Polymorphisms in miRNA processing genes and their role in osteosarcoma risk

Running title: miRNA-related SNPs and osteosarcoma risk

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Abstract

Background: The possible associations between genetic variants and osteosarcoma risk have been analyzed without conclusive results. Those studies were focused mainly on genes of biologically plausible pathways. However, recently, another pathway has acquired relevance in cellular transformation and tumorigenesis, microRNA (miRNA) processing pathway. Dysregulation of the expression levels in genes of this pathway has been described in cancer. Consequently, single nucleotide polymorphisms (SNPs) that change genes that codify proteins involved in miRNA processing pathway may affect miRNA, and therefore their target genes, that might be associated with cancer development and progression. The aim of this study was to evaluate whether SNPs in miRNA processing genes, confer predisposition to osteosarcoma.

Procedure: We analyzed 72 SNPs in 21 miRNA processing genes in a total of 99 osteosarcoma patients and 387 controls.

Results: A total of three SNPs were associated with osteosarcoma susceptibility. Interestingly, these three SNPs were located in three miRNA processing genes (*CNOT1*, *CNOT4* and *SND1*) part of the RISC complex. Among them, the association of rs11866002 in *CNOT1* remained significant (p=0.08) after Bonferroni correction.

Conclusions: For the first time, this study indicates that SNPs in RISC complex genes, especially rs11866002 in *CNOT1* may represent novel markers of osteosarcoma susceptibility.

Introduction

Osteosarcoma is the most common primary malignant bone tumor, mainly occurring in children and adolescents (1). Several studies have provided evidence of an inherited genetic risk for osteosarcoma. Most of these studies focused on biologically plausible pathways such as cyclic AMP signaling cascade (GRM4), growth related genes (VDR, IGF2R), or DNA repair (TP53, MDM2, etc) (2-4).

Recently, another pathway that has acquired relevance in cellular transformation and tumorigeneisis is microRNA (miRNA) processing pathway (Kumar et al. 2007, Zhang et al., 2013). In this pathway, primary double-stranded miRNA transcripts (pri-miRNA) are processed in the nucleus by DROSHA RNase and the double-stranded RNA binding protein, DGCR8. The resulting precursor miRNA molecule of 70-100 nucleotides (pre-miRNA) is then translocated into the cytoplasm by RAN GTPase and XPO5. In the cytoplasm, the pre-miRNA terminal loop is cleaved by DICER in collaboration with TARBP2, yielding ~22-nt RNA duplexes. One strand of the duplex is preferentially incorporated into the RNA-induced silencing complex (RISC), that includes EIF2C1, EIF2C2, SND1, GEMIN3, GEMIN4 and CCR4-NOT complex (3). In the RISC, miRNAs target mRNAs for translational repression, deadenylation, or degradation (Li et al., 2014). An alteration in any step of this pathway could affect miRNAs production, as has been shown in recent studies (Melo and Esteller 2014, Melo et al., 2009, Iliou 2013, Wu et al. 2014), and this might lead to the deregulation of cancer related genes. In fact, dysregulation of the expression levels in genes of this pathway has been described in several types of cancer, for example, the down-regulation of *DROSHA* and *DICER* expression in breast cancer and the up-regulation of *EIF2C2* and *TARBP2* in prostate cancer (refs Huang 2014).

It is widely known that single nucleotide polymorphisms (SNPs) could affect protein synthesis or function. Consequently, SNPs in genes of the miRNA processing pathway migth lead to changes in miRNA-mediated regulation (Mishra et al., 2009). In fact, the SNP rs640831 in DROSHA has been associated with the dysregulation of 56 miRNAs in lung cancer (Rottuno et al., 2010). Therefore, SNPs in this pathway may be associated with cancer development and progression (Horiwaka et al., 2008), as has been found in some studies. For instance, rs2740348 in *GEMIN4* has been associated with prostate cancer risk (Liu et al., 2012), rs417309 in *DGCR8* with breast cancer risk (Jiang et al., 2013), rs197412 in *GEMIN3* with renal cell carcinoma risk (Horiwaka et al., 2008), and recently, our group found rs139919 in *TNRC6B* to be associated with acute lymphoblastic leukemia susceptibility (Gutierrez-Camino et al., 2014),

However, in spite of all these evidences, SNPs in miRNA processing genes have not been studied in association with osteosarcoma risk.

Therefore, the aim of this study was to detect new genetic markers of osteosarcoma susceptibility, performing a deep analysis of SNPs in miRNA processing genes.

Methods

Patients

The patients included in this retrospective study were 99 Spanish children and young adults (<34 years) diagnosed with high-grade conventional osteosarcoma at the Oncology Unit of the Department of Pediatrics of the University Clinic of Navarra between 1985 and 2003. In addition, 387 healthy individuals of European origin with no previous history

of cancer from the collection C.0001171 registered in the Institute of Health Carlos III (ISCIII) were enrolled as controls (Table I). Informed consent was obtained from patients or their parents before sample collection and local institutional approval was obtained (Research Ethics Committee of the University of Navarra 105/2009)

Selection of genes and polymorphisms

We selected 21 genes in the miRNAs processing pathway after literature review and using the Patrocles database (14) (http://www.patrocles.org/) (University of Liège, Liège, Belgium). Using tagSNPs, we covered almost all the SNPs in each gene with potentially functional effects using International HapMap Project (release #24; http://www.hapmap.org), F-SNP (http://compbio.cs.queensu.ca/F-SNP/) (Queen´s University, Kingston, Canada), Fast-SNP (http://fastsnp.ibms.sinica.edu.tw) (Academia Sinica, Taipei, Taiwan), and Patrocles databases and the Haploview v4.2 software (http://www.broad.mit.edu/mpg/haploview/) (Broad Institute, Cambridge, USA). We considered functional SNPs those causing amino acid changes, alternative splicing, those located in the promoter region in putative transcription factor binding sites, or disrupting/creating miRNAs targets. We also selected SNPs from the literature described in association with cancer risk. All SNPs were selected with a minor allele frequency (MAF) greater than 5% (MAF≥0.05) in European/Caucasian populations.

Genotyping

Genomic DNA was extracted with conventional phenol-chloroform methods from EDTA-anticoagulated blood (Sambrook 2001).

SNP genotyping was performed using TaqMan OpenArray Genotyping technology (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) according to published Applied Biosystems protocols. The preliminary list of SNPs was filtered using suitability for the Taqman OpenArray platform as criterium. Initially, 76 SNPs were selected for analysis. After considering compatibility with the Taqman OpenArray platform, 72 SNPs in 21 genes involved in miRNA biogenesis were included in a Taqman OpenArray Plate (Applied Biosystems) (Supplementary Table SI).

Data analysis was carried out with Taqman Genotyper software (Applied Biosystems) for genotype clustering and genotype calling. As a genotyping control, duplicate samples were placed across the plates.

Statistical analysis

In order to identify any deviation from Hardy-Weinberg equilibrium (HWE) in the population of healthy controls (n = 387), $\chi 2$ test was used. The association between genetic polymorphisms in cases and controls was also evaluated using the $\chi 2$. Fisher´s exact test was used if a genotype class had less than 5 individuals. The effect sizes of the associations were estimated using odds ratio (OR) values obtained from univariate logistic regression. The most significant test among codominant, dominant, recessive, and additive genetic models was selected. In all cases, the significance level was set at 5%. The results were corrected for multiple comparisons using the conservative Bonferroni correction. In this case, the significance level was set at 10%. Analyses were performed using the R v2.11 software (http://www.R-project.org) (University of Auckland, New Zealand).

Results

Genotyping Results

A total of 99 patients with osteosarcoma and 387 unrelated healthy controls were available for genotyping. Successful genotyping was achieved for 427 DNA samples (87.86%) (samples with more than 20% missing genotypes were removed). Among the SNPs, 67/72 (93.05%) were genotyped satisfactorily. Failed genotyping was due to absence of PCR amplification, insufficient intensity for cluster separation or poor cluster definition. After removing failed samples and SNPs, the average genotyping rate was 97.22%. Furthermore, 10 SNPs out of the 67 genotyped SNPs were not in HWE in the population of 387 healthy controls and, therefore, were not considered for further analysis. In total, 15 SNPs were excluded from the association study (Supplementary Table SII), leaving 57 SNPs available for further analysis.

Analysis of the association with osteosarcoma risk

In order to investigate whether genetic variation in miRNA processing genes influences the risk of osteosarcoma, the frequencies of the 57 successfully genotyped polymorphisms were compared between cases and controls. As shown in Table II, three polymorphisms in miRNA-processing genes were associated with osteosarcoma risk ($p < 0.05$).

The most significant SNP was rs11866002, a SNP located in the *CNOT1* gene. Under the dominant genetic model, the CT+TT genotype was associated with a 0.44-fold decrease in osteosarcoma risk (95% CI: 0.27-0.73; $p = 0.001$) which remained statistically significant after Bonferroni correction ($p = 0.08$). The other two SNPs showing less

significant association with osteosarcoma risk were rs3812265 in *CNOT4*(p=0.025) and rs3823994 in *SND1* (p=0.041) (Table II).

Discussion

In this study, three SNPs in three miRNA processing genes (*CNOT1*, *CNOT4* and *SND1*), all of them were located in the RISC, were found to be associated with osteosarcoma risk. Remarkably, the association of rs11866002 in *CNOT1* remained statistically significant after Bonferroni correction. Our results suggest a role of SNPs in miRNA processing genes in osteosarcoma susceptibility.

In our study, the CC genotype of rs11866002 in CNOT1 was associated with a decreased risk of osteosarcoma. This SNP, which was the most significantly associated with osteosarcoma risk (p=0.001 and 0.08 after Bonferroni correction), is a synonymous change potentially affecting splicing regulation (20). Interestingly, this result is consistent with our previous study, in which the C allele was associated with a lower risk of acute lymphoblastic leukemia (Gutierrez-Camino et al., 2014), suggesting a relevant role of rs11866002 in cancer susceptibility. On the other hand, the rs3812265 CT+TT genotype in CNOT4 was associated with an increase in osteosarcoma susceptibility (p=0.025). This SNP is a missense variant that changes the sequence of the protein (Val>Ile) (20) , and that, as a result, could affect its function. In the RISC complex, both CNOT1 and CNOT4 are part of CCR4-NOT complex (18, 19), which removes poly(A) from mRNAs bound by miRNAs (17). It has been reported that the depletion of the components of the CCR4–NOT deadenylating complex prevents the decay of mRNAs (Behm-Ansmant 2006). Therefore, polymorphisms that affect CNOT1 and CNOT4 might alter mRNA

deadenylation and could alter the expression of genes involved in the origin and evolution of osteosarcoma.

Finally, TT genotype of SNP rs3823994 in SND1 gene showed association with a decreased risk of osteosarcoma. This SNP potentially affects splicing regulation. SND1 functions as a nuclease in the RISC complex (21) and controls the degradation of edited miRNAs (Li et al., 2008). It has been shown that SND1 is deregulated in hepatocellular carcinoma (Yoo et al., 2011) and primary cutaneous malignant melanoma (Sand et al., 2012). The deregulation of SND1 could affect miRNA levels, which could explain the increase in osteosarcoma risk. Indeed, it has been described that silencing of SND1 increases the expression of the mature miR-17-92a cluster members (Heinrich et al., 2013), a cluster overexpressed in osteosarcoma in association with proliferation, invasion and migration of osteosarcoma cells (Yang et al., 2014).

The remarkable finding in this study is that all the SNPs associated with osteosarcoma susceptibility were located in the RISC complex. This complex loaded with a miRNA (miRISC) mediates the repression of specific target mRNAs either by degrading or by inhibiting translation (Huang 2014). Several studies have provided evidence that deregulation of genes of this complex not only affects silencing of miRNA targets, but also miRNA expression levels. For instance, depletion of TNRC6A leads to the upregulation of many mRNA targets but it does not affect miRNAs expression levels (Eulalio et al., 2009) and the dysregulation of EIF2C2 has been correlated with an increase of mature miRNA levels in multiple myeloma (zhou et al., 2010, paper de winter). Therefore, SNPs in the components of RISC complex that affect their function may alter the miRNA-mediated mRNA regulation.

In conclusion, we have found for the first time that SNPs in RISC genes, especially rs11866002 in *CNOT1* may represent novel markers of osteosarcoma susceptibility. Further studies will be needed to confirm these results.

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Conflict of Interest Statement

The authors reported no potential conflicts of interest.

Tables

Table 1: Characteristics of the study population of osteosarcoma patients and controls.

	Patients	Controls
No. of individuals	99	387
Mean age ± SE, y	14.60 ± 5.23	51.2 ± 7.7
Sex, n (%)		
Males	55 (55.55)	199 (51.4)
Females	44 (44.44)	187 (48.3)

SE: standard error.

Table 2: Genotype frequencies of the SNPs in miRNA processing genes that were most significantly associated with osteosarcoma risk.

Gene	SNP	Genotype	Controls n (%)	Cases n (%)	Best fitting model	OR (95% CI)	P-value	P-value (Bonferroni)
CNOT1	rs11866002	CC	134 (38.7)	46 (59.0)	Dominant		0.001	0.08
		CT	174 (50.3)	26 (33.3)	CC CT/TT	1.00 0.44 (0.27-0.73)		
		TT	38 (11.0)	6 (7.7)				
CNOT4	rs3812265	CC	212 (63.1)	39 (49.4)	Dominant		0.025	N.S.
		CT	109 (32.4)	37 (46.8)	CC CT/TT	1.00 1.75 (1.07-2.87)		
		TT	15 (4.5)	3 (3.8)				
SND1	rs3823994	AA	163 (46.8)	47 (59.5)	Additive		0.041	N.S.
		AT	157(45.1)	28 (35.4)	AA vs AT vs TT	0.66(0.43-0.99)		
		TT	28 (8)	4 (5.1)				

NS: No significant

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