RESEARCH ARTICLE

Association of Thymidylate Synthase 5'-UTR 28bp Tandem Repeat and Serine Hydroxymethyltransfarase C1420T Polymorphisms with Susceptibility to Acute Leukemia

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Abstract

Background: The current study was aimed to elucidate the association of thymidylate synthase (TYMS) 5'-UTR 28bp tandem repeat and cytosolic serine hydroxymethyltransferase (cSHMT) C1420T polymorphisms with acute leukemia in South Indian subjects. A total of 812 subjects [523 healthy controls, 148 acute lymphoblastic leukemia (ALL) cases and 141 acute myeloid leukemia (AML) cases] were screened for TYMS 5'-UTR 28bp tandem repeat and cSHMT C1420T using PCR-AFLP and PCR-with confronting two-pair primers (CTPP) approaches. TYMS 5'-UTR 2R allele frequencies of controls, ALL and AML cases were 35.3%, 28.0% and 30.1% respectively. This polymorphism conferred protection against ALL (OR: 0.71, 95%CI: 0.53-0.96) while showing no statistically significant association with AML (OR: 0.79, 95%CI: 0.58, 1.07). The cSHMT variant allele (T-) frequencies of ALL and AML cases (6.42% and 5.68% respectively) were significantly lower compared to controls (58.3%). This polymorphism conferred protection against ALL (OR: 0.049, 95%CI: 0.029-0.081) and AML (OR: 0.043, 95%CI: 0.025-0.074). The TYMS 5'-UTR 2R2R genotype was associated with a lower total leukocyte count, smaller percentage of blasts, and more adequate platelet count compared to 2R3R and 3R3R genotypes in ALL cases. No such genotype-dependent differences were observed in AML cases. ALL cases carrying the cSHMT C1420T polymorphism showed higher disease free survival compared to those with the wild genotype. To conclude, the TYMS 5'-UTR 28bp tandem repeat reduces risk for ALL while cSHMT C1420T reduces risk for both ALL and AML. Both also influence disease progression in ALL.

Keywords: ALL - AML - thymidylate synthase - serine hydroxymethyltransferase - polymorphisms - progression

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Introduction

The etiology of acute leukemia is complex involving mutations (Abdel-Wahab et al., 2013), chromosomal rearrangements (Chapiro et al., 2013), epigenetic alterations (Estey, 2013) etc. Impaired synthesis of DNA either due to uracil misincorporation, oxidative DNA damage or due to defective DNA repair; and aberrant DNA methylation leading to activation of proto-oncogenes (global hypomethylation) and inactivation of tumor suppressor genes (focal hypermethylation) are one of the important etiological factors for different cancers including leukemias. Folate metabolic pathway plays a pivotal role in these processes.

Folic acid is an essential vitamin available through dietary sources in the form of folylpolyglutamates. Folate hydrolase 1 (FOLH1) catalyzes the hydrolysis of folylpolyglutamates to form folylmonoglutamates thus facilitating intestinal absorption of folate. Folate reductase catalyzes two-step reduction of folate to form dihydrofolate and then tetrahydrofolate. Serine hydroxymethyltransferase (SHMT) transfers methylene moiety from serine to tetrahydrofolate to form 5,10-methylene tetrahydrofolate. 5,10-methylene tetrahydrofolate is important substrate for both synthesis and methylation of DNA. Thymidylate synthase (TYMS) catalyzes the synthesis of thymidylate from uridylate using 5,10-methylene tetrahydrofolate. Methylene tetrahydrofolate reductase (MTHFR) catalyzes FAD-dependent reduction of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate. 5-methyl tetrahydrofolate carries the remethylation of homocysteine to methionine in the presence of methionine synthase-methionine synthase reductase (MTR-MTRR) holoenzyme complex. Methionine is precursor for the synthesis of S-adenosyl methionine (SAM), a universal methyl donor. DNA

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methyltransferases (DNMTs) regulate DNA methylation based on availability of SAM. Activities of TYMS and MTHFR enzymes determine whether flux of folate is towards methylation or synthesis. TYMS 5'-UTR 28-bp tandem repeat polymorphism in the immediate upstream of the ATG codon initiation start site, was found to reduce TYMS expression in the presence of 3R allele (Horie et al., 1995; Kawakami et al., 1999). The cSHMT C1420T polymorphism was found to induce futile folate cycle thus helping in maintaining one-carbon homeostasis depending upon cellular needs.

Several studies have been conducted worldwide on different genetic polymorphisms of the folate pathway for possible association with acute leukemias. Bolufer et al reported reduced risk for acute lymphoblastic leukemia (ALL) in subjects with TYMS 2R 3R genotype and risk reduction was more predominant in the presence of cSHMT C1420T polymorphism (Bolufer et al., 2006). Inhibition of TYMS is the key therapeutic strategy in leukemia through methotrexate administration (Rots et al., 2000). Methotrexate resistance was observed in subjects with TYMS 3R3R genotype due to over expression of TYMS (Welsh et al., 2000) with poor prognosis (Krajinovic et al., 2002). However studies on adult ALL indicated that 3R3R seemed to offer a higher level of protection against ALL risk than a double repeat (Skibola et al., 2002; Hishida et al., 2003). The possibility exists that an enhanced flux of methylene THF and resultant increase in dTMP production in the DNA synthesis pathway due to TYMS tandem repeat polymorphisms might work protectively against oncogenesis of lymphoid malignancies (Hishida et al., 2003).

The cSHMT C1420T polymorphism was shown to reduce risk for ALL in subjects with TYMS 3R3R or MTR 2756 AG genotype (Skibola et al., 2002). SHMT 1420 CC genotype was reported to be associated with malignant lymphoma (Hishida et al, 2003), esophageal squamous cell carcinoma (ESCC), gastric cardia adenocarconoma (GCA) (Wang et al., 2006; 2007) and breast cancer (Naushad et al., 2011) when compared to CT or TT genotypes. The association studies on functional polymorphisms of folate pathway are limited in relation to acute myeloid leukemia (AML). Kim et al demonstrated inverse association between TYMS 5'-UTR 28 bp tandem repeat and AML risk. None of the studies explored association of cSHMT C1420T with AML risk. Studies from Indian subcontinent in this aspect are very sparse. Nazki et al observed no association of TYMS 5'-UTR 28bp tandem repeat with ALL risk in Kashmiri population. In view of limited studies from India, discrepant results in association studies worldwide, we proposed to investigate the impact of TYMS 5'-UTR 28bp tandem repeat and cSHMT C1420T polymorphisms on the susceptibility to ALL and AML in South Indian subjects.

Materials and Methods

The age group of recruited cases was in the range of 5 to 40 years for ALL and 15 to 60 years for AML. Recruitment took place in the department of Medical Oncology, Nizam's Institute of Medical Sciences, Hyderabad, India. A combination of karyotyping, RT-PCR and fluorescence in situ hybridization was used to detect specific chromosomal abnormalities, including MLL lesions, TEL-AML1 translocations, and hyperdiploidy. In addition, peripheral blood samples were taken in remission from which DNA was extracted for this and other genetic studies. In total, DNA was available for 289 acute leukemia cases of which 148 were ALLs and 141 were AMLs. DNA was obtained from peripheral blood samples taken from 523 healthy controls (Age group between 5 to 60 yrs). The study protocol was approved by the ethics committee of Nizam's Institute of Medical Sciences, Hyderabad and in compliance with the Declaration of Helsinki. Informed consent was obtained from the subjects/parents / guardian of the subjects under study. All the participants of the study belonged to the same ethnic group and were unrelated. A questionnaire was used to collect demographic information, personal medical history and family history.

Whole blood samples were collected from all the subjects and genomic DNA was isolated using salting out method. (Nuremberg and Lahari 1991).

Analysis of TYMS 5'-UTR 28bp tandem repeat

The tandem repeat sequences in the 5'-terminal of the regulatory region of the TYMS gene were detected by using the following specific primers: forward (5,-CGT GGC TCC TGC GTT TCC - 3') and reverse (5'-GAG CCG GCC ACA GGC AT - 3'). PCR cycling parameters were: 10 min denaturation cycle at 95°C and 35 cycles of the following: 95°C for 30 sec (denaturation), 61°C for 30 sec (annealing), and 72°C for 45 sec (extension), then a final extension at 72°C for 5 min. Amplified PCR products were visualized on a 3% agarose gel with ethidium bromide. Homozygotes for the double repeat (2R2R) produced a single 210-bp band. Heterozygotes (2R3R) produced 210-bp and 238-bp fragments and homozygotes for the triple repeat (3R3R) produced a 238-bp fragment.

Analysis of cSHMT C1420T polymorphism

The SHMT1 C1420T polymorphism was analysed by PCR-with confronting two-pair primers (CTPP) (Hamajima et al, 2000). The primers were used as follows: F1: 5' CAG AGC CAC CCT GAA GAG TTC - 3' and R1: 5' - GCC AGG CAG AGG GAA GAG - 3' for C allele, and F2: 5'- GAG GTT GAG AGC TTC GCC TCT T- 3' and R2: 5'- GTG GGC CCG CTC CTT TA - 3' for the T allele. Polymerase chain reaction (PCR) cycling parameters were a 10 min denaturation cycle at 95°C and 30 cycles of the following: 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, then a final extension at 72°C for 5 min. The amplified PCR products were visualized on a 4% agarose gel. The SHMT1 C1420T polymorphism produces 115bp fragment for C allele and a 68bp fragment for T allele, as well as a 144bp common fragment.

All the PCR reactions were accompanied with a negative control (without genomic DNA) to rule out genomic DNA contamination in reaction mixture. Approximately 10% samples were reanalyzed to rule out genotyping discrepancies and 100% concordance was obtained.

Statistical analysis

All the genotype data was computed as 0, 1 and 2 based on number of variant alleles at each locus. Chi square test was performed for the observed and expected genotype frequencies to test whether genotype distribution is in accordance with Hardy-Weinberg equilibrium. This data was computed in 2×2 contingency table based on number of variant and wild alleles at each locus in cases and controls. Using this contingency table, Fisher exact test was performed to ascertain association of each genetic polymorphism with ALL and AML. The impact of each polymorphism on hematological parameters and disease progression was assessed using ANOVA or student-t-test. All the 'P' values were two sided and the level of significance was taken as p<0.05. All the statistical analyses were performed with Statistical Package for the Social Science (SPSS) 15.0.

Results

The genotype distribution of TYMS 5'-UTR 28bp tandem repeat (P_{HWE} values for controls, ALL and AML cases were 0.92, 1.00, 0.92 respectively) and cSHMT C1420T (PHWE values for controls, ALL and AML cases were 0.86, 0.80, 0.78 respectively) polymorphisms was in accordance with Hardy-Weinberg equilibrium.

As shown in Table1, the frequency of TYMS 5'-UTR 2R allele was 34.6% in controls while its corresponding frequencies in ALL and AML cases were 27.97% and 30.08% respectively. TYMS 5'-UTR 2R allele was found to reduce risk for ALL (OR: 0.71, 95%CI: 0.53-0.96). The frequency of cSHMT 1420 T-allele was higher in controls (58.26%) compared to ALL (6.42%) and AML (5.67%) cases. The cSHMT C1420T polymorphism conferred protection against ALL (OR: 0.05, 95%CI: 0.03-0.08) and AML (OR: 0.04, 95%CI: 0.03-0.07).

The impact of TYMS 5'-UTR 28bp tandem repeat on clinical variables was studied in ALL and AML as shown in table 2 and 3. ALL patients with TYMS 2R2R genotype showed lesser number of blasts compared to patients with 2R3R (p=0.04) and 3R3R (p=0.01) genotypes. All the ALL patients with 2R2R and 2R3R genotypes showed complete remission while 97.1% patients with 3R3R genotype showed complete remission. In AML, 2R2R genotype was associated with higher total leukocyte count compared to 2R3R and 3R3R genotypes. No impact of TYMS genotype was observed on other clinical parameters. However, AML patients with 2R2R genotype has lower rate of complete remission i.e. 16.67% compared to AML patients with 2R3R (63.41%) and 3R3R (67.39%) genotypes (p=0.053).

The impact of cSHMT C1420T polymorphism on

Table 1. Association of TYMS 5'-UTR 28bp Tandem Repeat and cSHMT C1420T with Acute Leukemia

Polymorphism	WW	WV	VV	VAF (%)	OR (95% CI)
TYMS 5'-UTR 28bp tandem repeat					
	3R3R	3R2R	2R2R		
Controls	214	249	60	34.6%	
ALL cases	115	95	29	27.97%	0.71 (0.53-0.96)**
AML cases	64	58	11	30.08%	0.79 (0.58-1.07)
cSHMT C1420T	CC	CT	TT		
Controls	82	245	163	58.26%	
ALL cases	186	48	10	6.42%	0.05 (0.03-0.08)***
AML cases	125	16	0	5.67%	0.04 (0.03-0.07)***

*WW: homozygous wild-genotype; WV: heterozygous; VV: homozygous mutant-genotype; VAF: variant allele frequency; OR: odds ratio; CI: confidence interval; **p<0.05; ***p<0.0001

Table 2. TYMS Genotype-based Association with Clinical Variables in ALL Group

Clinical variables	TYMS 2R2R Mean±SE	n	2R3R Mean±SE	n	3R3R Mean±SE	n
Mean Age (yr)	15.00±3.07	11	14.12±0.98	58	16.12±1.26	74
Mean TLC	18.78±4.17*	11	46.43±7.34	58	62.66±10.9	74
Mean blast%	26.18±7.60*	11	48.52±4.39	58	51.20±3.77	74
Mean platelet count (lakhs/μl)	1.31±0.19	11	0.91±0.09	58	0.77±0.08	74
Mean Hb (gm/dl)	9.36±0.82	11	8.98±0.31	58	8.57±0.30	74
Mean LDH (IU/L)	747.18±210.20	11	864.34±114.7	58	744.32±58.02	74
Mean DFS	29.82±4.24	11	27.81±2.20	54	27.41±2.61	74
CR	11/11		54/54		67/69	68

*TLC: total leukocyte count (Thousand/cu mm); Hb: hemoglobin; LDH: lactate dehydrogenase; DFS: disease free survival; CR: chronic remission: **p<0.05 (statistically significant)

Table 3. TYMS Genotype-Based Association with Clinical Variables in AML Group

Clinical variables	2R2R Mean±SE	n	2R3R Mean±SE	n	3R3R Mean±SE	n
Mean Age (yr)	34.64±5.44	11	31.56±1.90	58	30.71±2.00	62
Mean TLC	104.30±27.91**	11	48.34±9.71	58	48.36±9.55	62
Mean blast%	72.73±8.59	11	55.52±3.44	58	64.19±3.36	62
Mean platelet count (lakhs/μl)	0.35±0.14	11	1.09±0.18	58	1.07±0.16	62
Mean Hb (gm/dl)	8.47±0.68	11	8.30±0.31	58	8.35±0.34	62
Mean LDH (IU/L)	490.73±86.71	11	473.11±44.02	58	509.36±50.15	62
Mean DFS	5.25±3.06*	4	13.81±1.68	34	10.26±1.29	37
CR	1/6		26/41		31/46	

*TLC: total leukocyte count (Thousand/cu mm); Hb: hemoglobin; LDH: lactate dehydrogenase; DFS: disease free survival; CR: chronic remission: **p<0.05 (statistically significant)

Table 4. SHMT Genotype-Based Association with Clinical Variables in ALL Group

Clinical variables	CC Mean±SE	n	CT Mean±SE	n
Mean Age (yr)	15.78±0.87	129	15.16±2.34	19
Mean TLC	53.17±6.55	129	64.62±14.68	19
Mean blast%	49.78±2.97	129	48.53±7.85	19
Mean platelet count (lakhs/μl)	0.90±0.06	129	0.51±0.09*	19
Mean Hb (gm/dl)	8.93±0.23	129	8.54±0.64	19
Mean LDH (IU/L)	808.05±62.90	129	803.58±138.16	19
Mean DFS	26.02±1.64	118	35.44±4.54*	16
CR	117/121		16/17	

*TLC: total leukocyte count (Thousand/cu mm); Hb: hemoglobin; LDH: lactate dehydrogenase; DFS: disease free survival; CR: chronic remission: **P<0.05 (statistically significant)

Table 5. SHMT Genotype-Based Association with Clinical Variables in AML Group

Clinical variables	CC Mean±SE	n	CT Mean±SE	n
Mean Age (yr)	31.91±1.37	123	34.25±4.04	16
Mean TLC	54.48±7.03	123	35.00±15.43	16
Mean blast%	61.26±2.46	123	61.81±5.35	16
Mean platelet count (lakhs/μl)	0.94±0.11	123	1.01±0.26	16
Mean Hb (gm/dl)	8.11±0.21	123	9.04±0.74	16
Mean LDH (IU/L)	501.61±32.19	123	348.19±54.11	16
Mean DFS	11.15±1.04	71	12.82±2.48	11
CR	55/88		9/13	

*TLC: total leukocyte count (Thousand/cu mm); Hb: hemoglobin; LDH: lactate dehydrogenase; DFS: disease free survival; CR: chronic remission: **P<0.05 (statistically significant)

clinical variables was studied in ALL and AML as shown in Table 4 and 5. Mean platelet count in ALL patients with CT-genotype was lower compared to mean platelet count in ALL patient with CC-genotype (p=0.02). Disease free survival was more in ALL patients with CT genotype compared to those with CC genotype (p<0.05). No impact of cSHMT C1420T genotypes was observed on clinical parameters studied.

Discussion

In the present study, TYMS 5'-UTR 28 bp tandem repeat was found to reduce risk for ALL. During folate stress or as a result of TS inhibition, high level of uracil accumulates in the DNA, resulting in the degradation of newly synthesized DNA due to an active excision repair pathway (Ingraham et al., 1986). Early studies reported that the high levels of uracil misincorporation followed by extensive repair by uracil DNA glycosylase increase double strand DNA breaks that might contribute to chromosomal instability, translocation, and chromosomal aberrations which might contribute to leukemia risk (Melnyk et al., 1999). In addition, TYMS is a target for chemotherapeutic drugs such as 5-Flurouracil and TYMS mRNA and protein expression levels are considered as prognostic indicators for several cancers. Hence genetic variation and invivo regulations of TYMS are likely to be important in both cancer etiology and outcome (Hu et al., 2003; Molina et al., 2003).

Lesser number of blast percentages were observed in ALL cases with 2R/2R genotype suggesting that this polymorphism impairs disease progression also. TYMS 3R3R genotype, which was associated with higher number of blasts, previously reported to have poor clinical outcome compared to those with 2R3R and 2R2R genotypes due to increased expression of TYMS (Krazinovic et al., 2002). Colorectal cancer patients with the low expression TYMS genotypes (2R/2R), 2R/3R, and 3R/3R showed greater response and longer overall survival compared with other TYMS genotypes. (Marcuello et al., 2004). On the contrary, AML patients with 2R/2R genotype had elevated total leukocyte count and lesser rate of complete remission thus might be associated with poor prognosis.

In the current study, cSHMT C1420T polymorphism was found to confer protection against ALL and AML. Further, this polymorphism was observed to be associated with higher rate of disease free survival in ALL. Our results are consistent with the study of Skibola et al (2002) in reporting protective role of cSHMT C1420T polymorphism against ALL. Earlier studies reported protective role of this polymorphism in breast cancer (Naushad et al., 2011), coronary artery disease (Vijayalakshmi et al., 2011) and autism (Mohammad et al., 2009). This polymorphism located in exon 13 results in substitution of leucine by phenylalanine at codon 474 and 1420 CC-genotype was reported to be associated with reduced plasma folate and red blood cell folate. Naushad et al (2011) demonstrated elevated plasma folate levels in subjects with 1420 CT and TT-genotypes compared to those with 1420 CC-genotype. These observations were further supported by the dual role of cSHMT in forming 5,10-methylene tetrahydrofolate from serine and tetrahydrofolate; and forming 5-formyl tetrahydrofolate from 5,10-methylene tetrahydrofolate (futile folate cycle). Methenyl tetrahydrofolate synthetase (MTHFS) and cSHMT activities help in buffering 5,10-methylene tetrahydrofolate and 5-formyl tetrahydrofolate levels depending on the cellular requirements thus maintaining the critical balance between DNA synthesis and DNA methylation. TYMS and SHMT may share a common translational auto regulatory process that could couple the control of their expression, providing a mechanism to tightly regulate thymidylate and DNA synthesis (Snell et al., 2000).

To conclude, the current study demonstrates inverse association of cSHMT C1420T polymorphism with risk for ALL and AML. TYMS 5'-UTR 28 bp tandem repeat showed inverse association with ALL risk and its progression. Higher disease free survival was observed in ALL cases carrying cSHMT C1420T polymorphism.

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