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Original Article

Influence of variants of the *drosha*, *mir499a*, and *mir938* genes on susceptibility to acute lymphoblastic leukemia in an admixed population from the brazilian amazon

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Abstract: Acute Lymphoblastic Leukemia (ALL) is the most common type of cancer in children. Polymorphisms that alter the normal function of the microRNAs involved in the development of ALL have been widely investigated, although published data on these polymorphisms in admixed populations are scarce. We investigated the role of 10 polymorphisms in the microRNA and protein-coding genes of the microRNA synthesis complex in susceptibility to pediatric B-cell ALL. The study includes 100 pediatric ALL patients and 180 healthy individuals. The statistical analyses were run in SPSS v.25.0. In the case of the microRNA synthesizing genes, a significant pattern was found in only gene, that is, the rs3805500 polymorphism of *DROSHA*, in which the homozygous mutant (AA) genotype was associated with a threefold increase in the risk of developing ALL when compared to other genotypes (P=0.004, OR=2.913, CI=1.415-5.998). In the microRNA coding genes, the homozygous mutant rs3746444 genotype of the *MIR499A* gene was associated with a 17-fold increase in the risk of development of ALL (P<0.001, OR=17.797, CI=5.55-57.016). A protective effect against the development of ALL was also observed in the carriers of the wild homozygous rs2505901 genotype in the *MIR938* gene. Our findings highlight the potential of these polymorphisms in the genes involving in the coding of microRNAs for the evaluation of the risk of contracting ALL in the population of the Brazilian Amazon region. These findings contribute to a more complete understanding of the complex etiology of ALL.

Keywords: Acute lymphoblastic leukemia, microRNAs, single nucleotide polymorphism, susceptibility

Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common type of cancer in children and adolescents of up to 19 years of age, representing 28% of all malignancies in this age group and 75% of all cases of leukemia [1-3]. The etiology of ALL is considered to be multifactorial, involving both environmental and genetic factors, which have been investigated extensively. These factors include exposure to carcinogens, as well as chromosomal and molecular alterations [4, 5]. In particular, many single nucleotide polymorphisms (SNPs) in key genes of the regulatory pathways have been ascribed a fundamental role in the development of ALL [6].

The investigation of the SNPs found in the microRNA genes has emerged as a promising new field of genomic research. The principal function of these molecules is to regulate gene

expression via post-transcriptional silencing, the cleaving of messenger RNAs (mRNA) or inhibiting the initiation of translation through base pairing between the microRNA and its mRNA target [7]. The presence of SNPs in microRNA genes or the protein-coding genes involved in the synthesis of the microRNAs may affect their correct functioning, resulting in impacts on gene regulation processes [8]. Despite the important regulatory potential of these polymorphisms, few studies have focused on the influence of the SNPs located in non-coding genomic regions. In addition, evidence on the occurrence of these polymorphisms in populations with high levels of miscegenation, such as that of the Brazilian Amazon region, is particularly scarce. Given this, the present study investigated the role of 10 polymorphisms of the microRNA and microRNA synthesis genes in the susceptibility of pediatric patients from the Brazilian Amazon region to B-cell ALL.

Material and methods

Patients and controls

The case group of the present study included 100 patients diagnosed with B-cell ALL by immunophenotyping and/or molecular analysis. All these individuals were in treatment at the Otávio Lobo Hospital in Belém, a reference center for the treatment of pediatric cancer in the northern region of Brazil. The patients included in the study were between 1 and 18 years old. Recurrent patients or those with comorbidities were not included in the group. The control group consisted of 180 individuals with no diagnosis for B-cell ALL or any other type of cancer. None of these individuals were related to any of the patients in the case group, and they were all over the peak risk age for the development of ALL.

Ethical aspects

This study was approved by the Research Ethics Committee of the Research Center of Oncology of the Federal University of Pará (CAAE number 11433019.5.0000.5634). All participants (or their legal guardians) signed a term of informed consent authorizing the collection of samples and data.

Selection of the study polymorphisms

The SNPs selected for the present study were chosen due to their known association with susceptibility to ALL and different types of cancer, based on the published data. The references of the journal articles used for the selection of the polymorphisms are listed in the Table S1 [38-48]. A total of 10 polymorphisms were selected, seven in microRNA genes and three in the protein-coding genes essential to the synthesis of microRNAs: rs636832 (*AGO1*), rs10035440 e rs3805500 (*DROSHA*), rs213210 and rs107822 (*MIR-219-1*), rs2910164 (*MIR146a*), rs12894467 (*MIR300)*, rs3746444 (*MIR499a*), rs4919510 (*MIR608*), and rs2505901 (*MIR938*).

Extraction and quantification of the DNA

The DNA was extracted from peripheral blood using a commercial DNA extraction kit (Biopur Mini Spin Plus-250 extraction kit, Biopur, Brazil). The concentration of the genetic material was quantified in a NanoDrop 1000 spectrophotometer (Scientific Term NanoDrop 1000; NanoDrop Technologies Wilmington, DE).

Genotyping of polymorphisms

The 10 polymorphisms were genotyped by allelic discrimination using the TaqMan OpenArray Genotyping technology with a set of 32 customized assays, which were run in a QuantStudio™ 12K Flex Real-Time PCR system (Applied BiosystEM, Life Technologies, Carlsbad, USA), according to the manufacturer's protocol. This method is based on real-time Polymerase Chain Reaction (qPCR). The quality of the readings of the genotypes and other data were analyzed in the TaqMan Genotyper software.

Quality control

To ensure an adequate level of accuracy, polymorphisms were only included in the present study if they satisfied three criteria: (i) MAF≥ 1%; (ii) genotyping rate ≥80%, and (iii) were in Hardy-Weinberg (HWE). The HWE was performed using the Arlequin software (v.3.5.1.2). The significance of the HWE test was adjusted for multiple comparisons by the Bonferroni

Table 1. Primers used for RT-PCR in the chromosomal fusion analysis

Cromossomic Fusion	Genes	Primer (5'-3')
t(1;19)(q23;p13)	E2A	CTACTCCCCGGATCACTCAA
	PBX1	AGGCTTCATTCTGTGGCAGT
t(4;11)(q21;q23)	MLL	CGCCCAAGTATCCCTGTAAA
	AF4	GAGCATGGATGACGTTCCTT
t(9;22)(q34;q11)	BCR	TCGCAGAACTCGCAACAGT
	ABL	ACACCATTCCCCATTGTGAT
t(12;21)(p13;q22)	TEL	TCTCTCATCGGGAAGACCTG
	AML1	TGCGGTAGCATTTCTCAGC
del(1)(p32;p32)	SIL	TCCTACCCTGCAAACAGACC
	TAL1	AGGCGGAGGATCTCATTCTT

Table 2. Demographic parameters for the case (patients with B-cell ALL) and control groups analyzed in the present study

Variable	Case (100)	Control (180)	*p-value*
Sex (M/F)	60/40	53/127	<0.001[a]
Age*	5.53 ± 3.991	65.97 ± 16.021	<0.001[b]
*Genetic ancestry**			
European	0.429 ± 0.133	0.454 ± 0.170	0.189[c]
African	0.203 ± 0.089	0.241 ± 0.138	0.213[c]
Amerindian	0.361 ± 0.154	0.304 ± 0.149	0.004[c]

[a]Chi-square, [b]Studenta0 *t*, [c]Mann-Whitney9tr*U*. *Mean ± eStandard Deviation.

method (P≤0.001), and the HWE values are shown in the Table S2. As all 10 of the polymorphisms selected for the present study satisfied these criteria, they were all included in the association analyses (Table S2).

Fusion analysis by extraction of the RNA and the reverse transcriptase-polymerase chain reaction (RT-PCR)

For the cytogenetic analysis of the BCR-ABL, ETV6-RUNX1, MLL-AF4, SIL-TAL and E2A-PBX1 fusions, blood samples were collected from 84 patients via venipuncture and stored in EDTA. Ficoll Histopaque® (Sigma-Aldrich, USA) was added to the samples for the separation of the lymphocytes, following the manufacturer's instructions. The ARNsy MiniKit (Qiagen, USA) and High Capacity cDNA Reverse Transcription kits (Applied Biosystems, USA) were used to extract RNA and convert cDNA, respectively, both according to the manufacturers' protocols. A multiplex RT-PCR reaction was run using The Master Mix kit (Promega, USA) with the primers designed specifically for the five fusions mentioned above, also following the manufacturer's instructions. The primers used for RT-PCR are listed in **Table 1**.

Analysis of genomic ancestry

The genomic ancestry of the participants was analyzed following Santos et al. (2010) and Ramos et al. (2016), using a set of 61 Ancestry Informative Markers (AIMs). The individual and global proportions of European, Amerindian, and African genetic ancestry were estimated using STRUCTURE v.2.3.4 [9-11].

Statistical analyses

The Chi-square test was applied for the pairwise comparisons of the categorical variables between the case and control groups, while the quantitative variables were compared using Student's *t*. The multivariate analyses considered the sex of the participants and Amerindian ancestry as confounding variables. The Mann-Whitney test was used to compare the estimates of genetic ancestry between the groups. All statistical analyses were run in SPSS v.25.0 (SPSS, Chicago, IL, USA), considering a $P \leq 0.05$ significance level.

Results

Significant differences were found between the case (ALL) and control group ages, sex ratio, and genomic ancestry (**Table 2**). The mean age of the case group was significantly lower than that of the control group (P<0.001) and the sex ratios also varied significantly between groups (P<0.001), with a predominance of males in the case group and of females in the control group.

The genomic makeup of the case group was dominated by European ancestry (43%), followed by the Amerindian (36%) and African (20%) components. While the control group presented a similar makeup (European-45%, Amerindian-30%, African-24%), its Amerindian ancestry was significantly lower (P=0.004) than that of the case group. Given the observed variation, both sex and Amerindian ancestry

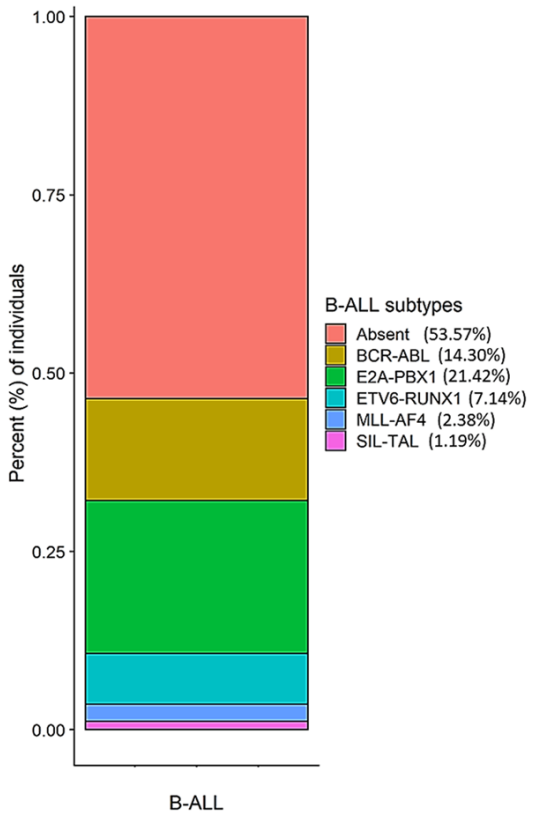

Figure 1. Distribution of the B-cell ALL subtypes in the study patients of the case group.

were controlled for in the analyses of the potential association between the polymorphisms and susceptibility to ALL.

The distribution of the B-cell ALL subtypes, based on the occurrence of the chromosomal fusions analyzed here, is shown in **Figure 1**. The most frequent fusions were E2A-PBX1 (21.42%) and BCR-ABL (14.30%), followed by ETV6-RUNX1 (7.14%), MLL-AF4 (2.38%), and SIL-TAL (1.19%).

Three SNPs were investigated further in the case of the genes involved in the synthesis of microRNAs, and a significant pattern was observed in the case of the recessive model (AA *vs.* GG + GA) of the rs3805500 variant of the *DROSHA* gene (**Table 3**). The mutant homozygote (AA) genotype of this gene was associated with a threefold increase in the risk of developing ALL (P=0.004, OR=2.913, CI=1.415-5.998).

Seven variants of the microRNA genes were analyzed, of which, two were related significantly to the susceptibility of the carrier to ALL (**Table 3**)-MIR499A (rs3746444) and MIR938 (rs2505901). The mutant homozygote rs3746444 (GG) genotype was associated with a 17-fold increase in the risk of developing ALL (P<0.001, OR=17.797, CI=5.55-57.016). By contrast, the wild homozygous rs2505901 (CC) genotype was associated with a lower risk for the development of ALL, apparently conferring a protective effect (P=0.013, OR=0.359, CI=0.160-0.805).

Discussion

The population of Brazil is one of the world's most genetically heterogeneous, and the pediatric ALL patients analyzed in the present study had a high level of admixture (42% European, 36% Amerindian, and 20% African). This is a very distinct genetic background in comparison with that of the other ethnic populations in which the association between SNPs in the microRNA genes have typically been evaluated [12].

We identified three variants with the apparent potential to influence the risk of developing ALL - the rs3805500 variant of the DROSHA gene, rs3746444 (MIR499A gene), and rs2505901 (MIR938 gene). The differential expression of these genes and their apparent role in the development of cancer has been demonstrated in a number of previous studies [13-15]. However, a number of both internal and external environmental factors may affect the levels of gene expression, which impedes the full understanding of the role of these variants in the susceptibility of the individual to ALL, which reinforces the importance of evaluating germline polymorphisms [16, 17].

The DROSHA gene is a key component of the synthesis of microRNAs. This gene encodes a type III RNase, which is essential for the maturation of pri-miRNAs and pre-miRNAs [18]. We found that the homozygous mutant (AA) rs3805500 genotype is associated with a threefold increase of the development of ALL. This is the first study to report the role of this polymorphism as a risk factor of ALL, although the rs3805500 mutant has already been identified as a factor in the susceptibility of individuals to other types of leukemia [19, 20]. A GWAS study [19] found that the rs3805500 mutant A allele, in a haplotype with two

Table 3. Distribution of the alleles and genotypes of the polymorphisms investigated in the B-cell ALL patients, in comparison with control individuals

Genotype ID	Case (%)	Control (%)	p	Model	OR (95% CI)
AGO1_rs636832	75	150			
AA	8 (10.7)	14 (9.3)			
AG	34 (45.3)	67 (44.7)			*AA + AG vs. GG*
GG	33 (44)	69 (46)	0.163	Recessive	1.687 (0.809-3.520)
Allele A	0.333	0.316			
Allele G	0.666	0.683			
DROSHA_rs10035440	80	148			
TT	60 (75)	98 (66.2)			
TC	17 (21.3)	44 (29.7)			*TT + TC vs. CC*
CC	3 (3.8)	6 (4.1)	0.434	Recessive	2.082 (0.331-13.085)
Allele T	0.856	0.810			
Allele C	0.143	0.189			
DROSHA_rs3805500	74	159			
GG	11 (14.9)	50 (31.4)			
GA	26 (35.1)	51 (32.1)			*GG + GA vs. AA*
AA	37 (50)	58 (36.5)	0.004	Recessive	2.913 (1.415-5.998)
Allele G	0.324	0.474			
Allele A	0.675	0.525			
MIR219-1_rs213210	82	166			*AA vs. AG + GG*
AA	64 (78)	134 (80.7)	0.813	Dominant	0.908 (0.409-2.015)
AG	16 (19.5)	19 (11.4)			
GG	2 (2.4)	13 (7.8)			
Allele A	0.878	0.864			
Allele G	0.121	0.135			
MIR219-1_rs107822	73	152			*CC vs. CT + TT*
CC	36 (49.3)	85 (55.9)	0.831	Dominant	0.929 (0.471-1.832)
CT	30 (41.1)	53 (34.9)			
TT	7 (9.6)	14 (9.2)			
Allele C	0.698	0.733			
Allele T	0.301	0.266			
MIR146A_rs2910164	81	160			*GG vs. GC + CC*
GG	47 (58)	68 (42.5)	0.091	Dominant	1.777 (0.912-3.462)
GC	24 (29.6)	74 (46.3)			
CC	10 (12.3)	18 (11.3)			
Allele G	0.728	0.656			
Allele C	0.271	0.343			
MIR300_rs12894467	76	175			*TT vs. TC + CC*
TT	22 (28.9)	75 (42.9)	0.062	Dominant	0.513 (0.254-1.035)
TC	42 (55.3)	80 (45.7)			
CC	12 (15.8)	20 (11.4)			
Allele T	0.565	0.657			
Allele C	0.474	0.342			
MIR499_rs3746444	75	167			
AA	5 (6.7)	126 (75.4)			
AG	43 (57.3)	36 (21.6)			*AA + AG vs. GG*
GG	27 (36)	5 (3.0)	<0.001	Recessive	17.797 (5.55-57.016)

Allele A	0.353	0.862			
Allele G	0.646	0.137			
MIR608_rs4919510	81	161			*GG vs. GC + CC*
GG	5 (6.2)	18 (11.2)	0.325	Dominant	0.566 (0.182-1.760)
GC	29 (35.8)	58 (36)			
CC	47 (58)	85 (52.8)			
Allele G	0.240	0.291			
Allele C	0.759	0.708			
MIR938_rs2505901	76	150			*CC vs. CT + TT*
CC	15 (19.7)	53 (35.3)	0.013	Dominant	0.359 (0.160-0.805)
CT	38 (50)	46 (37.7)			
TT	23 (30.3)	51 (34)			
Allele C	0.447	0.506			
Allele T	0.552	0.493			

OR: Odds Ratio; CI: Confidence interval. The *p* values refer to the logistic regression adjusted for sex and Amerindian ancestry.

other polymorphisms, was associated with an increased risk of Chronic Lymphocytic Leukemia (CLL), which is consistent with our finding that the rs3805500 mutant A allele contributes to an increased susceptibility to leukemia.

Polymorphisms in the DROSHA gene may influence shared pathways in the development of both ALL and CLL [19]. The rs3805500 mutant is also in linkage disequilibrium with the rs640831 variant of the same gene, which is related to a reduction in the expression of the DROSHA mRNA, and an alteration in the maturation of pri-miRNAs and pre-miRNAs, a condition linked to the progression of a number of different types of cancer [21-25].

Another variant analyzed here is the rs3746444, which is located in the seed region of the MIR-499a-3p gene, and may impede the microRNA from binding to its targets [12, 26]. Our results indicate an association between the homozygous mutant genotype (GG) and a 17-fold increase in the risk of developing ALL. Other studies have reported an association between the rs3746444 polymorphism and susceptibility to a number of malignant neoplasia, although these findings have been controversial [27-30]. The homozygous mutant (GG) rs3746444 genotype has been related to the risk of gastric and lung cancer in Asian populations, but not in Caucasians [29, 30]. The contradictory nature of these results is probably due to the distinct genetic backgrounds of the different types of cancer and interethnic differences between the Asian and Caucasian populations [12, 29, 30]. In the ALL, few studies have investigated the role of rs3746444 polymorphism in its development, which were carried out in homogeneous Caucasian populations [12, 26]. Hasani et al. (2014) showed no association of this variant with the risk of ALL [12], while Gutierrez-Camino (2014), described a protective effect of the G allele on the risk of ALL [26]. Further studies are needed to better elucidate the role of this variant in the ALL development. Furthermore, many findings indicate the potential influence of ethnicity in genetic associations, and reinforce the need for the analysis of ancestry to guarantee more conclusive studies.

The MIR938 gene is responsible for the regulatory pathways of the genes related to cell survival and apoptosis [31]. Variants of the SNP type present in the MIR938 gene have been associated with modifications in its biogenesis and stability [32, 33]. We investigated the rs2505901 variant, which corresponds to a C>T nucleotide swap in the intronic region of the MIR938 gene, and found that the wild homozygous (CC) genotype of this variant is associated with a 33% decrease in the risk of development of ALL. A decrease in the risk of gastric cancer was also observed in wild homozygous genotype carriers [34], which corroborates the protective effect of the rs2505901 variant. It is important to note here that only a few studies have investigated the role of this polymorphism in the development of

cancer, in general, and that the present study is the first to evaluate its role in ALL.

Chromosome abnormalities have an important role in the predisposition, prognosis, and treatment of ALL [35]. In the present study, a majority (53.57%) of the patients did not present any of the chromosomal translocations investigated. Even so, 45.24% of the translocations observed were of high or intermediate risk (BCR-ABL, E2A-PBX1, ETV6-RUNX1, MLL-AF4) [36, 37].

Conclusions

Overall, then, the present study provides convincing evidence of the influence of the rs3805500 (DROSHA), rs3746444 (MIR499), and rs2505901 (MIR938) variants on the susceptibility of the Brazilian Amazon population to ALL. From a methodological viewpoint, the use of SNPs as biomarkers is more practicable than the analysis of mRNA expression, given that germline variants can be investigated through the analysis of peripheral blood, requiring less costly laboratory procedures. Our findings are fundamental to the better understanding of the susceptibility of the population of the Brazilian Amazon region to ALL. This is an important advance, given the unique, ethnically diverse background of this population, which is quite distinct from the more homogeneous populations, which have been the focus of most previous ALL research.

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Disclosure of conflict of interest

None.

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Table S1. References used for selection of the investigated genes and polymorphisms

GENE	Variant ID	References
AGO1	rs636832	[38-40]
DROSHA	rs10035440	[20, 41]
	rs3805500	[19-21]
MIR219-1	rs107822	[42, 43]
	rs213210	[31, 43]
MIR146a	rs2910164	[12, 44]
MIR300	rs12894467	[45, 46]
MIR499a	rs3746444	[12, 26]
MIR608	rs4919510	[47, 48]
MIR938	rs2505901	[31, 34]

Table S2. Characterization of the polymorphisms analyzed in the present study and their quality control parameters

GENE	rs	ALLELE	FUNCTION	QUALITY CONTROL			
				MAF	Genotyping rate	HWE*	Status
AGO1	rs636832	A>G	Intron variant	36%	80%	0.33887	Included
DROSHA	rs10035440	T>C	Intron variant	15%	81%	1.0000	Included
DROSHA	rs3805500	G>A	Intron variant	49%	83%	0.20689	Included
MIR146a	rs2910164	G>C	Mature miRNA variant	29%	86%	0.12694	Included
MIR219-1	rs213210	A>G	Regulatory region variant	17%	89%	0.02196	Included
MIR219-1	rs107822	C>T	TF binding site	37%	80%	0.07143	Included
MIR300	rs12894467	T>C	Non-coding transcript exon variant	39%	90%	0.00204	Included
MIR499a	rs3746444	A>G	Mature miRNA variant	18%	86%	0.11173	Included
MIR608	rs4919510	G>C	Mature miRNA variant	36%	86%	1.0000	Included
PRE-MIR-938	rs2505901	C>T	Intron variant	40%	81%	0.00859	Included

*Significance adjusted for multiple comparisons by the Bonferroni correction (P≤0.001).