

Analysis of possible genetic risk factors contributing to development of childhood acute lymphoblastic leukaemia in the Latvian population

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Abstract

Introduction: Childhood acute lymphoblastic leukaemia (ALL) is a complex disease caused by a combination of genetic susceptibility and environmental exposure. Previous genome-wide association studies have reported several single nucleotide polymorphisms (SNPs) associated with the incidence of ALL. Several variations in genes encoding enzymes involved in carcinogenesis are suggested as being associated with an increased risk of ALL development.

Material and methods: We enrolled 77 paediatric ALL patients and 122 healthy controls, and in addition parental DNA was also available for 45 probands. SNPs rs10821936 (ARID5B), rs4132601 (IKZF1), rs2239633 (CEBPE), rs3731217 (CDKN2A) and rs1800566 (NQO1) and the presence of GSTT1 and GSTM1 null variants were detected. For statistical analysis the hybrid method of two designs 'Haplin' was used as well as linkage disequilibrium for family-based association studies.

Results: We identified the SNP rs10821936 in the ARID5B gene as being statistically significantly associated with childhood ALL, especially if the C allele is in a homozygous state, relative risk (RR) 4.65, 95% CI: 2.03–10.6, $p = 0.0006$. Statistically significant differences were not found in other SNPs. We found risk combinations including all five variations, the strongest association being found in a combination where all five genetic variants are in a homozygous state, CCTTTTTTCC, $p = 0.032$.

Conclusions: The identified SNP rs10821936 could serve as a potential risk marker for childhood ALL development. Further studies in an independent population are needed for verification.

Key words: childhood acute lymphoblastic leukaemia, single nucleotide polymorphisms, ARID5B, IKZF1.

Introduction

Acute lymphoblastic leukaemia (ALL) is the most frequently occurring haematopoietic malignancy in children worldwide [1], with cure rates increasing from less than 10% in the 1960s to over 80% nowadays [2]. Acute lymphoblastic leukaemia is a heterogeneous group of disorders that arise from the clonal proliferation of malignant, lymphoid cells in bone marrow, blood and other organs [3]. Approximately 75% of child-

hood ALL cases harbour a recurring chromosomal alteration detectable by karyotyping, fluorescent *in situ* hybridization (FISH), or molecular biology techniques. Although rearrangements are important in initiating events in leukemogenesis and are widely used in diagnosis and risk stratification algorithms, they are insufficient to fully explain leukemogenesis [4]. However, even if the genetic markers of ALL can be detected *in utero* (i.e., *ETV6-RUNX1* gene), this does not inevitably lead to ALL [5]. The development of childhood B-cell lineage ALL involves (at least) 2 genetic events (hits), the first of which frequently arises prenatally [6]. The development of leukaemia might be explained by a combination of genetic susceptibility and environmental exposure during early development in fetal life and infancy [7].

The heritable basis of susceptibility to ALL is further supported by recent candidate gene and genome-wide association studies (GWAS), suggesting that co-inheritance of multiple germline variants may contribute to the risk of the disease. Five GWAS have been performed with populations of 3 275 ALL cases and 4 817 healthy control individuals [8]. Four independent loci have been shown conclusively to be associated with ALL, and more specifically the B-cell precursor (BCP) ALL risk was associated with the particular SNPs located in 7p12.2 (*IKZF1*), 9p12 (*CDKN2A/CDKN2B*), 10q21.2 (*ARID5B*) and 14q11.2 (*CEBPE*). While the risk of ALL associated with these common variants is not insignificant (relative risk (RR) of 1.5–1.6), collectively they underscore 8–24% of the genetic variance in BCP-ALL risk [9]. The first GWAS performed by Trevino *et al.* evaluated 307 944 germline single nucleotide polymorphisms (SNPs) and identified 18 SNPs whose allele frequency differed ($p < 1 \times 10^{-5}$) between a paediatric ALL population and non-ALL controls. The strongest association was shown by the SNP rs10821936 located within intron 3 of the *ARID5B* gene [10]. AT-rich interactive domain 5B (*ARID5B*) belongs to the ARID family of transcription factors and plays an important role in the regulation of embryonic development and cell growth and differentiation [11]. However, the role of *ARID5B* in childhood ALL remains unknown, although alterations in gene function may contribute to an increased risk for this disease [12].

The second GWAS study showed that the IKAROS family zinc finger 1 (*IKZF1*) germline polymorphism rs4132601 is associated with inherited risk of paediatric ALL [13]. The product of the *IKZF1* gene is Ikaros, a sequence-specific DNA-binding protein with a zinc finger motif, which plays a role in immune homeostasis through the transcriptional regulation of the earliest stages of lymphocyte ontogeny and the differentiation by both gene

transcriptional activation and repression [14]. The third association seen was at 14q11.2, with rs2239633 in the vicinity of *CEBPE*, encoding the CCAAT/enhancer binding protein ϵ , a regulator of myelopoiesis [15]. Sherborne *et al.* reported an association between rs3731217 located in intron 1 of the gene *CDKN2A* (cyclin-dependent kinase inhibitor 2A) and childhood ALL [16].

One potentially important genetic pathway, besides the polymorphisms detected by GWAS that is suspected to play a role in the etiology of childhood ALL, is xenobiotic metabolism [17]. Polymorphisms in genes encoding xenobiotic-metabolizing enzymes are largely responsible for interindividual differences in the ability to activate and detoxify mutagenic/carcinogenic agents, and therefore may influence the susceptibility to cancer [18]. Environmental procarcinogens are activated by phase I enzymes, converting them into reactive intermediates, which are then detoxified by phase II enzymes including glutathione S transferase (GST) [19]. Functional polymorphisms have been reported in the genes coding GSTs; 20–50% of individuals do not express the enzyme *GSTT1* and/or *GSTM1* due to homozygous deletion [20]. Individuals with the *GSTT1* null and *GSTM1* null variant are unable to eliminate carcinogens effectively [21, 22]. For example, they are unable to detoxify tobacco-related carcinogens by conjugating glutathione to facilitate their removal [23], and can therefore be a candidate risk factor for childhood ALL.

The NQO1 (NAD(P)H dehydrogenase quinone 1) protein protects cells against quinine-induced oxidative stress. There have been more than 93 polymorphisms identified, the most widely studied being NM_000903.2:c.559C>T or previously c.609C>T (rs1800566). Homozygosity is associated with the loss of enzyme activity due to the instability of the protein product [24]. The heterozygous genotype showed a threefold decrease in enzymatic activity compared with the wild type allele [25]. NQO1 has been described as an anti-cancer enzyme because it plays a protective role in cancerogenesis by modifying internal exposure to bioactivated carcinogens [26], and in the case of a defective enzyme it might play a role in cancerogenesis.

Because of the possible roles of polymorphisms rs10821936 (*ARID5B*), rs4132601 (*IKZF1*), rs2239633 (*CEBPE*), rs3731217 (*CDKN2A*) – markers which showed the strongest association from GWAS data – and rs1800566 (*NQO1*), and null genotypes of genes *GSTT1* and *GSTM1*, we performed case control and case-parent triad studies to investigate the association between these genetic markers and childhood ALL susceptibility in the Latvian population.

Material and methods

Study subjects

Our cohort consisted of 76 patients with childhood B-cell progenitor cell ALL in complete remission, and 121 age- and gender-adjusted healthy controls. In addition, parental DNA was available for 45 probands. Out of 76 patients, 41 were male and 35 were female. Patients were diagnosed with ALL between 2005 and 2014, aged 1–18 years at that time. According to the data of the Latvian cancer patient registry, at the time of the study 85 patients with a diagnosis of ALL were alive (8 of them refused to take part in this study and 1 patient was not in full remission); therefore our study covered 89.4% of all patients. Parents signed an informed consent form, and the study was approved by the Central Medical Ethics committee of Latvia.

Genotyping

Peripheral blood from the patients and their biological parents and controls was used for DNA extraction. DNA isolation from white blood cells was performed using the standard phenol/chloroform method [27].

Genotyping of polymorphisms rs4132601, rs2239633, rs3731217, and rs1800566 was performed by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) assay; primer sequences and restriction enzymes are shown in Table I. Genotype of the polymorphism rs10821936 was detected by Sanger sequencing; for the initial PCR we used external primers (F: 5'-CAGTGAGAGCGAGACTGCAC-3'; R: 5'-TCCTGGCAATGTTTTTCCACA-3'), while for the second amplification an internal primer was used (F: 5'-ACACCACCCCATCTCAAT-3'). The direct sequencing of PCR products was performed using ABI Prism BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster city, USA) using the manufacturer's protocol.

Multiplex PCR was performed to detect the presence or absence of *GSTT1* and *GSTM1* null genotypes [28].

Statistical analysis

Statistical analysis using the hybrid method for the case-control study and case-parent study was performed using the R program package "Haplin" [29]. To analyse case-parent trios the family-based association test (FBAT) by the transmission distortion test (TDT) and the haplotype-based TDT were performed, as implemented in the software PLINK 1.07 [30]. To separately analyse the case-control model the software JavaStat (available online) was used, the Fisher exact test was used for statistical analysis, and a *p*-value < 0.05 was considered as statistically significant.

Results

All five analysed polymorphisms, rs10821936 (*ARID5B*), rs4132601 (*IKZF1*), rs2239633 (*CEBPE*), rs3731217 (*CDKN2A*) and rs1800566 (*NQO1*), were in Hardy-Weinberg equilibrium.

Linkage and case-control studies showed a significant association between ALL and rs10821936, which maps to intron 3 of the *ARID5B* gene. We did not find any significant association with rs4132601, which maps within the 3' UTR region of *IKZF1*, as well as rs2239633 in the locus of the *CEBPE* gene. When a hybrid statistical method (results shown in Table II) was used, statistical significance was more convincing than statistical analysis of separate association studies. The results of the family-based association study (TDT) are shown in Table III. The results of statistical analysis for case-control analysis are shown in Table IV. Separately analysing both designs showed the same results that the C allele of rs10821936 increases the risk of ALL, especially in a homozygous state.

Using the hybrid statistical method we also identified some risk-increasing combinations for

Table I. Polymerase chain reaction primer sequences and restriction enzymes

Polymorphism	Primer sequence	Annealing temperature [°C]	Restriction enzyme
rs4132601	Forward: 5'-TCATGGATTCTCTGCTCACA-3' Reverse: 5'-TTCTGGTTTGAGCTTTGTTGA-3'	59	<i>Mbol</i> (Thermo Fisher Scientific, Waltham, MA, USA)
rs2239633	Forward: 5'-GGGCTGGAAACGCACTAATA-3' Reverse: 5'-CCACTCTGAGCAACCTCCTC-3'	60	<i>Bsh1236I</i> (Thermo Fisher Scientific, Waltham, MA, USA)
rs3731217	Forward: 5'-CATGTAGATGGTGCCCTGA-3' Reverse: 5'-CCCCTCTCAAATATGCTGTCC-3'	55	<i>MvaI</i> (Thermo Fisher Scientific, Waltham, MA, USA)
rs1800566	Forward: 5'-TGAGAAGCCCAGACCAACTT-3' Reverse: 5'-TCTCCAGGCGTTTCTCCAT-3'	60	<i>HinfI</i> (Thermo Fisher Scientific, Waltham, MA, USA)

Table II. Hybrid analysis of SNP alleles in family trios and case-controls

Polymorphism	MAF	Dose	RR	95% CI	P-value
rs10821936	C	Single	1.3	(0.69–2.2)	0.44
		Double	4.65	(2.03–10.6)	0.0006
rs4132601	G	Single	1.03	(0.58–1.78)	0.92
		Double	1.36	(0.54–3.3)	0.5
rs2239633	T	Single	0.91	(0.52–1.63)	0.76
		Double	0.74	(0.33–1.65)	0.47
rs3731217	G	Single	0.77	(0.37–1.65)	0.5
		Double	–	–	
rs1800566	T	Single	0.94	(0.53–1.66)	0.7
		Double	0.57	(0.05–4.5)	0.33

Table III. Family-based association study TDT test

Polymorphism	OR (95% CI)	P-value
rs10821936	2.07 (1.07–4.03)	0.027
rs4132601	0.73 (0.4–1.32)	0.3
rs2239633	0.62 (0.35–1.12)	0.11
rs3731217	1 (0.32–3.1)	1
rs1800566	0.8 (0.38–1.67)	0.68

previously described SNPs (SNP order in genotype: rs10821936, rs4132601, rs2239633, rs3731217 and rs1800566); genotype CCTCCTTCC was sta-

tistically significantly more frequently observed in affected children ($p = 0.046$). Similar results were obtained for genotypes CCGGCCTTCC ($p = 0.049$) and CCTTTTTTCC ($p = 0.032$).

The *GSTT1* and *GSTM1* null allele detection method has a limitation in that this method only detects the presence of the null genotype in a homozygous state. For statistical analysis we could only compare the frequency of the null genotype versus the non-null genotype. There was no statistically significant association between the risk of childhood acute leukaemia and polymorphisms of *GSTM1* and *GSTT1*. The *GSTT1* null variant was observed in 27.6% of cases and in 18% of controls

Table IV. Frequency of SNPs in the patient and control groups

Polymorphism	Allele	ALL cases	Controls	OR (95% CI)	P-value
rs10821936	CC	14 (18.7%)	9 (7.5%)	2.83 (1.1–7.59)	0.02
	CC + CT	38 (50.6%)	40 (33.3%)	1.49 (0.8–2.78)	0.19
	C	52 (34.6%)	71 (29.6%)	1.67 (1.1–2.67)	0.028
rs4132601	GG	8 (10.6%)	7 (6.1%)	1.83 (0.56–5.9)	0.28
	GG + GT	40 (53.3%)	51 (44.7%)	1.41 (0.75–2.65)	0.3
	G	48 (32%)	58 (25.4%)	1.38 (0.85–2.23)	0.2
rs2239633	TT	13 (17.1%)	23 (19.1%)	0.87 (0.38–1.96)	0.85
	TT + CT	51 (67.1%)	75 (62.5%)	0.84 (0.43–1.64)	0.63
	T	64 (42.1%)	108 (45%)	0.89 (0.58–1.37)	0.6
rs3731217	GT	12 (16.2%)	26 (21.8%)	0.69 (0.3–1.56)	0.36
	G	12 (8.1%)	26 (10.9%)	0.72 (0.33–1.55)	0.48
rs1800566	TT	1 (1.3%)	5 (4.1%)	0.31 (0.01–2.8)	0.41
	TT + CT	22 (28.9%)	42 (30.6%)	0.82 (0.42–1.58)	0.54
	T	2	10	0.78 (0.44–1.38)	0.42

($p = 0.16$, OR = 1.69, 95% CI: 0.8–3.53), and the *GSTM* null variant was observed in 55.8% of cases and in 47.9% of controls ($p = 0.3$, OR = 1.37, 95% CI: 0.74–2.54).

Discussion

Acute lymphoblastic leukaemia is a multifactorial disease influenced by genetic and environmental factors. In this study we investigated whether SNPs which were previously reported in GWAS studies influence the risk of ALL in the Latvian population. We chose the most studied cases, because Xu *et al.* hypothesized that the genetic predisposition to ALL might be largely mediated by the robust effects of a modest number of key genes, rather than the cumulative effect of tens of thousands of variants with small effects [31]. We also studied the influence of xenobiotic metabolism involved genes as a possible risk factor. Although our cohort covered 89.4% of all patients, an important limitation of this study was the sample size. This may have influenced our ability to detect small associations. In this study we were able to replicate the results from previous studies, showing that rs10821936 increases the susceptibility to childhood ALL. As is shown in the literature, this SNP has a strong association with childhood ALL [32, 33]. In our study we used the hybrid method to improve statistical significance and join two designs, and statistical analysis showed a stronger association than the designs separately. Analysis of our data showed that individuals with genotype CC have a statistically significantly increased risk of development of ALL, whereas statistical significance was lost if the individual was heterozygous. It is thought that gene variants have additive effects; someone inheriting one copy of a variant will have a roughly 50% increased risk, whereas someone who inherits all four variants in homozygous form would have a 10-fold increased risk [34].

In our study, rs4132601 in *IKZF1* and rs2239633 in *CEBPE* did not seem to affect the susceptibility to childhood ALL, although an association was found in previous GWAS studies [13] using data from Asian colleagues Wang *et al.* in the Chinese population, and Lin *et al.* in Taiwanese children. They have published data where these polymorphisms do not show any significant difference between cases and controls [11, 35]. Moreover, Wang *et al.* reported results of a cohort which consisted of 570 individuals, so small sample size could not be a limitation of that study. Vijayakrishnan *et al.* reported on the racial differences between risk allele frequency in Asians and Caucasians [36]. As we belong to the European population our results should be similar to other European countries, but the only available published data are from Poland, Pastorczak *et al.*, who only iden-

tified rs4132601 as a possible risk variant, and they could not replicate the reported associations with rs2239633 or rs3731217, nor could we [37]. Our results could be explained by the limited sample size. Although we are located close to Poland geographically, our ethnic origin is not as closely related as shown in a paper published by Nelis *et al.* [38]. To reach more reliable conclusions, further investigations closer to our gene pool are needed. However, Healy *et al.*, from Canada, in a group of 284 childhood ALL patients, also were unable to replicate the reported associations with *IKZF1* and *CEBPE*. Their group explained the lack of replication by the limited power of their study to detect loci with weaker effects [39]. In our study we were unable to find statistically significant differences between the *GSTT1* and *GSTM1* null variants and the risk of childhood ALL. As reported in the literature, based on a meta-analysis performed by Xu *et al.* including 2 424 cases, the *GSTT1* null variant is strongly associated with the risk of childhood leukaemia in Asians, but there is no association in Caucasians [21]. Similar results about the *GSTM1* null variant were reported in a meta-analysis by Tang *et al.*, who found an overall association with ALL that was lost in Caucasians after stratification by ethnic groups [40], which might explain the lack of statistical significance in our study. Similarly, we could not find any association with another polymorphism involved in xenobiotic metabolism.

Although the meta-analysis performed by Han *et al.* showed an association with polymorphism 609 C>T (rs1800566), especially in a Caucasian population, they report that there is no association between *NQO1* polymorphism and childhood leukaemia [41]. These results showed a similarity with ours, especially taking into consideration that only 18 out of 77 children in our ALL cohort were older than 10 at the time of the ALL diagnosis. We might hypothesize that our cohort was too young to observe the possible impact of polymorphisms involved in xenobiotic metabolism.

In conclusion, overall, the *ARID5B* SNP rs10821936 has a significant association with the risk of childhood ALL – this polymorphism could serve as a genetic susceptibility marker for ALL. The cohort should be enlarged to estimate the real effect of other SNPs, or if Estonian and Lithuanian samples could be available to validate the effect of these SNPs in neighbouring populations.

Conflict of interest

The authors declare no conflict of interest.

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