.7	2.33	$1.60x10^{-05}$	0.00241
hsa-miR-127-3p	3977.2	2.01	$6.14x10^{-05}$	0.00661
hsa-miR-3681-5p	75.3	2.34	0.00016	0.01507
hsa-miR-370-3p	16.1	2.59	0.00041	0.02380
hsa-miR-4464	3.3	3.55	0.00117	0.04641
hsa-miR-129b-5p	27.5	2.88	0.00102	0.04641
hsa-miR-3928-3p	16.5	2.03	0.00113	0.04641
hsa-miR-192-5p	10375.5	-2.41	$1.60x10-^{07}$	$4.01x10^{-05}$

Positive Log2 Fold change represented the overexpression of microRNAs at diagnosis in patients with complete remission compared with refractory patients; and negative, overexpression in refractory patients; Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

Table 5. MicroRNAs significantly associated with prognosis.

MicroRNA	Base Mean	log2FoldChange	pvalue	padj
hsa-miR-4444	10.3	2.25	$6.20x10^{-05}$	0.0089
hsa-miR-449c-5p	9.8	2.22	$4.75x10^{-05}$	0.0089
hsa-miR-3681-5p	75.3	2.03	0.00021	0.014
hsa-miR-3928-3p	16.5	2.02	0.00018	0.014
hsa-miR-449b-5p	7.23	2.22	0.00036	0.021
hsa-miR-370-3p	16.1	2.12	0.00071	0.029
hsa-miR-4424	110.8	2.32	0.0010	0.041
hsa-miR-133a-2-3p	470.9	-3.8	$1.53x10^{-06}$	0.001
hsa-miR-133a-1-3p	69.29	-2.52	$6.78x10^{-05}$	0.0089
hsa-miR-208b-3p	12.94	-3.08	0.00014	0.013
hsa-miR-205-5p	62.4	-3.36	0.0002	0.014

Positive Log2 Fold change represented the overexpression of microRNAs in patients with long term remission compared with relapsed patients; and negative, overexpression in relapsed patients. Base Mean: represented the average of the normalized count values, dividing by size factors, taken over all samples.

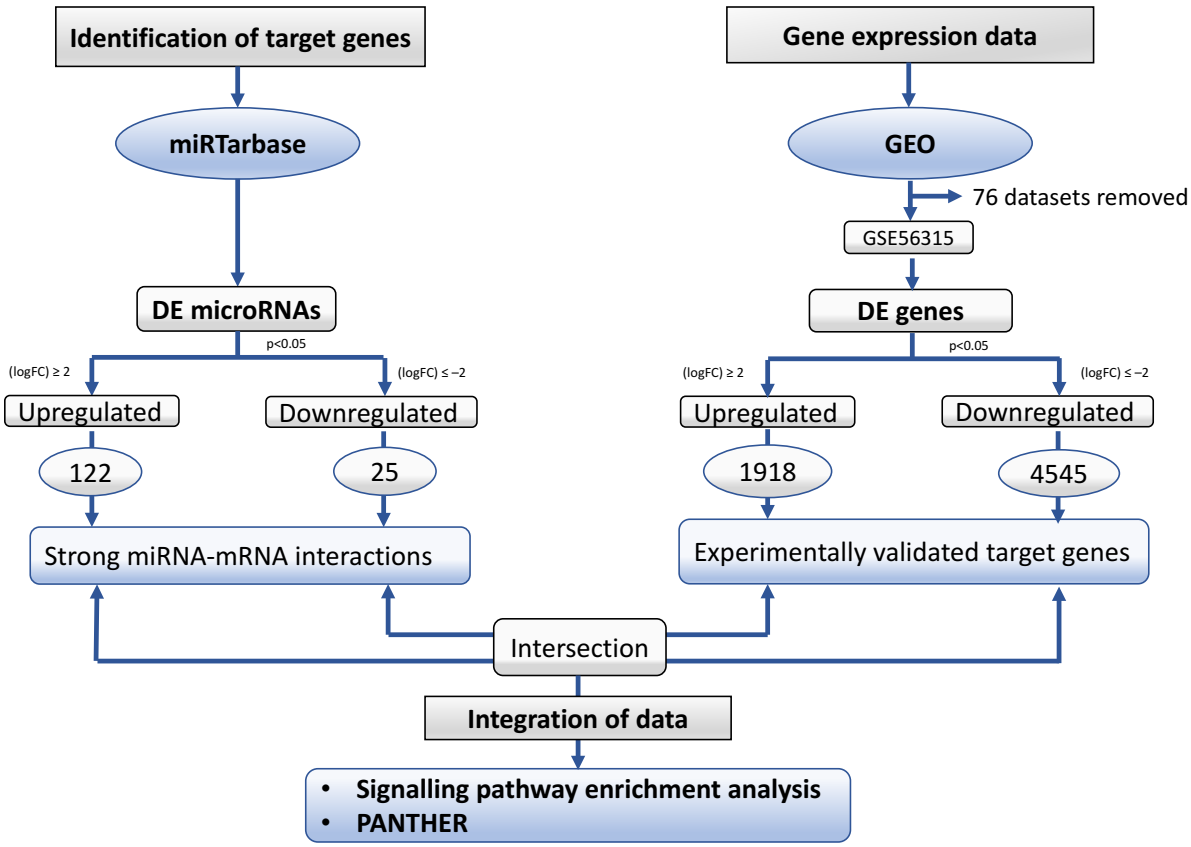

Figure 1: Workflow of microRNA-mRNA network construction

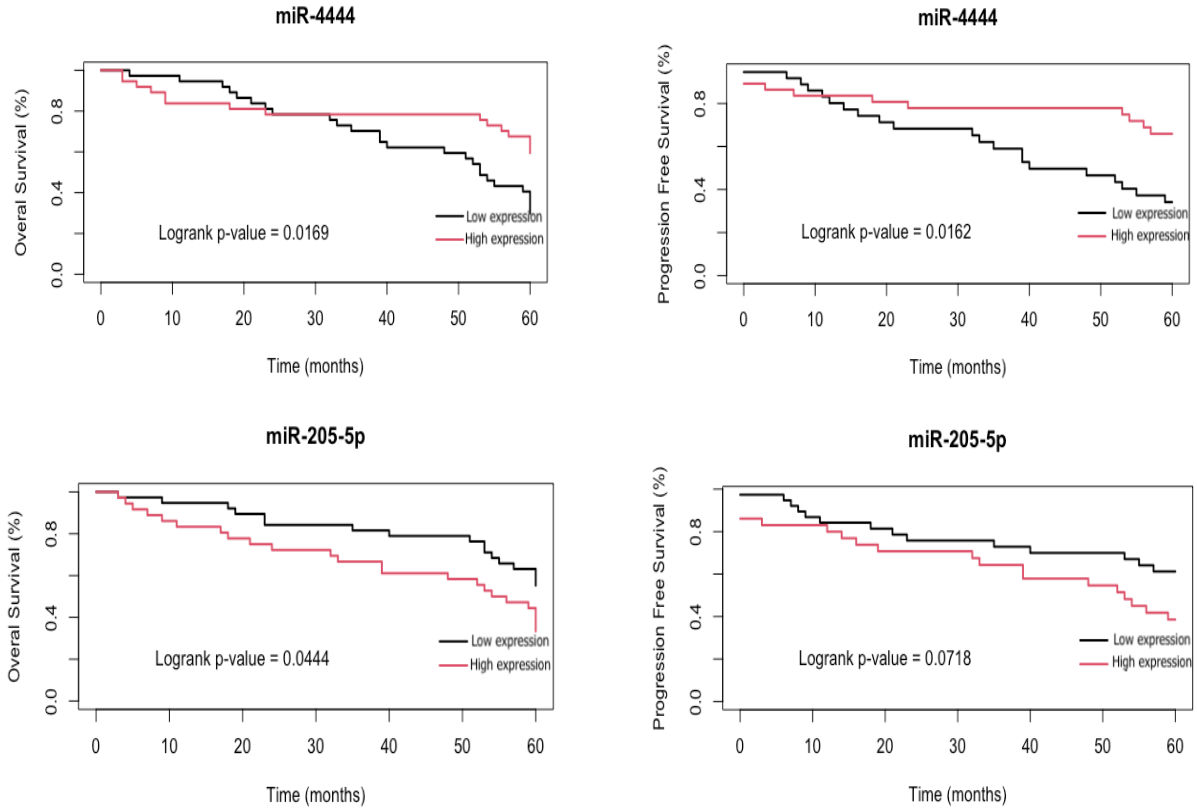

Figure 2: Survival analysis of the microRNAs significantly associated with prognosis.

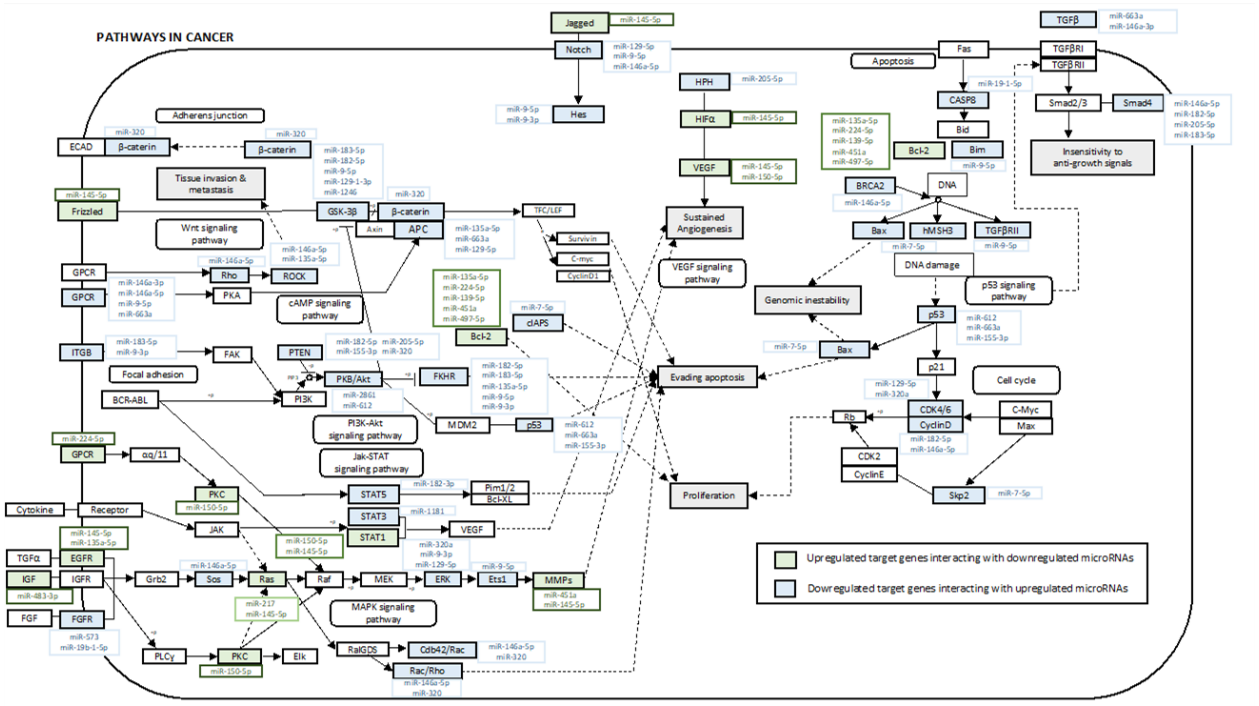

Figure 3: Genes of Pathway in cancer targeted by downregulated and upregulated microRNAs (adapted from KEGG database)