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Functional Polymorphism of the Thymidylate Synthase Gene in Colorectal Cancer Accompanied by Frequent Loss of Heterozygosity

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The thymidylate synthase (*TS*) gene has a polymorphic repeated sequence in its 5′-untranslated region. The repeat length is associated with TS protein expression, which suggests that we may be able to predict the efficacy of 5-fluorouracil (5-FU)-based chemotherapy from a patient's *TS* genotype determined through analysis of normal tissue obtained non-invasively. However, it is not yet elucidated whether the *TS* genotype is identical in tumor and normal tissue. In this study, we investigated the *TS* genotype in 151 matched tumor and normal DNA samples isolated from colorectal cancer and adjacent normal tissues by PCR analysis. The results showed that *TS* genotypes are identical in normal and tumor tissues of homozygous individuals, suggesting that the repeat sequence is stable through carcinogenesis. However, in heterozygous samples, an imbalance between the 2R and 3R alleles in the tumor DNA was frequently observed, suggesting loss of heterozygosity (LOH) at the *TS* locus. Detailed LOH analysis revealed that 62% (31 of 50) of 2R/3R-heterozygous samples had LOH. Frequent LOH at the *TS* locus was confirmed by RT-PCR of *TS* mRNA and microsatellite analysis using the marker D18S59, located on 18p11.3. There was no difference in the expressions of *TS* mRNA and TS protein between LOH and non-LOH samples. However, when the heterozygous genotype bearing LOH was subdivided according to the number of repeats, the cancer tissue with 2R/loss genotype expressed a significantly lower level of TS protein than did that with 3R/loss genotype. The results suggest that the difference in *TS* genotype between tumor and normal tissue due to LOH should be considered when the genotype is analyzed with normal tissue, such as peripheral blood cells, because it is important for TS protein expression.

Key words: Thymidylate synthase — Loss of heterozygosity — Gene polymorphism — Pharmacogenomics — Cancer chemotherapy

Thymidylate synthase (TS) catalyzes the reductive methylation of dUMP by 5,10-methylenetetrahydrofolate to form dTMP and dihydrofolate. TS has been an important target for cancer chemotherapy because of its central, rate-limiting role in the *de novo* synthesis of dTTP.[1] 5-Fluorouracil (5-FU) inhibits TS by forming a stable ternary complex among 5,10-methylenetetrahydrofolate, TS and fluoro-dUMP, the metabolite of 5-FU. Based on this mechanism, the TS expression level in cancer tissue has been expected to be a predictor of response to 5-FU-based chemotherapy, and indeed, recent studies have shown that the sensitivity of various tumors to 5-FU-based chemotherapy is associated with the intratumoral level of TS.[2–5]

The *TS* gene is known to have a unique tandemly repeated sequence in the 5′-untranslated region (5′-UTR) and is polymorphic in the numbers of this repeat.[6] The double (2R) or the triple (3R) repeats are the most common, although higher numbers are also found less frequently. We previously reported that this polymorphism was associated with TS protein expression in human gastrointestinal cancers.[7] The cancer tissue with 3R/3R genotype showed significantly higher TS protein expression than did that with 2R/3R genotype. This association between *TS* genotype and TS expression, together with the role of TS expression in 5-FU-based chemotherapy, suggest that the *TS* genotype might be a novel predictor of efficacy for 5-FU-based chemotherapy. Some clinical evidence has been reported to support this potential of the *TS* genotype,[8–11] although the studies had relatively small numbers of subjects, and validation by a larger-scale clinical study is needed.

One of advantages of clinical use of *TS* genotype would be that the genotype can be determined through a blood test, and so the strategy would be applicable to patients with cancer that is not easily accessible. This expectation is based on the assumption that the genotype in normal tissue, i.e. peripheral blood cells, is identical with that in cancer tissue. However, this assumption has not yet been validated in the case of the *TS* genotype. Theoretically, the *TS* genotype in cancer tissue could be changed by genetic alterations, including instability of repeat length and allelic loss. To test this possibility, we analyzed the *TS* genotype

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in 151 matched tumor and normal tissues from colorectal cancer patients. The results suggest that the repeat length is stable but the *TS* locus has a relatively frequent loss of heterozygosity (LOH) in cancer tissue, which alters the functional *TS* genotype in cancer tissue when it is heterozygous in the corresponding normal tissue.

MATERIALS AND METHODS

Samples and nucleic acid isolation A total of 151 matched tumor and adjacent normal tissue samples were obtained by surgical resection from 145 patients with primary colorectal adenocarcinoma. Patients were all Japanese and comprised 86 males and 59 females, ranging in age from 39 to 93 years, with a mean age of 66.0 years. Ethical approval for the project was obtained from the Kanazawa University School of Medicine Ethics Committee. Approximately 2 g of the surgically removed tissue was frozen immediately in liquid nitrogen and stored at −80°C until DNA, RNA and protein isolation. Genomic DNA was isolated by the standard method of proteinase K digestion and phenol-chloroform extraction.[12] Total RNA was isolated by the single-step guanidinium isothiocyanate method.[13]

PCR and sequencing of the PCR products PCR with the template of genomic DNA was performed for *TS* genotyping under the conditions previously described.[7] The amplified DNA fragments were analyzed by electrophoresis on a 4% agarose gel followed by staining with ethidium bromide. To determine the sequences of newly observed PCR products in this study, the fragments were subcloned using the pGEM-T Vector System (Promega, Madison, WI). Subsequently the cloned PCR products were sequenced with a Thermo Sequenase Cy5.5 Terminator Sequencing Kit (Amersham-Pharmacia, Piscataway, NJ).

For the LOH analysis we used the same PCR conditions except that the reverse primer TS18 5′-TCCGAGCCGGCCACAGGCAT-3′ was labeled with fluorescein 5′-isothiocyanate (FITC). The PCR products were quantified with a digital image analyser, FluorImager, and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) following electrophoresis on Spreadex gel (Elchrom Scientific, Cham, Switzerland). In the control study for LOH analysis, we used cloned PCR fragments from 2R and 3R sequences as templates, which were created in a previous study.[7]

Microsatellite analysis was carried out with the marker D18S59,[14] the forward primer for which was labeled with FITC. The PCR products with this marker were electrophoresed in Spreadex gel followed by quantitation of the products using the FluorImager and ImageQuant software. The relative ratio of normal and tumor alleles was normalized and compared. LOH was defined as either the absence of one allele or a decrease in intensity of one allele by at least 50%.

RT-PCR The total RNAs were treated with RNase-free DNase, followed by phenol-chloroform extraction. DNase-treated RNAs were reverse-transcribed with *TS* specific primer, TS6 5′-ATCCATTGGCATCCCAGATT-3′, in 10 μl of reaction mixture containing 50 m*M* Tris HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 0.25 μg of bovine serum albumin, 4 units of RNAguard (Amersham-Pharmacia), 1 m*M* dNTPs, 2.5 μM primer and 200 units of Moloney murine leukemia virus reverse transcriptase. The cDNA was then amplified using the forward primer TS25 5′-AGGCGCGCGGAAGGGGTCCT-3′ and reverse primer TS18 in 40 μl of reaction mixture containing 10 m*M* Tris HCl (pH 8.8), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 10% dimethyl sulfoxide, 0.2 m*M* dNTPs, 0.5 μM of each primer, 4 μl of cDNA and 0.5 units of *Taq* DNA polymerase. The amplified DNA fragments were analyzed by electrophoresis on a 4% agarose gel followed by staining with ethidium bromide. For every sample, a negative control experiment was performed, using the same procedure as above-mentioned, except that the control experiment excluded reverse transcriptase.

Quantitation of *TS* mRNA and protein The quantitation of mRNA levels was carried out by means of a real-time fluorescence detection method as described previously.[15] The quantity of *TS* mRNA was expressed in terms of the ratio between *TS* mRNA and β-actin mRNA. The primer and probe sequences are as follows: (a) for *TS*, forward primer, GGCCTCGGTGTGCCTTT, reverse primer, GATGTGCGCAATCATGTACGT, probe, 6-carboxyfluorescein-AACATCGCCAGCTACGCCCTGC-6-carboxytetramethylrhodamine; and (b) for β-actin, forward primer, TGAGCGCGGCTACAGCTT, reverse primer, TCCTTAATGTCACGCACGATTT, probe, 6-carboxyfluorescein-ACCACCACGGCCGAGCGG-6-carboxytetramethylrhodamine.

TS protein was measured by means of a [^{3}H]fluoro-dUMP binding assay as described previously.[16] Briefly, a cytosolic fraction from cancer tissue was incubated with an excess amount of [^{3}H]fluoro-dUMP and methylenetetrahydrofolate, forming a ternary complex among [^{3}H]fluoro-dUMP, methylenetetrahydrofolate and TS. The ^{3}H-labeled ternary complex was then counted with a scintillation counter and the amount of TS protein was calculated. The total protein concentration in the cytosolic fraction was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and the TS protein level was expressed as pmol/mg protein.

Statistical analysis The results of the quantitation of *TS* mRNA and TS protein are presented as scatter plots in the figures. Groups were compared by means of the Mann-Whitney *U* test. $P<0.05$ was taken as the criterion of significance.

RESULTS

Repeat length of *TS* is stable in colorectal cancer We analyzed the *TS* genotype in 151 matched tumor and normal DNA samples isolated from colorectal cancer and adjacent normal tissues using a PCR assay. We observed four different PCR fragments of about 210, 240, 270 and 300 bp in length (Fig. 1). The 210 and 240 bp fragments are known to represent the 2R and 3R sequences, respectively.[7] The 270 and 300 bp fragments were newly observed in this study. Thus, we cloned and sequenced these PCR fragments. The sequencing results showed that these PCR fragments had the same sequences as the other PCR fragments with the exception that the 270 and 300 bp fragments contained four and five copies of the 28 bp repeat sequence, respectively (data not shown). *TS* genotypes in normal tissues were classified into 2R/2R homozygote, 3R/3R homozygote, 2R/3R heterozygote, 3R/4R heterozygote, and 3R/5R heterozygote. The incidences of these genotypes were 6 (4.0%), 92 (60.9%), 50 (33.1%), 1 (0.7%), and 2 (1.3%), respectively. The genotypes were all identical between tumor and normal DNA, when the genotype was homozygous (2R/2R and 3R/3R genotype, n=98). This result showed that the length of the repeated sequence in *TS* 5′-UTR is stable during colorectal carcinogenesis.

LOH analysis at the *TS* locus by PCR amplification of polymorphic tandem repeated sequence We frequently observed unequal amounts of PCR products of two different lengths, each of which represented one of the heterozygous *TS* alleles, as shown in case 4 in Fig. 1. Although this observation strongly suggests the presence of LOH at the *TS* locus, it could also be caused by a difference in PCR efficiencies among various lengths of repeat sequence. To test this possibility, we determined whether the amounts of PCR products reflect the quantities of starting DNA in the PCR reaction.

We first quantified the PCR products from templates of heterozygous normal DNA, expecting that the amounts of PCR products of the two alleles should be the same. However, the allele ratio (2R/3R) was 0.70 to 0.76 in all normal DNA examined by agarose gel electrophoresis and ethidium bromide staining (Fig. 2A). This result is not canonical in that the longer PCR target, the 3R sequence in this case, is more effectively amplified. Therefore, we

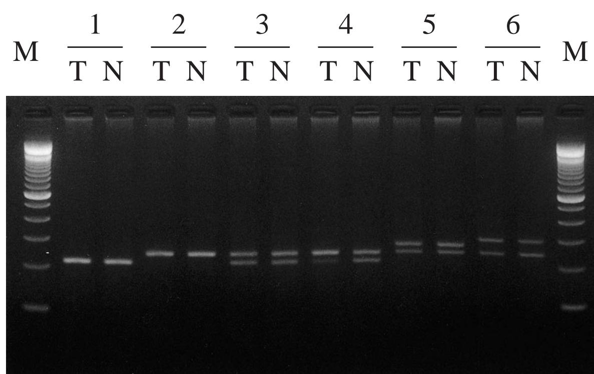

Fig. 1. The analysis of *TS* genotype with tumor (T) and normal (N) DNA from colorectal cancer patients. Numbers are case numbers. *TS* genotypes are as follows: case 1, 2R/2R; case 2, 3R/3R; cases 3 and 4, 2R/3R; case 5, 3R/4R; case 6, 3R/5R. Lane M contains the molecular marker (a 100-bp ladder).

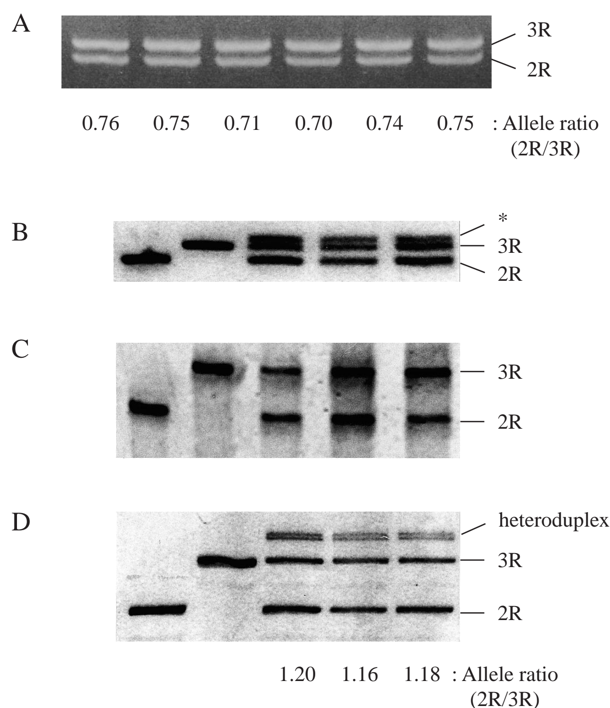

Fig. 2. The presence of heteroduplex product between 2R- and 3R-derived PCR fragment. A. The quantitative ratio between 2R- and 3R-derived PCR products was analyzed with DNA samples isolated from normal tissues by agarose gel electrophoresis and ethidium bromide staining. The allele ratio (2R/3R) was 0.70 to 0.76, which is different from the expected value of 1. B. The PCR experiment using FITC-labeled primer revealed an unexpected band indicated by an asterisk in the figure. This band was not observed in homozygous DNA samples. C. The unexpected PCR product in B disappeared on urea-containing polyacrylamide gel, in which PCR products are separated into single strands. D. The quantitative ratio between 2R- and 3R-derived PCR products was analyzed with DNA samples isolated from normal tissues using FITC-labeled primer and Spreadex gel. Heteroduplex product was negligible. The allele ratio (2R/3R) was 1.16 to 1.20, and this is consistent with the kinetics of PCR, in that the shorter amplicon is more effectively amplified.

further analyzed the allele ratio of normal DNA by the use of FITC-labeled primer and a digital image analyzer in order to quantify it more accurately. The high resolution of PCR products with the FITC-labeled primer revealed an unexpected band just above the product derived from the 3R sequence (Fig. 2B, ∗). This band was not seen in the product from homozygous DNA and disappeared upon urea-denatured polyacrylamide gel electrophoresis (Fig. 2C). Therefore, we concluded that the band was a heteroduplex product between 2R- and 3R-derived PCR products. We then employed Spreadex gel to separate out the heteroduplex product and quantify the allele ratio accurately. The quantitative ratio between 2R- and 3R-derived PCR products from normal DNA was 1.16 to 1.20 when the products were separated by Spreadex gel and quantified without the influence of heteroduplex products (Fig. 2D). The somewhat higher amount of PCR product from the 2R-allele can be explained by the higher efficiency of the PCR reaction with a shorter amplicon.

We then used a cloned PCR fragment as a template and performed PCR with reaction mixtures known to have various ratios of the two DNA templates differing in number of repeats. Fig. 3A shows the PCR results with the templates of 2R and 3R sequences using Spreadex and agarose gel. All the products were quantified using an image analyzer and the allele ratio was calculated. Each amount of PCR product was normalized by using the factor that caused the amounts of the products to become the same when the template ratio was 1 to 1. Fig. 3B shows the correlation between real template ratio and calculated allele ratio from the results of PCR. The quantitative difference between the two DNA templates was reflected in the difference in the amounts of PCR products when Spreadex gel was used. On the other hand, the allele ratio was underestimated when using agarose gel, probably due to

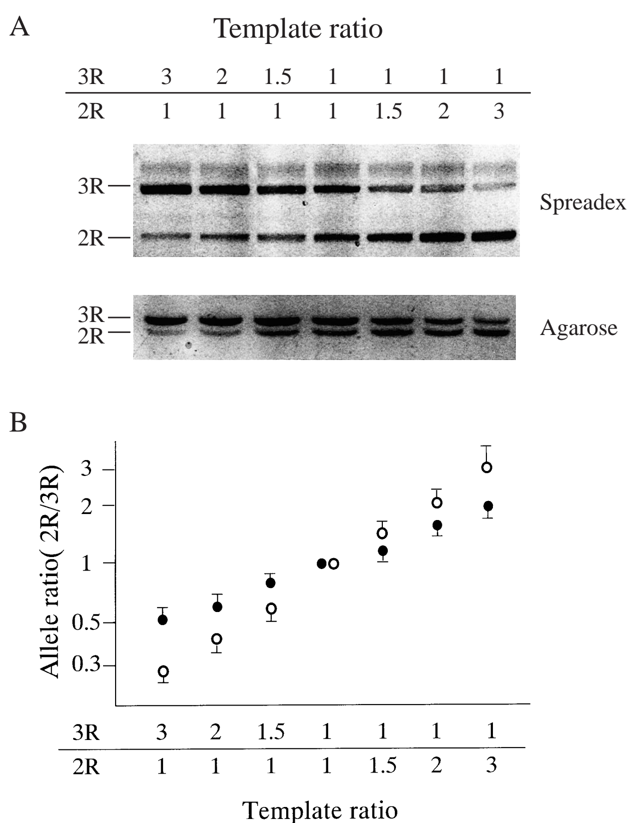

Fig. 3. The accuracy of LOH analysis using FITC-labeled primer and Spreadex gel. A. PCR with reaction mixtures known to have various ratios of two DNA templates containing 2R or 3R sequence. The known template ratio is indicated at the top of the figure. The results of electrophoresis using Spreadex and agarose gel are shown and the type of gel is indicated on the right side. B. The correlation of the known template ratio and the allele ratio from the results of electrophoresis. The allele ratio was calculated after normalization of each PCR product by using the factor that caused the product amounts to become the same when the template ratio was 1 to 1. The allele ratio from the Spreadex gel (open circle) was almost the same as the template ratio over a broad range. On the other hand, the allele ratio from the agarose gel (closed circle) was always lower than the real template ratio, probably due to interference by the heteroduplex product.

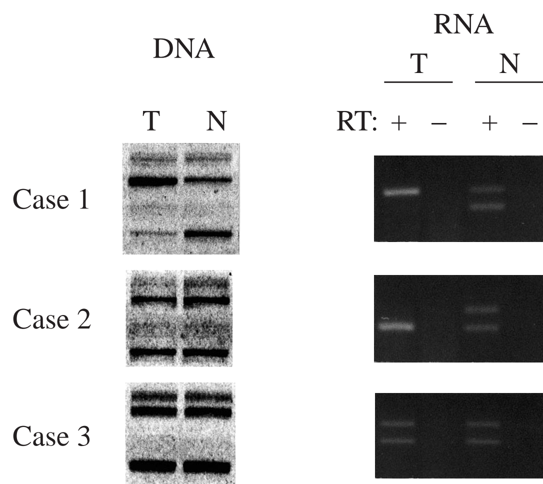

Fig. 4. Representative cases showing the relationship between DNA-based and RNA-based LOH status of *TS*: case 1, the results are identical (LOH positive, allele ratio in DNA-based analysis is 0.31); case 2, LOH is not observed with DNA-based analysis (allele ratio is 1.17). However, RNA-based analysis clearly demonstrates LOH; case 3, the results are identical (LOH negative, allele ratio in DNA-based analysis is 1.12). RT-PCR procedures with reverse transcriptase (RT) and without RT were performed in order to exclude the possible amplification of contaminating DNA.

the influence of the heteroduplex product. These results suggest that the cancer tissues heterozygous at the *TS* locus have a true imbalance between the two PCR products, and that our method using FITC-labeled primer and Spreadex gel is applicable to LOH analysis of the *TS* locus.

Frequent LOH of the *TS* locus in colorectal cancer We re-analyzed all the 2R/3R-heterozygous samples and quantified the allele ratio using FITC-labeled primer, Spreadex gel, and the digital image analyzer. The samples with 3R/4R or 3R/5R genotype were excluded for further LOH analysis because of their infrequency. All the PCR results on the *TS* genotype were reproducible when they were compared with the photos of the previous analysis. We defined a decrease in intensity of one allele by at least 50% as LOH at the *TS* locus. Under this definition, 31 of 50 2R/3R-heterozygous samples (62%) were determined to have LOH at the *TS* locus. The 2R allele was lost in 19 samples and the 3R allele in 12.

In order to obtain further evidence for LOH at the *TS* locus, we analyzed the genotype of expressed *TS* mRNA in cancer tissue by RT-PCR analysis with 2R/3R-heterozygous samples. We amplified the polymorphic repeated sequence of *TS* mRNA by RT-PCR with 46 available matched RNAs isolated from the same cancer and adjacent normal tissues as used for DNA-based analysis. The PCR products from the lost allele were barely detectable in LOH samples by RT-PCR, though they were easily detected in all cases by DNA-based analysis due to contamination of normal tissues. Therefore, the results were clear enough to determine LOH status without quantitation of the allele ratio. LOH of expressed *TS* mRNA was observed in all the RNAs (30 samples) isolated from those cancer tissues that had been determined to have LOH at the *TS* locus by DNA-based analysis. Furthermore, 3 RNA samples isolated from cancer tissues, which did not appear to have LOH by DNA analysis, showed LOH by RT-PCR. The DNA-based results on LOH may have been falsely negative in these 3 cases. In total, 33 of 46 RNA samples (71.7%) from cancer tissues were revealed to have LOH in expressed *TS* mRNA. Representative cases in regard to the relationship between DNA-based and RNA-based LOH status are shown in Fig. 4.

Although frequent LOH at the *TS* locus was evident in the cases of 2R/3R-heterozygous *TS* genotype, LOH status in the remaining two-thirds of cases that had the homozygous genotype could not be examined by the above methods. Therefore, we verified the frequent LOH at the *TS* locus by using microsatellite analysis. We employed the marker D18S59 on 18p13.1, the locus of which is close to the *TS* gene. The LOH analysis was performed using FITC-labeled primer and Spreadex gel with the samples, except for 3R/4R or 3R/5R genotype (Fig. 5). Twenty-two cases were homozygous and 4 cases showed microsatellite instability with the marker (these were taken as non-informative cases). Consequently, the informative cases were 122 of 148 matched DNA samples. The results of LOH analysis are summarized in Table I. Frequent LOH was consistently observed in all groups of *TS* genotype. The results confirmed frequent LOH at the *TS* locus in colorectal cancer.

Functional polymorphism of *TS* accompanied by LOH Once the LOH at *TS* locus was evident, we wanted to determine whether LOH status affects the expression of the *TS* gene. We thus quantified *TS* mRNA isolated from the 127 colorectal cancer tissues by real-time reverse transcription PCR and TS protein in 95 available samples by Fluoro-dUMP binding assay. The values were compared with the LOH status determined through the analysis of *TS* repeat sequences or microsatellite analysis. There was no association of LOH status with *TS* mRNA or TS protein level in either analysis (Fig. 6, A and B).

We then analyzed the cases with 2R/3R genotype in normal tissues to examine whether the residual allele type of *TS* as a result of LOH had any effect on the expression

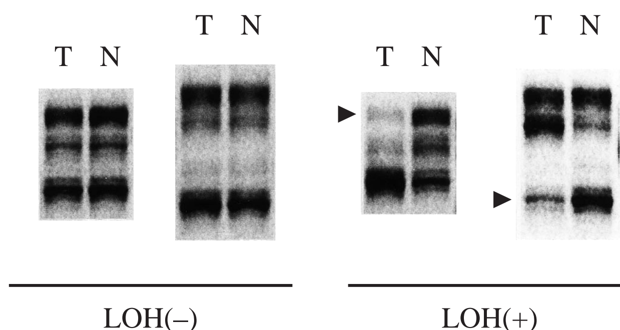

Fig. 5. LOH analysis using microsatellite marker D18S59. LOH was defined as either the absence of one allele or a decrease in intensity of one allele by at least 50%. The decreased intensity of one allele is indicated by an arrowhead in LOH-positive cases.

Table I. Summary of LOH Analysis by *TS* Repeat Polymorphism and Microsatellite Marker D18S59

	Analysis type	
	TS polymorphism	D18S59
2R/2R	NI	2/4 (50.0)
2R/3R	31/50 (62.0)	27/40 (67.5)
3R/3R	NI	47/78 (60.3)
Total	31/50 (62.0)	76/122 (62.3)

NI: not informative.
Numbers in parentheses show percent.

of *TS* mRNA and TS protein in cancer tissue. The result showed that the tumor with 2R/loss genotype expressed a significantly lower level of TS protein than that with 3R/loss genotype, indicating the influence of the LOH on TS protein expression in this specific type of allele loss (Fig. 6C). No association was observed between *TS* genotype and *TS* mRNA level. The results support our previous finding[17] that the translational efficiency of *TS* mRNA is responsible for the functional polymorphism, and thus the type of expressed *TS* mRNA is an important factor for TS protein expression. The LOH of *TS* in tumor tissue influences TS protein expression through alteration in the type of expressed *TS* mRNA, when the genotype is heterozygous in normal tissue. The effect of the type of expressed *TS* mRNA on TS protein expression was more evident when the *TS* homozygous samples were included in the analysis (Fig. 6D).

DISCUSSION

In this study we analyzed the *TS* genotypes in 151 matched colorectal cancer and adjacent normal tissues in order to answer the question of whether the *TS* genotype is identical in tumor and normal tissue. The results showed that the genotypes are all identical when they are homozygous. Our samples with homozygous *TS* genotype included 9 cancer tissues that showed instability among 5 microsatellite markers, BAT25, BAT26, D2S123, D5S346, and D17S250 (data not shown). Therefore, we concluded that the *TS* repeat sequence is stable in cancer cells even with an unstable microsatellite phenotype.

TS genotypes were qualitatively identical in tumor and normal tissue also in the heterozygous samples. However, a quantitative allelic imbalance was frequently observed in tumor DNA. Because this observation suggested the presence of LOH at the *TS* locus, we developed a method by which the 2R and 3R allele ratio of the *TS* gene could be quantified accurately. One obstacle in quantifying allele ratio by PCR can be the presence of a heteroduplex product formed by the different alleles. In the case of *TS*, indeed, a heteroduplex between 2R- and 3R-derived PCR product that electrophoresed close to the 3R-derived product was problematic for quantitation of the allele ratio. The use of a high separation gel, Spreadex, which is made from a new synthetic polymer, allowed us to ignore the heteroduplex band and to quantitate the allele ratio accurately. Although the manufacturer has not disclosed the details of this new gel, the heteroduplex product was successfully separated from the 3R-derived PCR product. This method needs no additional handling after or during

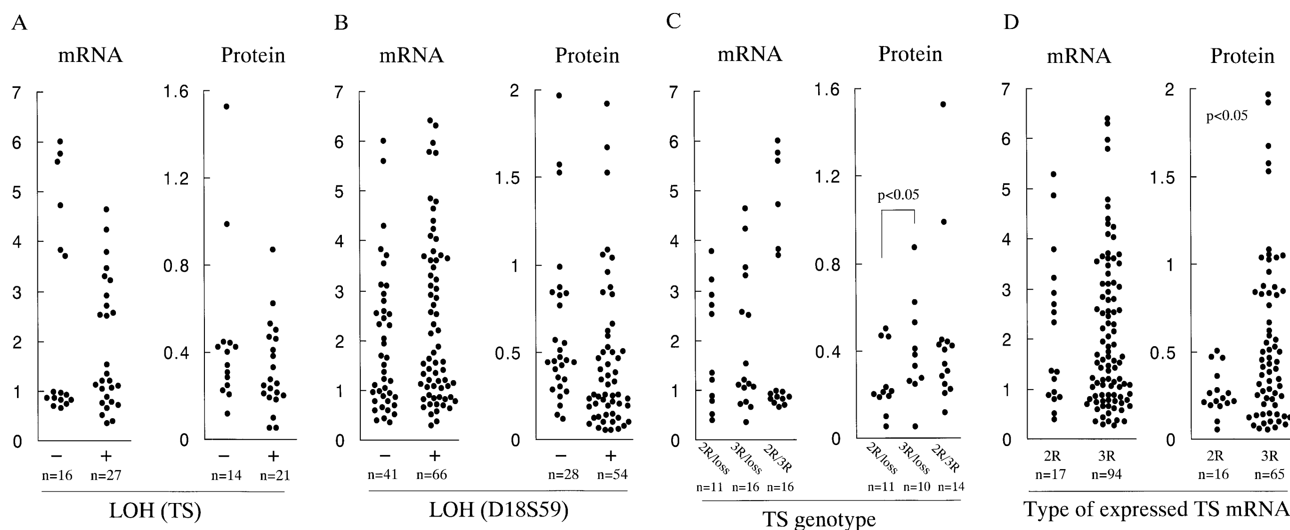

Fig. 6. Analysis of the relation of *TS* genotype and LOH status with *TS* mRNA and TS protein expression. *TS* mRNA level was expressed as the ratio between *TS* mRNA and β-actin mRNA. TS protein level was expressed as pmol/mg protein. There was no association of LOH status with *TS* mRNA or TS protein level in analysis by *TS* repeat sequence (A) or microsatellite marker D18S59 (B). C. The analysis of TS expression in tumor of the cases whose genotype is 2R/3R in normal tissues, considering the LOH status and residual allele. The tumor with 2R/loss genotype expressed a significantly lower level of TS protein than that with 3R/loss genotype (Mann-Whitney's *U* test, $P<0.05$). D. The relation between TS expression and the type of expressed *TS* mRNA. The tumor expressing *TS* mRNA of 2R showed a significantly lower level of TS protein than that expressing 3R. However, there was no association between mRNA expression level and the type of expressed *TS* mRNA.

PCR and is appropriate for future clinical application. LOH analysis with this method revealed that 62% of the 2R/3R-heterozygous samples had LOH. In addition, the LOH was confirmed by both RT-PCR and microsatellite analysis. The results verified frequent LOH in the region of *TS* in colorectal cancer. Frequent LOH on chromosome 18p has been reported in lung, brain, breast,[14] and esophageal squamous cell cancer.[18] These findings suggest that an unknown tumor suppressor gene on the short arm of chromosome 18 may be involved in the genesis of various types of cancer.

Once the LOH of *TS* locus became evident, it was important to determine whether the presence of LOH had any effect on TS expression. We found no association of the LOH status with either *TS* mRNA or protein expression through the *TS* repeat and microsatellite analysis. On the other hand, the LOH of *TS* indirectly affected the TS protein level through alteration of the functional *TS* genotype. For those cases in which the normal tissue was found to have the heterozygous 2R/3R genotype, the matched cancer tissue was divided into three *TS* genotypes (2R/loss, 3R/loss, and 2R/3R) depending on the status of LOH and the residual allele. The data showed that the cancer tissue with the 2R/loss genotype expressed a significantly lower level of TS protein than that of other genotypes. The functional polymorphism was attributed to the translational efficiency of *TS* mRNA since the genotype did not correlate with mRNA expression, but rather with protein expression. In other words, the type of expressed *TS* mRNA is critical with regard to functional polymorphism of *TS* in protein expression. Therefore, the *TS* LOH status and residual allele in cancer tissue are important because they determine the type of expressed *TS* mRNA.

The results in the present study were consistent with our previous observation that the repeat length of *TS* is associated with translational efficiency, but not with transcription of the gene.[17] Our previous results showed significant differences in TS protein expression, but not in *TS* mRNA expression, between cancers with the 2R/3R genotype and with the 3R/3R genotype. We could not compare the 2R/2R genotype and 3R/3R genotype, since the former is quite rare in the Japanese population. Direct comparison in this study between the 2R/loss and 3R/loss genotypes, i.e. between 2R mRNA and 3R mRNA, provides further evidence for the association of the repeat length of *TS* with mRNA translational efficiency. In contrast to our results, an association between *TS* genotype and *TS* mRNA expression level was reported in another study.[10] However, the patients included in that study were of different ethnic origin than the Japanese population of the present study. The expression of *TS* mRNA can be controlled by many factors, including E2F,[19] rTS[20] and NF-TS3.[21] The influence of *TS* genotype on mRNA expression might be masked by the variations of these factors among different population groups.

Table II. Summary of the Relation among *TS* Genotype, LOH Status, and the Type of Expressed *TS* mRNA

Genotype			Functional type (Type of expressed *TS* mRNA)		
Normal	LOH	Tumor	T	versus	N
2R/2R	+	2R/loss	2R	vs.	2R
	−	2R/2R			
2R/3R	+	2R/loss	2R	vs.	2R/3R
	−	2R/3R	2R/3R	vs.	2R/3R
	+	3R/loss	3R	vs.	2R/3R
3R/3R	+	3R/loss	3R	vs.	3R
	−	3R/3R			

The relation among *TS* genotype, LOH status, and the type of expressed *TS* mRNA is summarized in Table II. It is noteworthy that the cancer tissues can be classified into different functional groups even if the matched normal tissue is the same 2R/3R genotype. This finding suggests that the status of LOH in cancer must also be taken into consideration when the *TS* genotype found in normal tissue, such as peripheral blood cells, is heterozygous. Recent data suggests that the *TS* genotype may be a promising factor for predicting response to 5-FU-based chemotherapy,[8–11] although it needs to be validated by further large-scale clinical studies. A simple blood test in which peripheral blood cells are used for *TS* genotyping is an attractive method in the clinical setting because it is less expensive and invasive, but a blood test cannot distinguish the sub-groups classified by LOH in the cancer tissue. Our data suggest that a screening of *TS* genotype by blood test should be followed by LOH analysis when the genotype is heterozygous.

Although the presence of LOH adds a complication to the concept of determining *TS* genotype in blood, it may allow more effective prediction of the effectiveness of and adverse drug reactions to 5-FU-based chemotherapy. According to previous studies on *TS* genotype and clinical outcome,[10] it is suspected that cancer with the 2R/2R genotype responds well to 5-FU, although the concurrent adverse drug response is also substantial. On the other hand, cancer with the 2R/3R genotype is expected to show a poorer response and less side effects compared with the 2R/2R genotype. Taking these expectations together, the patient whose cancer is 2R/loss and normal tissue is 2R/3R genotype might obtain substantial benefit from 5-FU-based chemotherapy with less adverse drug

reaction, and this might be the most favorable *TS* genotype for 5-FU-based chemotherapy. With respect to the contrast of functional *TS* genotypes between tumor and normal tissue, patients can be stratified into 5 different types (Table II, T versus N). This classification might be beneficial in regard to the prediction of the so-called therapeutic index that represents not only the sensitivity of the cancer, but also the adverse effect on normal tissue. The clinical value of comprehensive information on *TS* polymorphism and LOH should be evaluated in a further study.

In conclusion, we showed that the *TS* locus has frequent LOH in colorectal cancer. This genetic alteration results in functional polymorphism of *TS* in cancer tissue when the genotype in matched normal tissue is heterozygous. Comprehensive information of the *TS* genotype and LOH status might allow more effective prediction of the effects of 5-FU-based chemotherapy.

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