RESEARCH ARTICLE

Lack of Association between Polymorphisms in Genes *MTHFR* and *MDR1* with Risk of Childhood Acute Lymphoblastic Leukemia

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is a complex disease caused by interactions between hazardous exogenous or/and endogenous agents and many mild effect inherited susceptibility mutations. Some of them are known, but their functional roles still requireinvestigation. Age is a recognized risk factor; children with disease onset after the age of ten have worse prognosis, presumably also triggered by inherited factors. Materials and Methods: The *MDR1* gene polymorphisms rs1045642, rs2032582 and *MTHFR* gene polymorphisms rs1801131 and rs1801133 were genotyped in 68 ALL patients in remission and 102 age and gender matched controls; parental DNA samples were also available for 42 probands. Results: No case control association was found between analyzed polymorphisms and a risk of childhood ALL development. Linkage disequilibrium was not observed in a family-based association study either. Only marginal association was observed between genetic marker rs2032582A and later disease onset (p=0.04). Conclusions: Our data suggest that late age of ALL onset could be triggered by mild effect common alleles.

Keywords: Lymphoblastic leukemia - childhood cases - MDR1 - MTHFR - polymorphisms

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood (Tharnprisan et al., 2013). Its annual incidence rate is approximately 9-10 cases per 100.000 people in childhood, with peak incidence between the age of two and five years (J Yan et al., 2012). More than 80% of children diagnosed with ALL can be cured with current multiagent regimens, however, subsets of patients experience significantly worse outcomes; one of the risk factors for worse prognosis is age of onset - 10 years and older (Bhojwani et al., 2009). There are some studies where single nucleotide polymorphisms have been studied as possible risk factors associated with the later age of onset. Hattori and colleagues found statistically significant difference from *MDR1* gene polymorphism G2325A and later age of onset in a group of 157 patients from Japan, and their hypothesis suggested that after the age of six the expression level of *MDR1* is sufficiently high and may be considerably altered by polymorphisms (Hattori et al., 2007). Gorniak et al. reported association between age of onset and polymorphism rs4132601 in the gene *IKZF1* in a group of 508 Polish patients (Gorniak et al., 2014).

Despite numerous studies very little is still known about etiology of ALL. It is thought that initiation of leukemogenesis occurs during fetal life or in early infancy and is likely caused by interactions between exogenous or endogenous exposures, genetic (inherited) susceptibility (Healy et al., 2010; Inaba et al., 2013). One potentially important genetic pathway suspected in playing a role in childhood ALL is xenobiotic metabolism. When an individual is exposed to various foreign chemicals, enzymes involved in xenobiotic metabolism are responsible for the elimination of these compounds through phase I (e.g. oxidation) and phase II (e.g. conjugation) (Chokkalingam et al., 2012; Nousome et al., 2013). One of the physiological roles of P-glycoprotein is the protection of the organism against toxic xenobiotics. A proportion of xenobiotics transported by P-gp are associated with mutagen activity. It can be hypothesed that genetically based differences in P-gp may result in varying exposure to environmental carcinogens, with lower activity linked to increased risk of malignancy (Semsei et al., 2008). A number of single nucleotide polymorphisms (SNPs) in *MDR1* gene were shown to result in altered expression and function of P-glycoprotein (Zhai et al., 2012). *MDR1* is a polymorphic gene and more

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than 50 SNPs have been reported (Ren et al., 2012) the most frequently reported SNPs G2677T/A (rs2032582) leads to amino acid exchange from alanine to serine or threonine and the silent mutation C3435T (rs1045642) (Wang et al., 2013). The SNP rs1045642 was found to be associated with altered P-glycoprotein activity in vitro and in vivo (Wu et al., 2014).

The folate metabolic pathway is suspected to play an important role in development of childhood ALL as it is critical for the synthesis, repair and methylation of DNA (Lupo et al., 2012). A critical component of the folate metabolic pathway is an enzyme methylene tetrahydrofolate reductase (*MTHFR*), which controls the balance between DNA methylation and synthesis via the irreversible conversion of 5, 10- methylenetetrahydrofolate (5,10-MeTHF) (Lightfoot et al., 2010). *MTHFR* gene is localized on chromosome 1p36.3. Two most commonly described SNP's are: *MTHFR* C677T (rs 1801133) resulting in alanine to valine substitution and A1298C (rs 1801131), causing glutamate to alanine substitution (Zintzaras et al., 2012). Studies showed that heterozygous individuals of polymorphism C677T have 70% of normal enzyme activity, but homozygous - only 30% of normal enzyme activity (Jain et al., 2012).

Materials and Methods

Study subjects

Our cohort consisted of 68 patients with childhood B cell ALL in a complete remission and 102 age and gender adjusted healthy controls. In addition, parental DNA was available for 42 probands. Out of 68 patients 35 were males and 33 females. 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 moment of study 78 patients with the diagnosis of ALL were alive (10 of them refused to take part in this study), therefore our study covered 87.1% of all patients. Parents signed an informed consent, and the study was approved by Central medical Ethics committee of Latvia.

To evaluate the age-related effect of the polymorphism on the development of ALL, patients were divided according to the age of the onset. As there were no children younger than one year, we divided children in two groups: younger than 10 years and aged 10 years and older, there were 50 and 18 patients in the groups, respectively.

Genotyping

Peripheral blood from the patients and their biological parents and controls was used for DNA extraction. DNA isolation was performed using standard phenol/chloroform method (John et al., 1991).

Genotyping of polymorphism was performed using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) assay used primer sequences and restriction enzymes shown in table 1.

Statistical analysis

Data analysis and quality control for the genotyping data were performed by PLINK software (Purcell et al., 2007). For cases and controls allelic and genotyping association tests were performed. Linear regression method was used to detect genotype impact on the age of onset. The allele and genotype frequency in case and control groups were compared by the χ^2 (chi-square) test, significance threshold applied when $p<0.05$. For haplotype analysis - haplotype based C/C test was used.

To analyze case - parent trios Family Based Association test (FBAT) - by transmission distortion test (TDT) and haplotype based TDT was performed, as implemented in PLINK 1.07.

Results

All four analyzed polymorphisms in genes *MTHFR* (rs1801131, rs1801133) and *MDR1* (rs1045642, rs2032582) were in Hardy-Weinberg equilibrium.

Table 2 shows the frequency of the analysed alleles and genotypes in patients with ALL and in the healthy control subjects. No significant differences were observed in allelic frequencies between cases and controls. There were no statistical differences when haplotype statistical analysis was performed, possible risk haplotypes were made from two SNPs localized in one chromosome.

We were unable to find significant results in allele transmission distortion test using linkage studies.

The allele frequency of each polymorphism was compared between patients and controls in age group younger than 10 years and group aged 10 years and older. Patients aged 10 years or older showed higher A allele frequency (G2667T/A) than younger age group (OR 0.26 (0.06-1.022); p=0.04 Table 3). However, linear regression analysis did not confirm finding p=0.09, p value adjusted by sex p=0.16. Frequency of A allele was statistically significantly different between age group ≥10 and controls (MAFaffected = 0.14, MAFunaffected = 0.03, OR 6.29

Table 1. PCR Primers and Restriction Enzymes

Polymorphism	Primer sequence	Restriction enzyme	Reference
C3435T	Forward: 5' TGT TTT CAG CTG CTT GAT GG 3' Reverse: 5' AAG GCA TGT ATG TTG GCC TC 3'	MboI (Thermo Fisher Scientific, Waltham, MA, USA)	(Kimura et al., 2005)
G2667T	Forward: 5' TTT GCA GGC TAT AGG TTC CAG 3' Reverse: 5' TTT AGT TTG ACT CAC CTT CCC G 3'	BanI (Thermo Fisher Scientific, Waltham, MA, USA)	(Ayaz et al., 2013)
G2677A	Forward: 5' TCA GAA AAT AGA AGC ATG AGT TG 3' Reverse: 5'AGC AGT AGG GAG TAA CAA AAT AAC 3'	BSrI (Thermo Fisher Scientific, Waltham, MA, USA)	(Alpman et al., 2010; Kim et al., 2006)
C677T	Forward: 5' TGA AGG AGA AGG TGT CTG CGG GA 3' Reverse: 5 AGG ACG GTG CGG TGA GAG TG 3	HinfI (Thermo Fisher Scientific, Waltham, MA, USA)	(Safarinejad et al., 2012)
A1298C	Forward: 5'CTT CTA CCT GAA GAG CAA GTC 3' Reverse: 5'CAT GTC CAC AGC ATG GAG 3'	MboII (Thermo Fisher Scientific, Waltham, MA, USA)	(Hanson et al., 2001)

Table 2. Comparison of Genotype and Allelic Frequencies in the MTHFR and MDR1 Genes in ALL Children and Healthy Controls

SNP	Genotype/ Allele	ALL (n=68)	Controls (n=102)	OR (95% CI)	P value
rs1801133	CC	0.47 (32 /68)	0.47 (48/102)		
	CT	0.46 (31/68)	0.43 (44/102)		0.84
	TT	0.07 (5/68)	0.1 (10/102)		
	T	0.3	0.31	0.94 (0.59-1.51)	0.81
rs1801131	AA	0.52 (35/68)	0.45 (46/102)		
	AC	0.41 (28/68)	0.42 (43/102)		0.48
	CC	0.07 (5/68)	0.13 (13/102)		
	C	0.28	0.34	0.76 (0.47-1.22)	0.25
rs1045642	CC	0.31 (21/68)	0.26 (27/102)		
	CT	0.43 (29/68)	0.5 (51/102)		0.64
	TT	0.26 (18/68)	0.24 (24/102)		
	T	0.48	0.49	0.97 (0.22-0.63)	0.89
rs2032582	AT	0.07 (5/68)	0.05 (5/102)		
	AG	0.06 (4/68)	0.03 (3/102)		
	GT	0.37 (25/68)	0.42 (43/102)		
	TT	0.16 (11/68)	0.2 (21/102)		Other*/TT 0.68
	GG	0.34 (23/68)	0.31 (32/102)		Other**/GG 1
	A	0.07	0.04	1.74 (0.65-4.6)	0.26

*All possible identified genotypes (AT, AG, GG, GT) versus TT genotype; ** All possible identified genotypes (AT, AG, GT, TT) versus GG genotype

Table 3. Impact of the Polymorphisms in different Age Groups

SNP	Minor allele	Patients <10 years of age	Patients ≥10 years of age	OR (95% CI)	p value
rs 1801131	C	0.29	0.25	1.22 (0.51-2.92)	0.64
rs 1801133	T	0.29	0.33	0.82 (0.36-1.85)	0.62
rs1045642	C	0.44	0.58	0.56 (0.26– 1.21)	0.14
rs2032582	A	0.04	0.14	0.26 (0.06-1.022)	0.04

p=0.007), while no significant difference was observed between age group <10 and controls younger than 10 years (MAFaffected = 0.04, MAFunaffected = 0.06, OR 0.66 p=0.54).

Discussion

Acute lymphoblastic leukemia is a multifactorial disease influenced by genetic and environmental factors. In this study we investigate whether functional polymorphisms in genes coding P glycoprotein and methylenetetrahydrofolate reductase might alter the susceptibility to childhood ALL or influence age of onset.

It must be noted that our study had some limitations. One of them is that our study population is biased, as patients who died or underwent bone marrow transplantation before our sample - collection period are underrepresented, thus it is possible that the conclusions of this study would have been modified if all the patients had been involved. The second is a small number of patients: although we had high patient compliance (87.1%), the population of our patients was very small and this factor could modify study results.

There is a number of possible causes that could explain discrepant results of research on ABC transporters polymorphisms and susceptibility to childhood ALL. Some of these causes are simply related to the nature of these candidate gene approach studies. It is clear that individual variations in environmental exposure may mask the effects of genetic polymorphisms (Jamroziak et al., 2008).

In recent years, many articles have reported about the *MTHFR* polymorphism and susceptibility to ALL, but the results are still controversial. Most of the studies had bigger cohort than ours, but Li and colleagues included 98 patients in their study and found that risk of ALL with the 1298C allele carriers (AC+CC) was elevated by 1.1 times compared with AA genotype (Li et al., 2014). Giovannetti et al. showed in their study ethnic differences in polymorphisms (Giovannetti et al., 2008). There are publications about several meta-analyses focused on the associations between *MTHFR* C677T and A1298C polymorphisms and ALL risk. They support the idea that polymorphisms might influence susceptibility; two groups published their meta-analysis in the same year (2012) and both of them found marginally significant association for the C677T variant and susceptibility to ALL (H Wang et al., 2012; Zintzaras et al., 2012). Wang et al. already showed ethnical differences in susceptibility. More clearly it is pointed out in most recent meta-analysis performed by Jiang et al. in 2013, where individuals with homozygous TT genotype had decreased risk of ALL in Caucasians, however the results among Asians did not show any association (Jiang et al., 2013). In Eastern Europe data for this study was only available in Slovenia no data from the Baltic States that might explain statistical differences are available. To our knowledge, this is first attempt to evaluate an association between *MTHFR* polymorphisms and leukemia risk in children in Latvia and in the entire Baltic region, and we were unable to find any published data about Lithuanian and Estonian population.

Allele frequencies of the *MDR1* C3435T polymorphism have been evaluated around the world, and significant interpopulation differences have been detected (Leal-Ugarte et al., 2008). Although association studies suggest statistically significant association between polymorphism C3435T, we did not find any association in our study, it should be considered that the apparent inconsistency of results may be caused by differences in disease prevalence, as well as possible limitations due to relatively small sample size (Wang et al., 2012; Wang et al., 2013).

Some studies suggest that functional polymorphisms in gene *MDR1* might influence the course of the disease, as it has been reported that children carrying the CT (C3435T) genotype are more prone to develop oral mucosistis (Bektas-Kayhan et al., 2012).

Meta-analysis performed by Yan et al., suggested that there was no association between *MDR1* G2677T polymorphism and leukemia risk in overall populations (Yan et al., 2014), but this analysis included also adult patients, and there were no data available about the third possible allele. A allele presence is very rarely detected in the studies, although A allele frequency was 7 % in our study. Several studies including ours did not find any difference in A allele frequency between cases and controls (Urayama et al., 2007). Rao and colleagues reported age related association of *MDR1* gene polymorphism C3435T, where TT genotype frequency was increased with age onset more than 10 years (Rao et al., 2010). To our knowledge, this is the first study where polymorphism G2677T/A is studied in association with age of the onset. As a consequence, this is the first time when A allele is found as a risk allele for later onset of the disease (age >10 years). Although statistical significance is not strong (p value is less than 0.05, but OR includes 1, and statistical significance is lost when linear regression analysis is performed) and larger amount of cases is required to make conclusions, there is a trend that this polymorphism may be a trigger for high risk ALL. In conclusion, we did not find significant association between polymorphisms in genes *MTHFR* and *MDR1* and increased risk of childhood ALL, but we found age-related association. This study needs to be continued in adult patient group and, if possible, in other Baltic countries.

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