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Variants in vincristine pharmacodynamic genes involved in neurotoxicity at induction phase in the therapy of pediatric acute lymphoblastic leukemia

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Abstract

Vincristine is an important drug of acute lymphoblastic leukemia (ALL) treatment protocols that can cause neurotoxicity. Patients treated with LAL/SHOP protocols often suffer from vincristine-related neurotoxicity in early phases of treatment. A genetic variant in *CEP72*, a gene involved in vincristine pharmacodynamics, was recently associated with neurotoxicity after prolonged vincristine treatment. This association was not replicated in our Spanish population during induction phase. To test the possibility that other variants in genes involved in vincristine pharmacodynamics were associated with vincristine neuropathy in early phases of the treatment, we evaluated the correlation with toxicity of 24 polymorphisms in 9 key genes in a large cohort of 152 Spanish children with B-ALL homogeneously treated. Results showed no association between any genetic variant in the *TUBB1*, *TUBB2A*, *TUBB2B*, *TUBB3*, *TUBB4, MAPT*, MIR146a, MIR202 and MIR411 genes and vincristine-related neurotoxicity. These results are in line with the hypothesis that there are different mechanisms causing pheripheral neurotoxicity after prolonged and short-term vincristine treatments.

Keywords

Vincristine, polymorphism, neurotoxicity, acute lymphoblastic leukemia, pharmacodynamics

Introduction

Vincristine is an important drug of treatment protocols for acute lymphoblastic leukemia (ALL), the most common pediatric cancer.[1,2] It is used in different phases of treatment (induction, intensification and reinductions). However, vincristine can cause sensory and motor neurotoxicity,[3] a disabling adverse effect that can affect the quality of life of the patients during and after treatment. This neurotoxicity can lead to dose reduction or treatment discontinuation, [4] which can impact survival.[3] Therefore, one of the challenges of the medicine today is to predict which patients will have greater risk of adverse drug effects in order to adjust the treatment.

The mechanism of action of vincristine is based on its binding to beta-tubulin which causes the inhibition of microtubule assembly and cell cycle arrest, leading to the death of the actively dividing leukemic cells.[3,5-7] In the nervous system, the binding of vincristine to beta-tubulin leads to severe alterations in axonal microtubules, resulting in axonal swelling and nervous fiber damage that disturbs both sensory and motor functions, that are characteristics in neurotoxicity.[3] Consequently, polymorphisms in genes coding beta-tubulin proteins and/or in microtubule-associated proteins could alter the stabilization of microtubules, modifying the cells sensitivity to vincristine and provoking differences in the effect of this drug among patients.

In this line, one polymorphism in the promoter of *CEP72* gene, involved in spindle pole and microtubule formation, was recently associated with an increased risk of vincristine-induced neuropathy in children[8] and adults[9] after prolonged vincristine treatment for ALL (up to 39 doses). This association was explained considering that rs924607 T variant created a binding site for the NKX-6.3 transcription repressor, markedly enhancing NKX-6.3 binding to the risk allele, leading to lower *CEP72* mRNA expression.[8] The reduction of *CEP72* expression was shown to increase sensitivity to vincristine in human

neurons derived from induced pluripotent stem cells and in leukemia cells from patients with CEP72 TT genotype.[8]

When we analyzed the effect of this polymorphism in our Spanish cohort, treated with LAL/SHOP protocols, we found no association during the induction, phase in which our patients developed neurotoxicity.[10] This result was also found by the same previous authors (Diouf *et al)* in their pediatric cohort, in which no association was found between *CEP72* polymorphism and neuropathy during induction.[11] In this context, the analysis of additional genetic variants in other genes involved in vincristine pharmacodynamics (Figure 1) could help to explain the predisposition to neuropathy in early phases of the ALL treatment.

Additionally, nowadays, it is known that microRNAs (miRNAs) regulate more than 50% of human genes, including genes involved in vincristine pharmacodynamic pathway. Consequently, polymorphisms affecting the levels or function of miRNAs targeting those genes could affect their expression, having in turn a role in the vincristine-related neurotoxicity. In fact, several SNPs in the premature mir-453, miR-5189, miR-595, and miR-6083, whose putative target genes are involved in drug transport (*ABCC1*, *ABCB1*, *ABCC2*, *ABCC4*, *SLC46A1*, *SLC19A1* and *SLCO1A2*) have been associated with methotrexate toxicity in ALL patients in previous studies. [12,13]

Therefore, in the present study we aimed to test the role of polymorphisms in genes involved in vincristine pharmacodynamic pathway in the risk of development vincristine-related neurotoxicity during the induction phase. With that objective, we analyzed 21 SNPs in genes coding beta-tubulin proteins (*TUBB1*, *TUBB2A*, *TUBB2B*, *TUBB3*, *TUBB4*) and microtubule-associated proteins (MAPT) and 3 SNPs in 3 miRNAs with the potential to affect the function of those genes in a large cohort of Spanish children with B-ALL homogeneously treated with LAL/SHOP protocol.

Patients and methods

Patients

This is a retrospective study including 152 Spanish children, mainly Caucasian, all diagnosed with B-ALL from 2000 to 2011 at the Pediatric Oncology Units of 3 reference hospitals (Hospital University Cruces; Hospital University Donostia; and Hospital University La Paz). The study included all the consecutive patients diagnosed and treated in the context of LAL/SHOP treatment protocols for which neurotoxicity data were available. Patient characteristics are reported in Table 1. Written informed consent was obtained from all patients or their parents before sample collection. University of the Basque Country (UPV/EHU) Ethics Committee board approval (CEISH/102R/2011) was obtained.

Treatment and toxicity evaluation

All the patients included in the study were homogeneously treated with the Spanish subsequent LAL-SHOP 94/99/2005 protocols. These protocols include 4 doses of vincristine (1.5 mg/m^2) given on a weekly basis at induction and between 6 and 16 doses (1.5 mg/m^2) in later phases of treatment (given on a monthly or weekly basis depending on phase and risk group).

In induction, in addition to vincristine, these treatment protocols also included daunorubicin (120mg/m^2 for 2 days), prednisone (60 mg/m^2/day for 28 days, 30 mg/m^2/day for 4 days and 15 mg/m^2/day the last 4 days), cyclophosphamide (1 or 2 doses of 1000 mg/m^2/dose depending on risk group), asparaginase (10 doses of 5000-10000 u/m2), and intrathecal methotrexate-cytarabine-hydrocortisone therapy (2 or 3 age-adjusted doses depending on risk group). High risk patients in the LAL/SHOP 99 also received a single dose of 3g/m^2 of methotrexate on day +15. The later phases of treatment were more heterogeneous based on risk group segregation.

Toxicity data were collected blinded to genotypes from the patients' medical files by the same two expert researchers in all cases. Patients were evaluated at 3 hospitals by physicians trained in the assessment of vincristine neuropathy, using the same standards and same frequency of evaluation among the different centers. Monitoring of neurotoxicity after vincristine treatment is required specifically in the treatment protocol and is more common during induction, when vincristine doses are more frequent. Neurotoxicity was graded according to the WHO criteria (Supplementary table 1); three phenotypes were considered: neurotoxicity (grades 1-4), low grade neurotoxicity (grades 1-2) and high grade neurotoxicity (grades 3-4). Other relevant data including age, sex, number of vincristine doses and risk group were systematically collected from the clinical records. In the present study, neurotoxicity was examined in all phases of the treatment (excluding consolidation, the only phase in which vincristine is not included). Nevertheless, since only 5% (9 out of 152) showed toxicity in later phases (insufficient sample size to detect statistically significant differences between groups), the study was focused on neurotoxicity during induction phase.

Gene and polymorphism selection

Six candidate genes reported to be involved in vincristine pharmacodynamics were selected based on the information available in the Pharmacogenomics Knowledge database (PharmGKB) (www.pharmgkb.com) (Stanford University, Stanford, CA) (16) and the literature. These genes encode tubulin genes expressed in the brain or the blood (*TUBB1*, *TUBB3*, *TUBB2A*, *TUBB2B*, *TUBB4*) and tubulin-stabilizing proteins with a role in the neurons (*MAPT*) (Figure 1).

A total of 21 SNPs ranging from 10-kb upstream of the translation initiation site to 10-kb downstream of the translation stop site of the 6 selected genes were included. SNPs were selected based on the following criteria: (i) tagSNPs, defined using the International HapMap Project (release #24; http://hapmap.ncbi.nlm.nih.gov/) (The HapMap Data Coordination Center (DCC), Bethesda, MD) and

Haploview software v.4.2 (http://www.broad.mit.edu/mpg/haploview/) (Broad Institute, Cambridge, USA) with an r^2 threshold of 0.8 and a minimum minor allele frequency (MAF) of 0.10 (ii) SNPs with potential functional effects (amino acid changes, alternative splicing, promoter regions, putative transcription factor binding sites, CpG sites or miRNAs targets) identified using bioinformatics tools: Ensembl (http://www.ensembl.org/) (Welcome Trust Genome Campus, Cambridge, UK), 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), polymirTS (http://compbio.uthsc.edu/miRSNP/) (University of Tennessee Health Science Center, Memphis, TN),[14] Patrocles ((http://www.patrocles.org/) (University of Liège, Liège, Belgium)[15] (iii) SNPs previously reported to be associated with drug responseor metabolism in the literature.

In addition, 96 miRNAs that potentially target those 6 genes were selected using miRanda algorithm (http://www.microrna.org/microrna/home.do) (Memorial Sloan-Kettering Cancer Center, New York, NY). SNPs in their corresponding pre-miRNAs were searched using miRNASNiPer database (http://www.integratomics-time.com/miRNA-SNiPer) (University of Ljubljana, Ljubljana, Slovenia).[16] From the total, we selected those SNPs with a MAF > 0.01 in European/Caucasian populations in dbSNP database (http://www.ncbi.nlm.nih.gov/snp/) remaining 3 SNPs in 3 miRNAs.

A final number of 24 SNPs was included in an oligonucleotide pool assay for analysis using the Illumina Veracode technology (Illumina Inc., San Diego, CA) (Supplementary Table 2 and 3).

Genotype analyses

Genomic DNA was extracted from remission peripheral blood or bone marrow using the phenol-chloroform method as previously described.[17] DNA was quantified using PicoGreen (Invitrogen Corp., Carlsbad, CA).

For each sample, 400 ng of DNA were genotyped using the GoldenGate Genotyping Assay with Veracode technology according to the published Illumina protocol. A final number of 24 SNPs was included in an oligonucleotide pool assay for analysis using the Illumina Veracode technology (Illumina Inc., San Diego, CA) (Supplementary Table 2).

Data were analyzed with Genome Studio software for genotype clustering and calling. Duplicate samples and CEPH trios (Coriell Cell Repository, Camden, NJ) were genotyped across the plates. SNPs showing Mendelian allele-transmission errors or showing discordant genotypes were excluded from the analysis.

Statistical analysis

The association between neurotoxicity and genetic polymorphisms was evaluated by the $\chi 2$ or Fisher's exact test. The effect sizes of the associations were estimated by the OR's from univariate logistic regression. The most significant test between dominant and recessive genetic models was used to determine the statistical significance of each SNP. The results were adjusted for multiple comparisons by the False Discovery Rate (FDR).[18] Univariate and multivariate logistic regressions were also performed to account for the possible confounding effect of sex, age and the number of vincristine doses received. The Quanto 1.2.4 software (http://biostats.usc.edu/Quanto.html) was used to measure the statistical power of the associations. In all cases the significance level was set at 5%. Analyses were performed by using R v2.11 software. Haploview v.4.2 was used to determine haplotype block structure and to infer haplotype frequencies and differences between individuals with and without toxicity.

Results

Genotyping Results

Successful genotyping was obtained for 133 DNA samples (87.5%). All the 24 selected SNPs were genotyped satisfactorily (100%). The SNP rs17145779 in *TUBB2B* was monomorphic in our population, so it was removed from the association study since it was not informative.

Polymorphisms in association with neurotoxicity

In order to investigate if genetic variation may influence vincristine-related toxicity, we tested the association between the 23 informative polymorphisms and neurotoxicity.

We found that neurotoxicity was not significantly associated with the analyzed covariates (age, sex and number of vincristine doses received). Thus, we did not include these covariates in the subsequent association analyses.

When we considered any grade of neurotoxicity (1-4), no statistically significant association with neurotoxicity in induction ($p<0.05$) was found. When only patients with low grade toxicity (grade 1-2) were compared with those with no toxicity, rs12355840 in mir-202, a miRNA targeting *TUBB2B* was significantly associated with neurotoxicity in induction (Table 2). When only patients with high grade toxicity (grade 3-4) were considered, 1 SNP, rs11867549 in *MAPT*, was significantly associated with neurotoxicity in induction (Table 2). Similar results were found when neurotoxicity was considered at any phase during the treatment (Supplemental Table 4). After FDR correction, none of them remained statistically significant.

Haplotypes in association with toxicity

To test the association between haplotypes and vincristine-induced neurotoxicity, we first determined the linkage disequilibrium block structure for each gene (block definition was based on the study by

Gabriel et al.[19] *MAPT* was defined by two blocks that showed six haplotypes with frequencies higher than 1%; *TUBB3* was defined by one block that showed three haplotypes. *TUBB1*, *TUBB2A* and *TUBB4* showed no haplotype blocks.

Significant results of the association analyses comparing the frequency of each haplotype between patients with and without any toxicity are shown in Table 3. No significant association was found when all the patients were considered together. When only patients with low grade toxicity (grade 1-2) were compared with those with no toxicity, significant associations were found between 2 haplotypes in *TUBB3* formed by rs4395073-rs4558416 (CA and TG) and neurotoxicity in induction. When only patients with high grade toxicity (grade 3-4) were considered, significant associations were found between 1 haplotype in *MAPT* formed by rs1001945-rs11867549 (CG) and neurotoxicity in induction. After FDR correction, none of them remained statistically significant.

Discussion

The present study evaluated the correlation with vincristine-induced toxicity in the early phases of treatment of 24 polymorphisms in 9 genes, from which 6 were involved in the pharmacodynamic vincristine pathway and 3 were miRNAs that regulate them. The study was performed in a group of 152 children diagnosed with B-ALL and treated according to the standardized LAL/SHOP protocol. During the treatment, 30% of patients developed neurotoxicity during induction phase and only 5% showed toxicity in later phases. When we analyzed the association between the polymorphisms and the risk of developing vincristine-related total neurotoxicity in induction, no genetic variant was found significant after FDR correction.

The negative results shown in the present study were in line with two previous candidate-gene studies analyzing genetic polymorphisms in genes involved in vincristine pharmacodynamics:[4,20] *TUBB1* encoding for beta-tubulin, and *MAPT*, encoding a microtubule-associated protein Tau that plays a

fundamental role in stabilization of microtubules in the axonal compartments of proteins.[20,21] None of these two studies found significant results between vincristine-related neurotoxicity and the polymorphisms in the pharmacodynamic genes analyzed (*TUBB1* rs6070697 and rs10485828; *MAPT* rs1800547, rs17651549, rs17651754, rs10445337, rs17652121).[4,20]

When neurotoxicity was graded, two polymorphisms, *MAPT* rs11867549 and mir-202 rs12355840, were found associated with vincristine-related neurotoxicity, although these associations were not significant after FDR correction. *MAPT* rs11867549 AG+GG genotypes were associated with a decrease of severe neurotoxicity grades 3-4, while mir-202 rs12355840 CT+CC genotypes were associated with an increase of neurotoxicity grades 1-2. However, the associations found in the analysis were too weak to be considered positive.

The current study has some limitations such as the relatively small sample size of the analyzed population, due to the difficulty of collecting samples of ALL patients treated with standardized treatment protocol and with data about neurotoxicity. In this case, for a MAF=0.25 and assuming a dominant genetic model and OR=2.0, the statistical power of the present study to detect association was of 60% when neurotoxicity grades 1-4 versus no toxicity was compared. This was 50% when neurotoxicity grades 1-2 *versus* no toxicity were analyzed and 40% when grades 3-4 *versus* no toxicity were studied. Although the statistical power to detect a moderate or small degree of interaction was limited, error type I (false-positives) was corrected by multiple testing. However, we cannot exclude the existence of error type II (false-negatives). Moreover, since this study was focused on common polymorphisms with MAF>0.1, we cannot exclude the possibility of being rare variants affecting the VCR-induced neurotoxicity, as it was already demonstrated in *SLCO1B1,* where rare variants seemed to have an important effect on pharmacogenetic phenotype in relation to methotrexate clearance.[22]

In summary, this study shows no significant association between polymorphisms in genes involved in the pharmacodynamics of vincristine and neurotoxicity during induction in pediatric ALL patients. These results together with the fact that previous studies of our group identified two significant polymorphisms (from a total of 153 analyzed) in the vincristine transporter gene *ABCC2* associated with vincristine-related neurotoxicity during induction,[23] supports the following idea: neurotoxicity in early phases of the therapy could be modulated by little changes in pharmacokinetics genes, while in late phases, this could be influenced by changes in pharmacodynamic genes. Therefore, it is logical to think about the existence of different mechanisms causing peripheral neurotoxicity after prolonged and short-term treatments. Further studies are required to fully understand the biological role of the polymorphisms in genes involved in vincristine pharmacodynamics in the development of vincristine-related neurotoxicity at induction phase.

Table legends

Table 1. Characteristics of the study population.

Table 2. SNPs significantly associated with neurotoxicity in the induction phase.

Table 3. Haplotypes significantly associated with neurotoxicity in the induction phase.

Figure legends

Figure 1.Vincristine pharmacodynamics.

Supplementary table legends

Supplementary table 1. Neurotoxicity criteria (WHO criteria)

Supplementary table 2. Characteristics of the Single Nucleotide polymorphisms and selection criteria

Supplementary table 3. Single Nucleotide Polymorphisms in miRNAs

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Author disclosure and conflict of interest

The authors declare that they have no conflict of interest.

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