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Original article

Screening of polymorphisms in the folate pathway in Turkish pediatric Acute Lymphoblastic Leukemia patients

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ABSTRACT

Background and aim: Folate metabolic pathway plays a significant role in leukemogenesis because of its necessity for nucleotide synthesis and DNA methylation. Folate deficiency causes DNA damage. Thus polymorphisms of folate-related genes may affect the susceptibility to childhood Acute Lymphoblastic Leukemia (ALL). *MTHFR* (Methylenetetrahydrofolate Reductase), *DHFR* (Dihydrofolate reductase), *CBS* (Cystathionine β-synthase) and *TYMS* (Thymidylate Synthase) have an important role in folate pathway because their activated variants modulate synthesis of DNA and levels of folate. In this study, we aimed to investigate whether polymorphisms in genes related to folate metabolic pathway influence the risk to childhood ALL.

Subject and methods: The patient groups who were diagnosed with childhood ALL at Losante Pediatric Hematology-Oncology Hospital and healthy control groups were included in the study. *MTHFR* 677 C-T, *MTHFR* 1298 A-C, *CBS* 844ins68, *DHFR* 19-bp and *TYMS* 1494del6 polymorphisms were screened. Genotyping of these polymorphisms was performed by Restriction Fragment Length Polymorphism (RFLP) analysis and Real Time Polymerase chain Reaction (Real Time-PCR).

Results: In total, we have screened 5 polymorphisms in the studied genes. The results were compared between childhood ALL patients and healthy groups. Genotype frequencies of *MTHFR* 677 C-T, *MTHFR* 1298 A-C, *CBS* 844ins68 and *DHFR* 19-bp del were similar for childhood ALL patients and healthy groups. However, statistical results showed that *TYMS* 1494del6 may be associated with ALL pathogenesis ($p < 0.001$).

Conclusion: We showed that *TYMS* polymorphism (rs2853542) may be associated with ALL pathogenesis. In addition, our results demonstrated that *MTHFR*, *DHFR* and *CBS* do not affect development of leukemia. Our study displays also importance as it is the first screening results to identify association with the studied polymorphisms in Turkish patients with childhood ALL and determination of the frequency in Turkish population.

1. Introduction

Folate is a water-soluble B vitamin included in one-carbon metabolism that plays an essential role in synthesis, repair and methylation of DNA. Several genes encoding metabolizing enzymes are involved in the folate metabolic pathway. Polymorphisms in these genes alter the folate status or cause alteration in folate distribution. Deficiency of folate leads to DNA strand breaks, chromosomal damage, reduced DNA repair, and aberrant DNA methylation, thus affecting the susceptibility of ALL in children. In the literature, some of polymorphisms in folate-related genes have been described [1–4].

MTHFR is located on chromosome 1p36, consisted of 11 exons. It is a key enzyme in folate pathway that catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-methyltetrahydrofolate acts as a carbon donor for DNA methylation [5,6].

The gene coding the DHFR cytosolic enzyme is located at the long arm of chromosome 5 at position 11.2. DHFR is an enzyme

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which reduces dihydrofolic acid to tetrahydrofolic acid, using -NADPH as a cofactor. Tetrahydrofolate is required for de novo synthesis of varieties of crucial metabolites involved in amino acids, lipids, pyrimidines, and purines [7,8].

TYMS gene is located on chromosome 18q11.32, consisted of 7 exons. The gene which encodes TYMS enzyme converts deoksitimidin monofosfat (dTMP) to deoksiuridin monofosfat (dUMP). This reaction is performed in the biosynthesis of pyrimidine required for DNA replication and repair. 5′ end of TYMS doesn't contain CAAT and TATA boxes [9,10].

The gene coding the CBS enzyme is located on chromosome 21q22.3, consisted of 23 exons. CBS enzyme is a member of the pyridoxal 5′ phosphate (PLP) family. CBS catalyzes the formation of cystathionine from serine and homocysteine. CBS uses the PLP as a cofactor [11,12].

MTHFR, DHFR, TYMS and CBS genes were chosen for screening in this study because of their critical function in folate pathway. Polymorphism in these genes can effect their activity in folate pathway and susceptibility to development of leukemia. Therefore our aim was to determine genotype distribution and allele frequencies of common polymorphisms in these genes in the Turkish childhood acute leukemia patients and their association with ALL.

2. Subjects and methods

2.1. Subjects

The study population consisted of two main groups; the first group was composed of childhood acute lymphoid leukemia patients. The second group which is a control group was composed of healthy individuals. Study population consisted of 180 patients aged between 1 and 15 years who were admitted to Losante Pediatric Hematology and Children's Hospital with the diagnosis of childhood acute leukemia.

Healthy groups were selected among healthy unrelated subjects from Turkey (n: 296). An informed written consent was obtained from all the patients' parents. The study is carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.2. DNA isolation and Genotyping

Blood samples were collected with EDTA (Ethylenediaminetetraacetic acid)-containing tubes and DNA was extracted from peripheral blood and bone marrow leukocytes with MagNA Pure automatic DNA isolation instrument (Roche Diagnostics, Manheim, Germany).

Genotyping of *MTHFR* C677T (rs1801133) and A1298C (rs1801131) polymorphisms analysis were performed by real-time PCR (RT-PCR). Genotyping of polymorphisms were screened with real time PCR using fluorescence melting curve detection analysis by means of the Light Cycler 480 II System (Roche Diagnostics, Manheim, Germany).

Primers and probes used in MTHFR polymorphisms were as follows: *MTHFR* C677T; forward primer: 5′-TGG CAG GTT ACC CCA AAG G-3′;reverse primer: 5′-TGA TGC CCA TGT CGG TGC-3′; anchor hybridization probe: 5′-LC–640-CGG GAG CCG ATT TCA TCA T-3′-PHO; Mutation probe: 5′-TGA GGC TGA CCT GAA GCA CTT GAA GGA GAA GGT GTC T-3′-Flu) (TIB MOLBION, Berlin, Germany). *MTHFR* A1298C; forward primer:5′- CTT TGG GGA GCT GAA GGA CTA CTA C -3′;reverse primer: 5′- CAC TTT GTG ACC ATT CCG GTT TG -3′; anchor hybridization probe: 5′-LC–640- CTC CTC CCC CCA CAT CTT CAG CAG -3′-PHO; Mutation probe: 5′- CTT CAA AGA CAC TTT CTT CAC TGG TC -3′-Flu.) (TIB MOLBION, Berlin, Germany).

Determination of 19-bp deletion polymorphism (rs1222809) of *DHFR* and 68-bp insertion polymorphism of *CBS* were performed using the conventional polymerase chain reaction. Then PCR products were analyzed by 3% agarose gel electrophoresis and stained with ethidium bromide. The deletion/insertion was determined based on the length of PCR products. Primers used in for DHFR and CBS polymorphisms are as follows: *DHFR* 19-bp deletion; forward primer-1: 5′-CCA CCG TCG GGG TAC CTG GG-3′; forward primer-2: 5′-ACG GTC GGG GTG GCC GAC TC-3′; reverse primer: 5′-AAA AGG GGA ATC CAG TCG G-3′.

CBS 844ins68; forward primer-1: 5′-CTG AAC ATT TAG GTC ATT ACC-3′; forward primer-2: 5′-ACG GTC GGG GTG GCC GAC TC-3′; reverse primer: 5′-TTT CAC ACG TTT TCC CTG C -3′.

To determine the 1494del6 *TYMS (*rs2853542) polymorphism, conventional PCR and RFLP methods were used. PCR products were digested with the FastDigest *HaeIII* (Fermentas, Lithuania) restriction enzyme. Primer used for the detection of *TYMS* 1494del6 were as follows; forward primer-1: 5′-CCA ATC TGA GGG AGC TGA GT-3′; reverse primer: 5′-CAG ATA AGT GGC AGT ACA GA-3′.

2.3. Statistical analysis

The chi-square test was used to compare categorical variables. P value of <0.05 was considered statistically significant. Allelic frequencies were calculated by gene-counting method. Genotype distribution was analyzed for Hardy-Weinberg expectations by using X^2 and Fisher's exact tests.

3. Results

Genotype distributions and frequencies of gene polymorphisms of *MTHFR, DHFR, TYMS* and *CBS* in Turkish children with acute leukemia were given in Table 1.

The prevalence of *MTHFR* polymorphisms were determined for 180 childhood ALL patients and 296 healthy individuals. No significant difference for *MTHFR* 677 C-T genotypes frequency between childhood leukemia patients and healthy group was found [(OR: 1.0 (0.6–1.5), CI%95; p = 0.8)].Similarly no difference was observed in the distribution of *MTHFR* 1298 A-C genotypes frequency between childhood ALL patients and healthy group [(OR: 1.48 (0.7–2.8), CI%95; p = 0.2)].

The prevalence of *DHFR* 19 bp deletion polymorphism was determined for 63 childhood ALL patients and 296 healthy controls. The prevalence of *DHFR* 19-bp deletion polymorphism was similar in the study groups [(OR: 0.8 (0.4–1.6), CI%95; p = 0.5)].

For the 1494del6 *TYMS* polymorphism, we found that 124 (% 91.17) of the 136 patients were heterozygous. Heterozygosities of 1494del6 TYMS polymorphisms demonstrated a significant difference between childhood ALL patients and healthy group [(OR: 0.09 (0.04–0.2) CI%95; p < 0.001).

When the *CBS* 844ins68 polymorphisms were examined, we observed that there was no significant difference in the frequency of heterozygosity genotype of *CBS* 844ins68 polymorphism between childhood ALL patients and healthy group [(OR: 0,9 (0.5–1,8) CI%95; p: 0.9) .

4. Discussion

ALL is the most common type of childhood malignancy. Genetic and epigenetic alterations play a major role in ALL pathogenesis such as gene mutations, deletions, translocations, and DNA methylation [1,13,14]. Folates are required for many cellular processes including methylation and synthesis of DNA. Deficiency of folate causes imbalance in DNA precursors, misincorporation of uracil

Table 1
The genotype distribution of polymorphisms in childhood ALL and healthy groups.

MTHFR 677 C-T	Childhood ALL n: 180 (%)	Controls n:296 (%)	OR/CI (%95)	p
Genotype				
CC	86 (47%)	141 (47.6%)	1	
CT	78 (45.2%)	131 (44.2%)	1.0 (0.6–1.5)	0.8
TT	16 (7.5%)	24 (8%)	0.9 (0.4–1.8)	0.7
Allele (%)				
C	0.69	0.69	1	
T	0.31	0.31	1.7 (1.30–2.23)	0.0001
MTHFR 1298 A-C	Childhood ALL n: 152 (%)	Controls n:55 (%)	OR/CI (%95)	p
Genotype				
A/A	64 (42.1%)	19 (35%)	1	
A/C	75 (49.3%)	33 (60%)	1.48 (0.7–2.8)	0.2
C/C	13 (8.5%)	3 (5%)	0.7 (0.2–3.0)	0.9
Allele (%)				
A	0.66	0.64	1	
C	0.34	0.36	1.10 (0.70–1.75)	0.68
CBS 844ins68	Childhood ALL n:133 (%)	*Controls n: 296 (%)	OR/CI (%95)	p
Genotype				
wild/wild	117 (90%)	261 (88%)	1	
wild/ins	16 (9.7%)	35 (11.8%)	0.9 (0.5–1.8)	0.95
ins/ins	–	–	0.4 (0.008–22.7)	0.5
Allele (%)				
wild	0.93	0.94	1	
ins	0.07	0.06	1.52 (0.98−2.39)	0.06
TYMS 1494del6	Childhood ALL n:136(%)	Controls n:296 (%)	OR/CI (%95)	p
Genotype				
−6 bp/−6 bp	8 (5.8%)	93 (31.4%)	1	
+6bp/−6 bp	124(92%)	147 (58.7%)	0.09 (0.04–0.2)	0.000001
+6bp/+6 bp	4(1.9%)	56 (18.9%)	1.2 (0.3–4.1)	0.9
Allele (%)				
−6 bp	0.51	0.56	1	
+6 bp	0.49	0.44	0.82 (0.62–1.10)	0.18
DHFR 19-bp del	Childhood ALLn:63(%)	Controls n:296 (%)	OR/CI (%95)	p
Genotype				
wild/ wild	30 (47%)	162 (54.7%)	1	
wild/del	26 (41.2%)	117 (39.5%)	0.8 (0.4–1.6)	0.5
del/del	7 (11.1%)	17 (5.7%)	0.4 (0.1–1.1)	0.1
Allele (%)				
wild	0.68	0.74	1	
del	0.32	0.26	0.74 (0.48–1.11)	0.15

into DNA and chromosome breakage. Thus, fast proliferating cells need high amount of folate [15].

In the literature, numereos studies researched in Turkish population, showed correlation between genetic polymorphisms of folate metabolism pathway in different diseases [16–20]. However, there is inadequate studies in development of childhood ALL [2,5,8–11,13,21–25,28,31].

Therefore in this study we screened 5 polymorphisms in *MTHFR, TYMS, DHFR* and *CBS* genes which are critical enzymes involved in folate metabolic pathway and influence the risk of several diseases associated with folate deficiency. We also investigated the association of these polymorphisms with the development of childhood ALL.

MTHFR is an enzyme associated with folate metabolism along with DNA methylation and synthesis. MTHFR gene polymorphisms are 677 C > T and 1298 A > C on the catalytic domain that are responsible for a decrease in enzyme activity in variant allele carriers. Studies involved in this polymorphisms were shown that folate deficiency is associated with leukemogenesis. There are some of studies which demonstrated that there are significant correlation between *MTHFR* polymorphisms and childhood ALL patients [1,13,14,27–30,32,33]. Kuzelicki et al. reported that decreased enzyme activity of *MTHFR* 677 TT genotype was associated with Slovenian childhood ALL [32]. Another study preformed by Damnjanovic et al. revealed that there was an important association between CT + TT haplotype and reduced risk of Serbian childhood ALL. In addition, *MTHFR* 1298 polymorphism did not demonstrate significant statistical difference in their study groups [33].However, in our study, comparison of healthy group and childhood ALL patients revealed no statistically significant difference in the 677 C-T and 1298 A-C genotype distribution. Our findings have been supported by other studies which found no significant difference in *MTHFR* 677 C-T and 1298 A-C frequencies between childhood ALL patients and healthy group [21–24]. A meta-analysis study was presented by Wang et al. did not find evidence for a main role of *MTHFR* C677T in the leukemogenesis of childhood ALL [25].

DHFR is one of the key folate-metabolizing enzymes that is necessary for DNA synthesis and homocysteine remethylation. The 19 bp deletion polymorphism in intron 1 was associated with a lower plasma level of homocysteine. The 19-bp wild type genotype includes a binding site of Sp1 transcriptional factor at the intron 1 [8]. This polymorphism plays a role in the transcription of DHFR and affects the amount of produced protein [26,34]. In our study, screening of *DHFR* 19-bp del showed no statistically significant difference in childhood ALL patients compared to the healthy groups

[(OR: 0.8 (0,4–1,6), CI%95; p = 0.5)]. Alessia et al. reported that DHFR 19-bp deletion was brought 2.42 fold increased risk in adult ALL [35]. But, to the best of our knowledge, there is no study that investigates the association between childhood ALL and DHFR 19-bp deletion.

TYMS enzyme converts dUMP to dTMP required for DNA-synthesis [28]. The *TYMS* polymorphism, a 6-bp insertion/deletion (ins/del) is located at bp 1494 nucleotid in the region specifying the mRNA 3′UTR. This polymorphism has been proposed to affect mRNA stability and translation [9,30]. It has been proposed that the other polymorphism, tandem repeat 2R 3R, in 5′ UTR of *TYMS* have an association with development of leukemia [36]. In the present study, we found that the leukemia risk was significantly high in cases that carry heterozygote *TYMS* polymorphism (p = 0.001). Milne et al. found that homozygosities of *TYMS* 6-bp ins/ins genotype brought a risk of leukemia between Australian childhood ALL and healthy group (OR 1.8 (0.3–9.2) [30]. Therefore, our findings supported their statistical results. However, Krajinovic et al. screened *TYMS* polymorphisms in childhood ALL patients and found no correlation with the development of leukemia. *TYMS* 3R/3R genotype had an effect on EFS (Event Free Survival) in Canadian childhood ALL patients while 6 bp ins/del polymorphism did not affect EFS [9].

The CBS enzyme is associated with trans-sulfuration pathway, stimulating irreversible catalysis of homocysteine to cystathionine. Previous studies reported that *CBS* 844ins68 polymorphism affected activity of the enzyme and caused reduction in the levels of homocysteine. In the present study, we found no significant differences in the genotype frequency for 844ins68 polymorphism between cases and healthy group (p = 0.5). A study performed by Milne et al. have found that 55 (14%) of the 392 Australian childhood ALL patients carried heterozygous genotype (OR 0.79 0.53–1.17) [30]. Franco et al. displayed same genotype frequency between Brazilian childhood ALL patients and healthy group. OR 1(0.4–2.2) [31]. We suggested that the *CBS* 844ins68 polymorphism was not associated with childhood ALL patients.

In this study, we showed that polymorphisms of folate related genes except *TYMS* do not affect development of leukemia because the allele distributions are similar in childhood ALL patients and healthy group. Polymorphisms of *MTHFR, CBS* and *DHFR* genotype frequencies in study groups are similar. But importantly our results showed that *TYMS* polymorphism may bring a risk for leukemia. Doing further studies will be beneficial to verify these results with a larger group of patients and in different populations.

5. Conclusions

Our work carries scientific importance in three ways: First, the screened polymorphisms in this article seem to be the first results of studied genes in Turkish childhood ALL patients. Second, MTHFR 677 C-T, MTHFR 1298 A-C, CBS 844ins68 and DHFR 19-bp polymorphisms did not play an important role as genetic risk factors for the development and progression of ALL. We found that TYMS polymorphism (rs2853542) may be associated with development of ALL as genetic risk factor. Three, the present study are helpful to provide knowledge to other researchers about included gene variations analyzed in folate metabolic pathway in childhood ALL patients.

Conflict of interest

The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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