Associations between polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and susceptibility to malignant lymphoma

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Background and Objectives. Polymorphisms in thymidylate synthase (TS) 28-bp tandem repeats in the promoter region and in cytosolic serine hydroxymethyltransferase (*SHMT1 C1420T*) have been reported to modulate the risk of adult acute lymphocytic leukemia (ALL). We examined the associations between susceptibility to malignant lymphoma and these polymorphisms.

Design and Methods. A hospital-based prevalent case-control study was conducted in Aichi Cancer Center. One hundred and eight patients with histologically confirmed lymphoma and 494 control subjects without cancer were evaluated.

Results. In a risk estimation of each genotype, those who harbored at least one TS 2 repeat (2R) allele had a 1.6-fold increase in the risk of malignant lymphoma (OR=1.63; 95%CI, 1.05-2.53, p=0.030) when using those without the *TS* 2R allele as a reference. For the *SHMT1* C1420T polymorphism, those harboring at least one T allele showed a 2.2-fold decrease in risk (OR =0.46; 95% CI, 0.23-0.93, p=0.031). Moreover, combined analysis of *TS* and *SHMT1* polymorphisms revealed that the OR for lymphoma in patients with *SHMT1* 1420 CC and the *TS* 2R allele, which might be expected to provide the basis for the highest susceptibility, was 2.88 (95% CI, 1.26-6.58, p=0.013).

Interpretations and Conclusions. This study suggests that genetic traits involving low penetrance polymorphisms in folate-metabolizing genes may modulate the risk of malignant lymphoma.

Key words: malignant lymphoma, genetic predisposition to disease, *TS*, *SHMT*, gene polymorphism.

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Considerable information about the pathogenic aspects of lymphoid malignancies has been accumulated. Certain genetic events during cell differentiation, such as chromosomal translocation[1-3] and the methylation status of oncogenes or tumor suppressor genes,[4] have been elucidated to play important roles in their genesis. Lymphoid malignancies are supposed to be a result of multiple genetic events,[5] but details are still unknown. Low penetrance susceptibility gene polymorphisms and gene expression profiling are now under examination to clarify the mechanisms of lymphomagenesis.[6,7]

Folic acid is an important nutrient, which is required for DNA synthesis. The antifolic acid agent, methotrexate, has proven to be an effective chemotherapeutic drug for lymphoid malignancies, indicating an association between folate metabolism and the oncogenesis of such malignancies. Our previous report concerning methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms supports the possible association between folate metabolizing pathway and lymphomagenesis.[8] Similar observations in adult acute lymphocytic leukemia (ALL) by Skibola *et al.*[9] also support this hypothetic association in lymphoid malignancy. The same authors recently reported an association between the risk of ALL and the polymorphisms in methionine synthase (*MTR*) A2756G, serine hydroxymethyltransferase (*SHMT*)1 C1420T and thymidylate synthase (*TS*) tandem repeat,[10] all enzymes associated with folate metabolism.

Thymidylate synthase (TS), whose gene is located on chromosome 18p11.32, is a key enzyme in DNA synthesis, converting deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), with the simultaneous conversion of 5,10-methylenetetrahydrofolate (methyleneTHF) to dihydrofolate. Thus, TS plays a pivotal role in providing a nucleotide available for DNA synthesis and repair. Impairments of the TS were found to be associated with chromosome damage and fragile site induction.[11,12] TS is also known to be a target for chemotherapeutic drugs such as 5-fluorouracil. A genetic polymorphism in the TS gene was found in the tandem repeat sequence in the 5' untranslated region (UTR) immediately upstream of the ATG codon initiation start site, which consists of either 2 repeats (2R) or 3 repeats (3R) of 28-bp.[13] *In vitro* and *in vivo* studies reportedly found higher *TS* gene expression in association with the 3R than with the 2R.[14-17] This enhanced

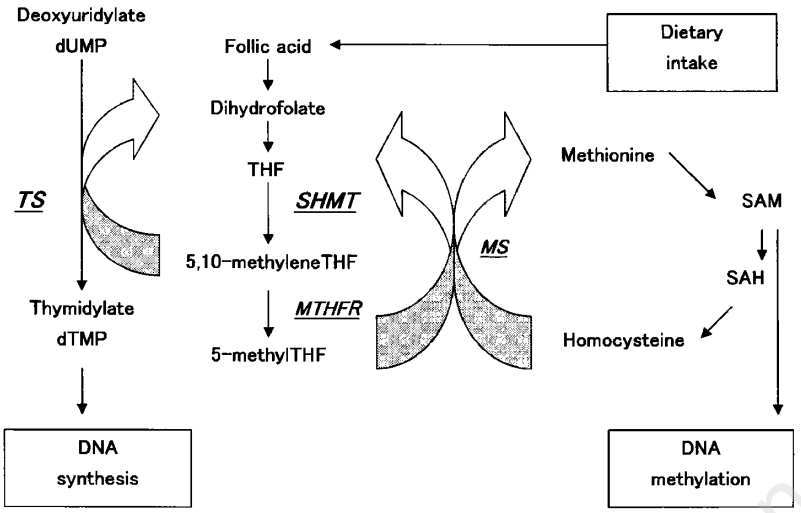

Figure 1. Overview of the human folate metabolic pathway. Thymidylate synthase (*TS*) binds methylenetetrahydrofolate (methyleneTHF), which serves as a hydroxymethyl donor in the conversion of dUMP to dTMP in the DNA synthesis pathway. Reductions in *TS* gene expression could likely affect the balanced supply of deoxynucleotides required for normal DNA synthesis. SHMT catalyzes the reversible conversion of serine and tetrahydrofolate (THF) to glycine and methyleneTHF.

expression may increase the conversion of dUMP to dTMP, reducing the chance of uracil misincorporation into DNA. This could limit DNA double-strand breaks in rapidly proliferating tissues such as hematopoietic stem cells, and might work protectively against the genesis of malignant lymphoma.

Serine hydroxymethyltransferase (SHMT) is a vitamin B6-dependent enzyme[18] that catalyzes the reversible conversion of serine and tetrahydrofolate (THF) to glycine and methyleneTHF (Figure 1). Two different SHMT isoenzymes are known: one is located in the cytoplasm, localized to the *SHMT1* gene on chromosome 17p11.2, and the other is in the mitochondrion localized to the *SHMT2* gene on chromosome 12q13.2.[19] SHMT1 plays a crucial role in generating one-carbon units for purine, thymidylate, and methionine synthesis in the cytoplasm.[20] Recently, a C1420T polymorphism in *SHMT1*, numbered from the translation initiation site, has been found. It leads to reduced plasma and red blood cell folate levels in 1420CC individuals.[21] Considering the role of SHMT in the provision of one-carbon units for multiple folate pathways, it is speculated that reduction in protein expression or enzyme activity caused by this polymorphism could imitate a folate deficiency by reducing the one-carbon moieties needed for both remethylation of homocysteine and DNA synthesis.

Thus, we hypothesized that the aberration in DNA synthesis due to these two polymorphisms involved in the folate metabolic pathway could modulate the risk of malignant lymphoma as well as ALL and we conducted a case-control study as an extension of a previous study.[8]

Design and Methods

The study population

Case subjects were recruited from the patients at Aichi Cancer Center Hospital who were histologically confirmed to have malignant lymphoma between October 1986 and February 2000. Those with a history of other types of malignancy were excluded. Control subjects meeting the criteria for *non-cancer* were selected from first visit outpatients at Aichi Cancer Center who were consecutively recruited to participate in the Hospital-based Epidemiologic Research Program at Aichi Cancer Center II (HERPACC II)[22] during the period from November 2000 to April 2001. Definition of *non-cancer* was no history or diagnosis of cancer. Information about the controls was confirmed by checking medical records, including pathology reports. All patients and control subjects were Japanese. Subjects who provided written informed consent to participation in this study were asked to complete a self-administered questionnaire and to provide blood from a peripheral vein. This study was approved by the Institutional Review Board of Aichi Cancer Center.

The characteristics of the study population were as follows:108 patients (age range, 19-79 years; mean age, 52.8 years; male, 54.6%) and 494 control subjects (age range, 18-81 years; mean age, 51.0 years; male, 41.3%) were recruited. Histologic types among patients were diffuse large B-cell lymphoma (n = 36), follicular lymphoma (n = 25), MALT lymphoma (n = 16), peripheral T-cell lymphoma (n = 6), Hodgkin's disease (n = 5), cutaneous T-cell lymphoma (n = 4), mantle cell lym-

phoma (n = 5), nodal marginal zone lymphoma (n = 2), lymphoblastic lymphoma (n = 2), angioimmunoblastic T-cell lymphoma (n = 1), anaplastic large cell lymphoma (n = 1), low-grade lymphoma, and not otherwise specified (n = 5).

Genotype analyses of the TS tandem repeats and SHMT C1420T

Figure 2 shows representative gels for the genotyping. DNA of each subject was extracted from the buffy coat fraction with a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA).

The tandem repeat sequences in the 5'-terminal of the regulatory region of the *TS* gene were detected by polymerase chain reaction (PCR) assay as follows. Genomic DNA (30ng to 100ng) was used in 25 μL of reaction mixture with 0.15mM dNTPs, 12.5 pmol of each primer, 2.5 μL of glycerol, 0.5 units of *AmpliTaq Gold*, 2.5 μL GeneAmp 10 μPCR buffer including 15mM $MgCl_2$ (Perkin-Elmer Corp., Foster City, CA, USA) and the forward (5'-CGT GGC TCC TGC GTT TCC-3') and reverse (5'-GAG CCG GCC ACA GGC AT-3') primers. Amplification conditions were a 10-minute denaturation cycle at 95°C and 35 cycles of the following: 95°C for 30 seconds, 61°C for 30 seconds, and 72°C for 45 seconds, then a final extension at 72°C for 5 minutes. Amplified PCR products were visualized on a 3% agarose gel with ethidium bromide. Homozygotes for the 2 repeats (2R2R) produced a single 210-bp band. Heterozygotes (2R3R) produced 210-bp and 238-bp fragments, and homozygotes for the 3 repeats (3R3R) produced a 238-bp fragment.

For the *SHMT1* C1420T polymorphism, genotyping was first conducted by PCR-with confronting two-pair primers (CTPP).[23] The primers used were as follows: F1: 5'-CAG AGC CAC CCT GAA GAG TTC -3' and R1: 5'-GCC AGG CAG AGG GAA GAG -3' for the 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 alleles. The underlining shows the bases of the single nucleotide polymorphism. Genomic DNA was used in 25 μL of reaction mixture with 0.15mM dNTPs, 12.5 pmol of each primer, 0.5 units of *AmpliTaq Gold*, and 2.5 μL GeneAmp 10 μPCR buffer including 15mM $MgCl_2$. Amplification conditions were a 10-minute initial denaturation cycle at 95°C and 30 cycles of the following: 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, then a final extension at 72°C for 5 minutes. The amplified DNA was visualized on a 4% agarose gel with ethidium bromide staining. Genotypes of the *SHMT1* C1420T polymorphism were distinguished as follows: a 115-bp band for the C allele, a 68-bp band for the T allele, as well as a 144-bp common band. However, although *SHMT1* 1420CC could be distinguished clearly from the other genotypes we were not able to detect the difference between *SHMT1* 1420CT and TT very well by this method. So, we then genotyped this polymorphism by PCR-restriction fragment length polymorphism (RFLP). PCR with the primers F1 and R2 was conducted under the same conditions described above. Amplified 144-bp PCR products were digested with a restriction enzyme, *EAR1*, which cuts the wild type sequence into 100- and 44-bp fragments. Accordingly the C allele produces 100-bp and 44-bp fragments, and the T allele a 144-bp band. Genotyping for this polymorphism was, in fact, slightly ambiguous, as described in the previous paper.[10] While the distinction between 1420TT and 1420CT was clear, there were some 1420CC samples indistinguishable from 1420CT, as demonstrated in Figure 2 (C). We speculated that this could be attributable to the relatively low specificity of the *EAR1* enzyme. For this reason, we first genotyped *SHMT1* C1420T polymorphism by PCR-

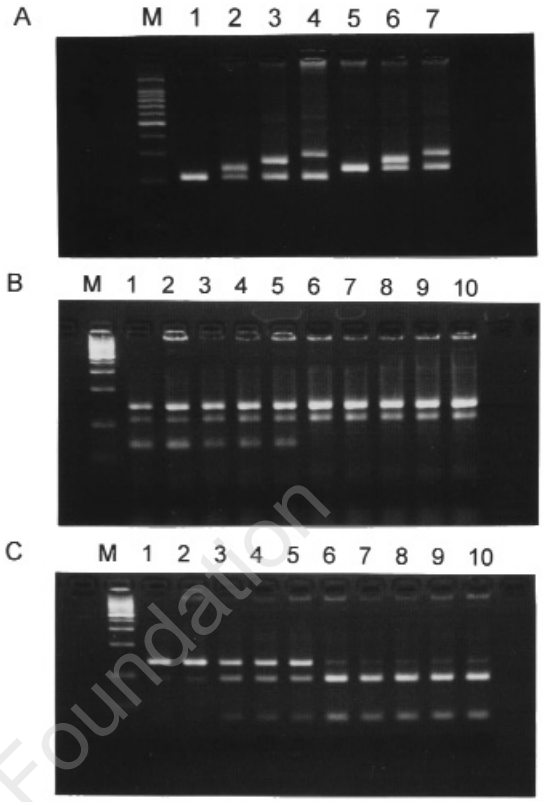

Figure 2. Polymorphisms of TS tandem repeats and SHMT1 C1420T. Lane M shows a 100-bp DNA ladder. (A) TS tandem repeat polymorphism. Lane 1, 2R2R; lane 2, 2R3R; lane 3, 2R4R; lane 4, 2R5R; lane 5, 3R3R; lane 6, 3R4R; lane 7, 3R5R. (B) PCR-CTPP for SHMT1 C1420T polymorphism. Lane 1 and 2, TT genotype (68- and 144-bp bands); lanes 3-5, TC genotype (68-, 115-, and 144-bp bands); and lane 6-10, CC genotype (115- and 144-bp bands). (C) PCR-RFLP for SHMT1 C1420T polymorphism. Lanes 1 and 2, TT genotype (144-bp bands); lanes 3-5, CT genotype (44-, 100-, and 144-bp bands); and lanes 6-10, CC genotype (44- and 100-bp bands) from the same 10 individuals as in (B).

CTPP, and determined the difference between 1420CT and 1420TT afterwards by PCR-RFLP.

Statistical analysis

All statistical analyses were performed using STATA (College Station, TX, USA) statistical software. Accordance with the Hardy-Weinberg equilibrium, which indicates an absence of discrepancy between genotype and allele frequency, was checked for among control subjects using a χ^2 test. Odds ratios (OR) and 95% confidence intervals (95% CI) were adjusted for sex and age using an unconditional logistic regression model; age as 3 binary variables for 4 age categories (younger than 45, 45 to 54, 55 to 64, and 65 or older). Adjustment for multiple comparisons was not performed because the analyses were conducted in an exploratory context, which requires careful interpretation of any *p* values.

Results

Genotyping for TS tandem repeats and SHMT C1420T

Table 1 shows the genotype frequencies of TS tandem repeats and SHMT1 C1420T. Among the 108 cases analyzed, the frequency of the TS 2R allele was 19.0% in cases and 14.5% in controls, and that of the TS 3R allele was 80.6% in cases and 84.3% in controls. When we determined the genotypes of the *TS* tandem repeats, we found 2 new forms of *TS* tandem repeat variant alleles, *TS* 4R and *TS* 5R. If we consider the number of *TS* 3R allele (no *TS* 3R, one *TS* 3R or two *TS* 3R alleles), the genotype frequency among the control subjects was not significantly different from the Hardy-Weinberg equilibrium (p=0.832). For *SHMT1* 1420, the frequency of the 1420T allele was 4.6% in patients and 8.9% in controls. The genotype frequency of SHMT1 C1420T among the controls was not significantly different from the Hardy-Weinberg equilibrium either (p=0.106).

Risk estimation for genotypes by the unconditional logistic model

Table 2 shows the genotype frequency, age- and sex-adjusted odds ratios and 95% confidence intervals for each polymorphism. When the state of being without the *TS* 2R allele was defined as the reference, subjects harboring at least one TS 2R allele showed a higher OR (1.63; 95% CI, 1.05-2.53, p=0.030). When the *SHMT1* 1420CC genotype was defined as the reference, the adjusted OR for the *SHMT1* 1420CT/TT genotype was significantly reduced (0.46; 95% CI, 0.23-0.93, p=0.031).

Table 3 shows the ORs for the combinations of the 2 genes, *TS* and *SHMT*. In this analysis, the cases without the *TS* 2R allele and with *SHMT1* 1420 CT/TT were redefined as the reference group because they were expected to have the lowest susceptibility. Adjusted OR for the group at risk (the patients other than the reference group taken together) was 1.83 (95% CI, 0.85-3.96; p =0.124), and the *ORs* for *SHMT1* 1420CC without the *TS* 2R allele and *SHMT1* 1420CT/TT with the TS 2R allele were 1.58 (95% CI, 0.72-3.48; p =0.26) and 0.59 (95% CI, 0.12-2.99; p = 0.53), respectively. The OR

Table 1. Genotype distributions for *TS* tandem repeats and *SHMT1* C1420T.

	TS tandem repeats						
Genotype	*2R2R*	*2R3R*	*3R3R*	*2R4R*	*2R5R*	*3R4R*	*3R5R*
Case (n=108)	1(0.9%)	39(36.1%)	67(62.0%)	0(0%)	0(0%)	0(0%)	1(0.9%)
Control (n=494)	10(2.0%)	121(24.5%)	348(70.4%)	1(0.2%)	1(0.2%)	1(0.2%)	12(2.4%)

	SHMT1 C1420T		
Genotype	*CC*	*CT*	*TT*
Case (n=108)	98 (90.7%)	10 (9.3%)	0 (0.0%)
Control (n=494)	407 (82.4%)	86 (17.4%)	1 (0.2%)

Table 2. Odds ratios and 95% confidence intervals (95%CIs) for *TS* tandem repeats and *SHMT1* C1420T.

Genotype	*Cases (n=108)*	*Controls (n=494)*	*OR*	*95% CI*	*p value*
TS tandem repeats					
2R (-)*	68 (63.0%)	361 (73.1%)	1	Reference	
2R (+)°	40 (37.0%)	133 (26.9%)	1.63	1.05-2.53	0.030
SHMT1 C1420T					
CC	98 (90.7%)	407 (82.4%)	1	Reference	
CT/TT	10 (9.3%)	87 (17.6%)	0.46	0.23-0.93	0.031

**Subjects without TS 2R allele. °Subjects with at least one TS 2R allele.*

Table 3. Odds ratios and 95%CIs for the combined status of *TS* tandem repeats (2R allele) and *SHMT1* C1420T.

TS tandem repeats	*SHMT1 C1420T*	*Cases (n=108)*	*Controls (n=494)*	*OR*	*95% CI*	*p value*
2R (-)	CT/TT	8 (7.4%)	62 (12.6%)	1	Reference	
	CC	60 (55.6%)	299 (60.5%)	1.58	0.72-3.48	0.26
2R (+)	CT/TT	2 (1.9%)	25 (5.1%)	0.59	0.12-2.99	0.53
	CC	38 (35.2%)	108 (21.9%)	2.88	1.26-6.58	0.013

**Test for interaction between TS genotypes with at least one 2R allele and SHMT1 1420CC genotype: OR=3.16 (p=0.177).*

for *SHMT1* 1420CC with the TS 2R allele was 2.88 (95%CI, 1.26-6.58; p=0.013). The interaction between genotypes harboring TS 2R and SHMT1 CC genotype resulted in an insignificant OR of 3.16 (95%CI, 0.59-16.85; p=0.177).

We performed subgroup analyses for diffuse large B-cell lymphoma and follicular lymphoma, which accounted for 33.3% and 23.1% of cases, respectively. The OR for those with the *TS* 2R allele compared to those without the *TS* 2R allele was 1.38 (95%CI, 0.67-2.85; p=0.384) for diffuse large B-cell lymphoma, and 1.81 (95% CI, 0.79-4.14; p =0.158) for follicular lymphoma. The OR for the *SHMT1* 1420CT/TT genotype was 0.55 (95% CI, 0.19-1.61; p =0.275) for diffuse large B-cell lymphoma and 0.66 (95% CI, 0.19-2.26; p =0.507) for follicular lymphoma.

Using our previously published data on the *MTHFR* 677, 1298 and *MTR*2756 polymorphisms, we tested the interactions of *TS* and *SHMT1* with *MTHFR* and *MTR*. No significant statistical interaction was found in these analyses (*data not shown*). Given the small sample size, the combined effects of more than 2 genotypes (ie, 3-way interactions) could not be examined.

Discussion

In the present study, we found that subjects with at least one *TS* 2R allele had an approximately 1.6-time higher susceptibility to lymphoma than those without the *TS* 2R allele. On the other hand, subjects with at least one *SHMT1* 1420 T allele had about 2.2-fold lower susceptibility than those without the T allele. Analyses in combination with *TS* tandem repeat and *SHMT1* C1420T also showed the highest susceptibility for *TS* 2R allele with the *SHMT1* 1420 CC genotype. We detected a positive interaction between *SHMT1* and *TS* although statistically insignificant. This suggests that a biological interaction might exist between these two genes. Subgroup analyses for diffuse large B-cell lymphoma and follicular lymphoma showed similar trends to those in overall analyses, although some attention should be paid to their interpretation.

For *TS* tandem repeats, the frequency of the 2R allele in this study was not significantly different from that in Chinese or Japanese in previous studies,[14,15,24] but lower than in Caucasians. There has been no report ever on the frequency of *SHMT1* C1420T in the Japanese population. The frequency of *SHMT1* 1420T allele in our study was relatively low compared with that found in other investigations. It is likely that discrepancies in allele frequency result from ethnic or regional differences.

Folic acid is important in the synthesis of DNA. Folate deficiency allows massive misincorporation of uracil into human DNA and chromosome breaks, which induce extensive chromosome damage, fragile site expression, micronucleus formation, and increased uracil levels in bone marrow cell DNA.[25] Taking this information into consideration, the concept that functional alterations due to polymorphisms within the folate metabolic pathway may induce certain pre-malignant conditions seems biologically plausible.

TS binds methyleneTHF, which serves as a carbon donor in the formation of dTMP from dUMP in the final step of the *de novo* synthesis of thymidine.[26] This enzyme is essential in the regulation of the balanced supply of four precursors for the replication and repair of DNA. Reduction in *TS* gene expression could affect this balanced supply, especially in rapidly proliferating cells such as hematopoietic stem cells. *TS* comprises a unique tandem repeat sequence in the 5' untranslated region (UTR) immediately upstream of the ATG codon initiation start site, this repeat sequence has been found to be polymorphic, containing either 2 or 3 28-bp repeats. The less common TS 2R allele is reported to lead to 2.6 times lower gene expression than the 3R allele in *in vitro* studies,[14] and 3.6-fold lower mRNA expression levels were observed in tumor tissue of *TS* 2R2R individuals than in tissue from *TS* 3R3R individuals.[16] Repeated sequences in *TS* are supposed to regulate *TS* gene expression by forming secondary structures in the 5'-terminal domain of *TS* mRNA.[15] On the other hand, a recent study also revealed that resistance to *TS* inhibitors was not associated with *TS* polymorphisms.[27] Thus, the actual influence of the *TS* polymorphism on its enzyme activity, including mRNA transcription and protein translation levels, in the real cell environment needs further clarification. Until recently, only two forms of this tandem repeat (2R and 3R) had been documented,[24,28] but lately, other variant forms (4R, 5R and 9R) have been reported.[29,30] In this study, we found 4R and 5R alleles in our Japanese subjects. The influence of these novel variant forms (4R and 5R) on *TS* gene expression is still unknown. We calculated the ORs of those with at least one 2R allele against those without the 2R allele as the reference, based on the hypothesis that gene expression would be greater the higher the number of *TS* 28-bp repeats. We also conducted univariate analysis of TS tandem repeats only between those with 2R2R, 2R3R and 3R3R genotypes. Those with at least one 2R allele (2R3R and 2R2R) had a significantly higher OR (1.62; 95% CI, 1.04-2.53; p=0.032). Our finding suggests the possibility that an enhanced flux of methyleneTHF and resultant increase in dTMP production in the DNA synthesis pathway due to *TS* tandem repeat polymorphisms might work protectively against oncogenicity of lymphoid malignancies.

SHMT is a major entry point for one-carbon units from serine into folate-dependent metabolism. Two different isoforms of SHMT are known; one is pre-

sent in the cytosol (*SHMT1*) and the other in the mitochondrion (SHMT2). SHMT is a pyridoxal phosphate-dependent enzyme, which catalyzes the reversible conversion of serine and THF to glycine and methyleneTHF. *SHMT1* also catalyzes another reaction, the irreversible conversion of 5, 10-methenyltetrahydrofolate (methenylTHF) to 5-formyltetrahydrofolate (5-formylTHF), which is called the *futile cycle*.[11,31] The conversion of serine and THF to glycine and methyleneTHF by SHMT provides the cell with most of the one-carbon units required for the synthesis of thymidine, purines, choline, and methionine. Our results indicate that the *SHMT1* C1420T polymorphism may influence the flux of one-carbon units required for the thymidylate synthesis, either by increasing formation of 5-formylTHF[32] or in combination with other genes involved in DNA synthesis such as TS or dihydrofolate reductase (*DHFR*).

Whereas subjects with the *TS* 2R allele demonstrated a significantly increased risk and those with *SHMT1* 1420 CT/TT had a markedly reduced risk, those with *SHMT1* 1420 CT/TT and the *TS* 2R allele had an insignificantly reduced OR of 0.59. These seemingly inconsistent results may be attributable to the small number of subjects in the study, or could be explained as follows: the significant ORs in the univariate analysis were just a reflection of the increased OR of those with *SHMT1* 1420 CC genotype and *TS* 2R allele, which are genetic traits conferring true susceptibility.

This is the first report on the association between the *TS* and *SHMT1* polymorphisms and susceptibility to malignant lymphoma. The effect of the two polymorphisms on the risk of malignant lymphoma had a similar trend to that in ALL, indicating that theses diseases are similar from the aspect of folate metabolism. The association between *TS* polymorphisms and the risk of colorectal adenomas in combination with folate intake has been examined.[29] None of the genotypes demonstrated significant ORs compared with the 3R3R genotype, while a statistically significant gene-nutrient interaction with dietary folate intake was observed. The reason for the discrepancy observed between these kinds of carcinogenesis remains unclear, but might be attributable to differences in gene-gene interactions and resultant cell transformation in each type of cancer.

It seems possible that the amount of folic acid intake and the status of the folate cofactors, vitamin B2, B6 and B12 may modulate the risk of malignant lymphoma. Folic acid supplementation to the mother is reported to be effective in the prevention of neural tube defects and acute lymphoblastic leukemia of their infants.[33,34] In colorectal cancer, an insufficient level of plasma folate was found to negate the protective effect of *MTHFR* 677.[35] In colorectal adenomas, high folate intake was found to be associated with a 2-fold decreased risk among TS 3R3R individuals, but with a 1.5-fold increased risk among 2R2R individuals.[29] Several previous studies on the association between fruit or vegetable intake and risk of non-Hodgkin's lymphoma yielded inconsistent results;[36-40] moreover, no association was observed between folate intake and risk of non-Hodgkin's lymphoma.[41] The reason for this inconsistency remains unclear, and further investigations should be conducted about this relation, for example by examining folate or vitamin B intake in combination with the polymorphisms in folate metabolizing enzymes. Results from such research might provide clues for the prevention of malignant lymphoma.

Recently, it was reported that there is an association between *TS* tandem-repeat polymorphism and the outcome of acute lymphoblastic leukemia.[42] Individuals with 3R3R genotypes had a poorer prognosis than those with other genotypes. The *TS* polymorphisms or *TS* levels have also been reported by several authors to have prognostic and predictive values in colorectal cancer.[43-46] Thus, polymorphisms of the *TS* and the *SHMT* genes might have implications for the treatment outcome of malignant lymphoma and other malignancies.

In this study, non-cancer outpatients (most were free from any kind of disease) were adopted as controls, and the genotype frequencies of the two polymorphisms were in accordance with the Hardy-Weinberg laws of equilibrium, indicating that no selective mechanisms for a specific genotype of these polymorphisms existed among the controls. We enrolled 108 cases and 494 controls in this study. The statistical power for this sample was more than 70% for an OR of 2 or 0.5 with a two-sided α error of 0.05, when a genotype frequency among the controls was between 30% and 70%. It was more than 50% when a genotype frequency among controls was between 20% and 80% under the same conditions. The statistical power for this sample is somewhat weak because of the relatively small sample size and the low frequency of the variant allele in the Japanese population. Although this was a prevalent case-control study, it is speculated that the effects of these *TS* and *SHMT* polymorphisms on the survival of our lymphoma cases might not be so strong, considering that TS inhibitors such as 5-FU are rarely used for the treatment of malignant lymphoma. However, we should be careful of this aspect in conducting such kind of study.

In conclusion, the present study found that genetic polymorphisms of *TS* and *SHMT1*, which are key enzymes in folate metabolism involved in thymidylate synthesis, are significantly associated with susceptibility to malignant lymphoma. This suggests that aberrations in the folate metabolic pathway cause imbalances in pyrimidine synthesis,

leading to an increased level of DNA double strand breaks, and resulting in lymphomagenesis. Further studies to confirm this association and to investigate the detailed biological mechanisms are required.

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Contributions

All authors contributed to the design of the study. AH, KM and NH contributed to the collection of data and samples, and were responsible for the laboratory data and statistical analysis; they principally wrote the article. HI contributed greatly to the collection of data and samples, and revised the article critically. MO, YK, HT and YM collected clinical data from the prevalent cases, were responsible for the interpretation of clinical data, and revised the article from the clinical point of view. NE and KT played major roles as senior authors in designing the study, provided supervision of the study, and also revised the article critically. All authors approved the final version of the paper. The authors are listed according to a criterion of decreasing individual contribution to the work. The authors are grateful to Ms. Michiyo Yagyu, Ms. Keiko Asai, and Ms. Hiroko Fujikura for their technical assistance.

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In the following paragraphs, Prof. Gaidano summarizes the peer-review process and its outcomes.

What is known in this field

The field of pharmacogenetics of human lymphoma is relatively unexplored. A previous study by the authors has suggested an association between methylenetetrahydrofolate reductase (MTHFR) variants and lymphomagenesis. In other lymphoid malignancies, though, namely acute lymphoblastic leukemia, a number of polymorphisms of several other folate metabolizing enzymes have been associated with increased risk of leukemia development.

What this study adds

The results of this case-control study indicate that the risk of lymphoma in the Japanese population is influenced by specific polymorphisms in two folate metabolizing enzymes, i.e. thymidylate synthase and cytosolic serine hydroxymethyltransferase. Because folate is essential for DNA replication, the authors speculate that alterations in folate metabolism caused by such polymorphic variants of folate metabolizing enzymes may favor the development of preneoplastic conditions in the replicating compartment of mature lymphoid cells.

Caveats

Given the epidemiological peculiarities of lymphoma in the Japanese population, as compared to other areas of the world, these results need to be reproduced in different ethnic background. Also, because of the relatively small size of the lymphoma panel investigated, it is unclear whether polymorphisms of folate metabolizing enzymes modify the risk of all clinico-pathologic categories of lymphoma, or, rather, specifically predispose to a given lymphoma type.