

## Interaction of thymidylate synthase polymorphism with MTHFR variants modify the risk of childhood acute lymphoblastic leukemia

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**Abstract.** A genetic susceptibility to acute lymphoblastic leukemia (ALL) has been suggested. The present study was conducted to examine the influence of interaction between methylenetetrahydrofolate reductase (MTHFR) variants and thymidylate synthase (TS) 28- base pair (bp) repeat polymorphism on the risk of pediatric ALL. Sixty eight ALL children with the mean age of 8.19±4.0 years and 70 age- and sex-matched healthy children were studied for TS 28-bp repeat and MTHFR C677T and A1298C variants using PCR and PCR-RFLP, respectively. The presence of TS 2R allele had a protective effect on the risk of ALL that did not reach a statistical significance [OR=0.5 (95% CI 0.19- 1.24, p=0.13)]. Also, the effect of MTHFR 677T allele on the risk of ALL was similar to TS 2R allele [OR=0.5 (95% CI 0.18- 1.4, p=0.19)]. However, there was a trend toward increased risk of ALL in the presence of both TS 2R and MTHFR 677T alleles [OR=1.92 (95% CI 0.7- 5.3, p=0.2)]. In contrast, the overall distribution of interaction between TS 2R and MTHFR 1298C alleles in ALL patients compared to controls was not significant. Our results suggest that the interaction between polymorphisms of different genes instead of the one polymorphism in a single gene might determine the susceptibility to pediatric ALL.

**Key words:** ALL, thymidylate synthase, MTHFR, Western Iran, leukemia.

### Introduction

Acute lymphoblastic leukemia (ALL) is a common pediatric malignancy accounting for 25-30% of all cancers among children with relatively high rate of mortality (Giovannetti et al. 2008, Amirghofran et al. 2011). It has been suggested that the interaction between genetic and environmental factors might be involved in the pathogenesis of ALL (Alcasabas et al. 2008, Kamel et al. 2007). Folate is a key element in the one-carbon group metabolism that is required for DNA synthesis and methylation. Its deficiency has been associated with the malignancies including susceptibility to leukemia. Thymidylate synthase (TS) converts dUMP to dTMP required for DNA synthesis. The most common polymorphism in TS is a double (2R) or triple (3R) 28-base pair (bp) repeat sequence in the promoter enhancer region of TS gene influences protein expression in cancer cells (Skibola et al. 2004). The role of TS variants on the susceptibility to ALL has been reported in various populations with inconsistency (Skibola et al. 2002, Lauten et al. 2003, Petra et al 2007, De Jong et al. 2009).

Also, the possible association between the two most common polymorphisms of 5, 10-methylenetetrahydrofolate reductase (MTHFR) gene, namely MTHFR C677T and A1298C and the risk of pediatric ALL has been examined in several studies. Some studies reported a protective role for these variants on the risk of pediatric ALL (Kamel et al. 2007, De Jong et al. 2009, Zintzaras et al. 2006, Franco et al. 2001). In contrast, others reported a role for these polymorphisms on the susceptibility to ALL (Alcasabas et al. 2008).

Previously, we reported the absence of association between TS variants and susceptibility to childhood ALL among Kurdish population from Western Iran (Rahimi et al. 2012). Also, recently we indicated the lack of association between MTHFR variants and the risk of ALL in our

population (Azhar et al. 2012). It seems different gene polymorphisms instead of single genetic defects play a role in the susceptibility to childhood ALL. In the study of de Jong et al (De Jong et al. 2009) the interaction between carriers of TS 2R and MTHFR 677T allele resulted in a 1.4-fold reduction in ALL risk ( $p < 0.05$ ). In the study of Giovannetti et al (Giovannetti et al. 2008) only the frequency of both polymorphisms has been reported among Indonesian ALL children without examination of their interaction on the risk of ALL.

The aim of the present study was to determine if both variants of MTHFR and TS 28-bp repeat in combination with each other modulate the risk of childhood ALL in the population of Western Iran.

### Patients and methods

Patients consisted of 68 ALL children (44 males and 24 females) with the mean age of 8.19±4.0 years newly diagnosed with ALL according to French-American-British classification (Rahimi et al. 2012) and 70 age- and sex-matched healthy children including 40 males and 30 females with the mean age of 8.24±6.0 years. Cases were selected from the files of patients who received hematological diagnosis by the Hematology Unit of Kermanshah University of Medical Sciences clinics during June 2002 and September 2009. At diagnosis 50% of ALL patients had normal white blood cells (WBC), 15% had increased WBC ( $>50000 \times 10^9/l$ ), and 35% had decreased WBC ( $<4000 \times 10^9/l$ ). There were 80-90% blast cells in bone marrow of ALL patients. Around eighty percent of patients were of B-cell ALL type (All were pre-B-cell). The remaining patients were T-cell ALL type. Both groups were from Kermanshah Province of Iran with Kurdish ethnic background. ALL patients were treated according to UKALL10 protocol. According to this protocol during induction phase patients received E-coli L-asparaginase 6000U/m<sup>2</sup> at two day intervals for nine doses, daily prednisone (40 mg/ m<sup>2</sup>) for 28 days (days 1-29), weekly vincristine (1.5 mg/m<sup>2</sup>) for 5 weeks, daily adriamycin (45 mg/m<sup>2</sup>) for 2 days, intrathecal methotrexate (adjusted dose for age) on days 1, 15, 29, 36, 43, 50. The patients had

bone marrow aspiration 3 times on days 1, 15, 29 post treatment. In re-induction (intensification) phase patients received vincristine (1.5 mg/m<sup>2</sup>) in the first day of therapy, daily prednisone (40 mg/m<sup>2</sup>) for 7 days, daily adriamycin (45 mg/m<sup>2</sup>) for 2 days, intrathecal methotrexate on the first day of phase and on weeks 5 and 20, twice a day cytosar (100 mg/m<sup>2</sup>) for 5 days, etoposide (100 mg/m<sup>2</sup>) for 5 days and thioguanine (75 mg/m<sup>2</sup>) for 5 days. The patients had bone marrow aspiration in the first day of therapy. In the maintenance phase which prolongs 3 to 3.5 years children received, daily 6-mercaptopurine (50 mg/m<sup>2</sup>), weekly methotrexate (20 mg/m<sup>2</sup>), prednisone (40 mg/m<sup>2</sup>) at 4 week intervals for 5 days and vincristine (1.5 mg/m<sup>2</sup>) at 4 week intervals.

Informed written consent was obtained from each individual or their parents before participation. The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences and was in accordance with the principles of the Declaration of Helsinki II.

DNA was extracted from leukocytes of EDTA treated whole blood using the phenol-chloroform method (Rahimi et al. 2010).

#### Genotyping

##### MTHFR

To detect MTHFR C677T a 198-bp region in exon 4 was amplified using the forward primer of 5' TGA AGG AGA AGG TGT CTG CGG GA 3' and the reverse primer of 5' AGG ACG GTG CGG TGA GAG TG 3'. The PCR products were digested with HinfI as described by Frosst et al. (Frosst et al. 1995). The HinfI treated PCR fragments were analyzed by 3% agarose gel. MTHFR A1298C was identified by amplifying a 163-bp region using the forward primer of 5' CTT TGC GGA GCT GAA GGA CTA CTA C 3' and the reverse primer of 5' CAC TTT GTG ACC ATT CCG GTT TG 3' and subsequent digestion of PCR products with MboII as previously described (Mtiraoui et al. 2007). The digested PCR products were resolved on a 12% polyacrylamide gel.

##### Thymidylate Synthase

For detection of the tandem repeated sequences in the 5'-terminal of the regulatory region of the TS gene, polymerase chain reaction using the forward primer of 5' GTG GCT CCT GCG TTT CCC CC 3' and the reverse primer of 5' CCA AGC TTG GCT CCG AGC CGG CCA GCA TGG CGC GG 3' was performed as previously described (Skibola et al. 2002).

##### Statistics

The allelic frequencies were calculated by the chromosome counting method. The distribution of the genotype frequencies in both groups did not deviate from the Hardy-Weinberg Equilibrium. The significance of the difference of alleles and genotype frequencies between the groups was tested using the chi-square method. Data on quantitative characteristics are expressed as means  $\pm$  standard deviations. Odds ratios (OR) were calculated as estimates of relative

risk for disease and 95% confidence intervals were measured by logistic regression using SPSS software. A two-tailed Student's t test analysis was used to compare quantitative data. Statistical significance was assumed at the  $p < 0.05$  level. All of the statistical analyses were performed using SPSS statistical software package version 16.0.

## Results

The interaction of TS and MTHFR variants in ALL patients and controls are demonstrated in Table 1. In 33.8% of ALL patients and 14.3% of controls there was both TS 2R and MTHFR 677T alleles compared to the lack of both alleles in 26.5% of patients and 21.4% of controls. Overall distribution of interaction between TS 2R and MTHFR 677T alleles in ALL patients compared to controls was significantly different ( $\chi^2 = 9.87$ ,  $df=3$ ,  $p=0.02$ ).

As shown in Table 1, the presence of TS 2R had a protective effect on the risk of ALL that did not reach to a statistical significance [OR=0.5 (95% CI 0.19- 1.24,  $p=0.13$ )]. Also, the effect of MTHFR 677T on the risk of ALL was similar to TS 2R [OR=0.5 (95% CI 0.18- 1.4,  $p=0.19$ )]. However, the concomitant presence of both TS 2R and MTHFR 677T alleles was associated with non significant increased risk of ALL [OR=1.92 (95% CI 0.7- 5.3,  $p=0.2$ )].

Also, the synergistic effects of MTHFR A1298C and TS 28-bp repeat was examined and no significant difference was found in the overall distribution of interaction between TS 2R and MTHFR 1298C alleles in ALL patients compared to controls ( $\chi^2 = 1.88$ ,  $df=3$ ,  $p=0.6$ ).

## Discussion

There are controversial reports related to the role of TS 28-bp repeat on the susceptibility to ALL (Skibola et al. 2002, Lauten et al. 2003, De Jong et al. 2009, Lightfoot et al. 2010). Some studies suggested a protective role for TS 3R or 2R with reduction of the risk of ALL (Skibola et al. 2002, De Jong et al. 2009). However, other studies failed to indicate any association between TS 28-bp repeat and risk of ALL (Lauten et al. 2003, Lightfoot et al. 2010). It has been reported that TS 28-bp repeat polymorphism interacts with folate intake which in 3R3R genotype carriers with high folate

**Table 1.** The carrier odds ratios of interaction between TS 28-bp repeat and MTHFR C677T alleles with respect to 3R or C alleles, in ALL patients and controls.

TS2R	MTHFR 677T	ALL patients, n=68 n (%) [OR (95% CI, p)]	control group, n=70 n (%)
-	-	18 (26.5) Reference group	15 (21.4) Reference group
+	-	16 (23.5) [0.5 (0.19- 1.24, $p=0.13$ )] ( $\chi^2=2.27$ , $df=1$ , $p=0.13$ )	27 (38.6)
-	+	11 (16.2) [0.5 (0.18- 1.4, $p=0.19$ )] ( $\chi^2=1.7$ , $df=1$ , $p=0.19$ )	18 (25.7)
+	+	23 (33.8) [1.92 (0.7- 5.3, $p=0.2$ )] ( $\chi^2=1.61$ , $df=1$ , $p=0.2$ )	10 (14.3)

Overall distribution of interaction between TS 2R and MTHFR 677T alleles in ALL patients compared to controls ( $\chi^2 = 9.87$ ,  $df=3$ ,  $p=0.02$ ).

intake the risk of ALL is decreased (Ulrich et al. 2002). There are several studies reporting the possible association between MTHFR variants and the risk of pediatric ALL. While most of them reported a protective role for both MTHFR C677T and A1298C variants on the risk of pediatric ALL (Kamel et al. 2007, De Jong et al. 2009, Zintzaras et al. 2006, Franco et al. 2001), others failed to indicate any protective effect for these polymorphisms (Giovannetti et al. 2008, Thirumaran et al. 2005, Oliveira et al. 2005) or found opposite results and an evidence on the role of these polymorphisms on the susceptibility to ALL (Alcasabas et al. 2008). The protective effects of MTHFR C677T and A1298C has been attributed to the lower enzyme activity resulting in increased availability of 5, 10-methylenetetrahydrofolate and reducing the uracil misincorporation and hence reduced risk of leukemia (Kamel et al. 2007). In our study in the presence of either TS 2R or MTHFR 677T allele there was a trend toward decreased risk of ALL. However, the risk of ALL tended to increase in the combined presence of both alleles. Conversely, in only one available study, De Jong et al. (De Jong et al. 2009) examined the interaction between the TS 2R and MTHFR 677T allele and found a reduction in ALL risk.

The conflicting results related to the association between TS and MTHFR variants and ALL risk might be due to different ethnicities, sample size, the gene-environment interactions, the folate status of the patients and their mothers during pregnancy and the complexity of the folate metabolic pathway since more than 30 enzymes were involved in the pathway (Lightfoot et al. 2010).

Briefly, the present study indicates that each variant of MTHFR C677T and TS 28-bp repeat tended to have a protective role on the susceptibility to pediatric ALL that is modified by the concomitant presence of both of them with increase risk of ALL. Our results suggest that the interaction between polymorphisms of different genes instead of the one polymorphism in a single gene might determine the susceptibility to pediatric ALL.

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