

# Thymidylate synthase locus LOH in combination with genotype has prognostic and predictive significance in colorectal cancer

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Received September 29, 2020; Accepted July 22, 2021

DOI: 10.3892/mco.2021.2398

**Abstract.** The aim of the current study was to investigate the prognostic and predictive significance of polymorphisms in the thymidylate synthase (TS) gene, alongside the loss of heterozygosity (LOH) at this gene locus in patients with colorectal cancer. Genotyping was carried out for a variable number tandem repeat (VNTR) polymorphism in the TS 5'-untranslated region, a G/C single nucleotide polymorphism (SNP) located within this VNTR, and for TS LOH status in 246 colorectal cancer and paired normal DNA samples. The results were analyzed in relation to clinicopathological features, including the prognostic and predictive significance of TS genotype in patients who underwent curative surgery. Complete VNTR, SNP and LOH information for TS was obtained in 226 cases. No significant associations were observed between normal tissue TS genotype status and clinicopathological features. LOH of TS was observed in 58% of tumor samples and was associated with poor prognosis independently of clinical stage.

Cases exhibiting TS LOH were classified into the three groups of 2R/loss, 3G/loss and 3C/loss. Patients with 3C/loss genotype status had poor outcomes when treated by surgery alone, but their survival was similar to patients with other genotypes following Fluorouracil (5-FU)-based adjuvant chemotherapy. The results suggested that LOH of the TS locus may be a significant prognostic factor in colorectal cancer, with the genotype of the residual allele also demonstrating an influence on prognosis. In conclusion, LOH status should be considered when TS genotype is explored as a potential prognostic and predictive marker for 5-FU-based adjuvant chemotherapy in colorectal cancer.

## Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide. Although most localized cases of the disease are treated surgically, a considerable number of patients experience disease recurrence. Adjuvant chemotherapy after curative surgery has been shown to reduce the recurrence rate and therefore all CRC patients with clinical stage III disease are recommended to receive adjuvant chemotherapy (1), even though only a proportion of these derive a benefit. Patients with clinical stage II disease are mostly treated with surgery alone, however some may benefit from adjuvant therapy because of a high risk of recurrence (2,3). To maximize the efficacy of adjuvant chemotherapy, accurate predictive markers are needed to select patients who will benefit most from treatment.

5-FU-based chemotherapy is the current standard of care for adjuvant therapy following surgical treatment of CRC (4). Thymidylate synthase (TS) is a target enzyme for 5-FU (5), leading to extensive studies of TS mRNA expression (6), TS protein expression (7,8) and TS gene polymorphisms (9,10) as potential predictive factors for the efficacy of 5-FU-based adjuvant chemotherapy. Currently however, no information regarding TS status is recommended for routine clinical use in the selection of patients to receive 5-FU-based chemotherapy (11).

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**Abbreviations:** CRC, colorectal cancer; LOH, loss of heterozygosity; PE, primer extension; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; TS, thymidylate synthase; 5'-UTR, 5'-untranslated region; VNTR, variable number tandem repeat

**Key words:** 5-fluorouracil, colorectal cancer, genetic polymorphism, loss of heterozygosity, thymidylate synthase

TS shows unique genetic variants comprising a variable number of tandem repeat (VNTR) and a single nucleotide polymorphism (SNP) in its 5' untranslated region (5'UTR) (12-14). These may be predictive markers for 5-FU efficacy and for adverse events from this treatment. We previously reported that VNTR and SNP can give rise to four TS allele types: 2G, 2C, 3G and 3C. These may affect the translational activity of TS mRNA, thus influencing TS protein expression and therefore constitute a marker for the efficacy of 5-FU-based adjuvant chemotherapy (14-16). In addition to these four allele types, other rarer alleles comprising more than three repeats and novel SNPs in the 2R allele have also been reported (17), thus giving rise to a larger number of allele types. Furthermore, we observed that frequent loss of heterozygosity (LOH) of the TS locus in tumors can affect the genotype, thereby indirectly influencing the TS expression level in tumors (18,19). This potential change in genotype status due to LOH should be considered in studies of the TS genotype as a predictive marker. The status of VNTR, SNPs in both 2R and 3R, and LOH must all be evaluated before TS genotype information can be introduced into the clinical setting.

In this study, we analyzed the TS VNTR, the SNPs in both the 2R and 3R alleles, as well as the LOH status of the TS locus in order to explore their potential significance as prognostic and predictive markers of 5-FU-based adjuvant chemotherapy in CRC.

## Materials and methods

**Patient cohort and DNA isolation.** Matched tumor and normal tissue samples were obtained following surgical resection for primary colorectal adenocarcinoma in 246 patients. The patients were all Japanese and comprised 146 males and 100 females, ranging in age from 33 to 93 years (mean age 66.0 years). The resected tissues were fixed in formalin and embedded in paraffin followed by H&E staining and histological diagnosis. Tumor tissue was dissected manually from 10  $\mu$ m sections of formalin-fixed, paraffin-embedded tissue blocks. After deparaffinization using xylene and ethanol, genomic DNA was isolated using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) following the protocol provided by the manufacturer. Approval for this project was obtained from the Kanazawa University Genome/Gene Analysis Research Ethics Committee.

**Genotyping of TS VNTR and SNP.** TS genotypes for the VNTR and the SNP in the 3R allele were determined by PCR and PCR-restriction fragment length polymorphism (RFLP) using the forward primer TS25: 5'-AGGCGCGGGAAGGGTCTCCT-3' and reverse primer TS18: 5'-TCCGAGCCGCCACAGGCAT-3' as described previously (14) with a modification of PCR conditions. PCR with the genomic DNA template was performed in reaction mixtures containing 1X TaKaRa HS Taq buffer (TaKaRa Bio, Otsu, Japan), 200  $\mu$ M deoxyribonucleoside triphosphates, 500 nM of each primer, 0.5 unit of TaKaRa HS Taq DNA polymerase (TaKaRa Bio) and 100 ng of genomic DNA. The cycling conditions were: one cycle at 95°C for 3 min, 35 cycles at 98°C for 10 sec and 68°C for 60 sec, with a final extension at 72°C for 5 min. Aliquots

of amplified fragments were separated on 3% agarose gels to determine the TS VNTR genotype.

Samples showing the 2R/3R or 3R/3R genotypes were analyzed further for the G/C polymorphism in the 3R allele by using the RFLP method. *Hae*III digestion of the 3R fragment produced 66-, 37-, 28- and 10-bp bands for the 3G allele, and 94-, 37- and 10-bp bands for the 3C allele after separation on 3% agarose gels. For samples with 2R/2R or 2R/3R genotypes, the G/C polymorphism in the 2R allele was determined by PCR-PERFLP method, consisting of PCR followed by primer extension (PE) and RFLP analysis with *Hae*III digestion. The PCR reaction was performed using reverse primer TS21: 5'-CAGCTCCGAGCCGCCACAG-3' instead of TS18. Five microliters of PCR product was mixed with extension primer TS105: 5'-TCCGAGCCAGCCACAGGCAT-3' labeled with fluorescein 5'-isothiocyanate to a total volume of 7.5  $\mu$ l. The mixture was denatured for 5 min at 98°C, annealed for 10 min at room temperature and then combined with 2.5  $\mu$ l of PE reaction mixture containing 0.5 unit of Vent (exo-) DNA polymerase (New England BioLabs, Ipswich, MA), 200  $\mu$ M deoxyribonucleoside triphosphates, 1X ThermoPol Reaction Buffer provided by the manufacturer, followed by incubation for 10 min at 72°C. The product of primer extension was digested with *Hae*III, separated on 3% agarose gels and visualized with the Typhoon fluoroimager. The 2G allele produced a 48 bp fragment and the 2C allele a 76 bp fragment with the fluorescein 5'-isothiocyanate label. The TS genotype was thus classified into 2G/2G, 2G/2C, 2C/2C, 2G/3G, 2G/3C, 2C/3G, 2C/3C, 3G/3G, 3G/3C, or 3C/3C by comprehensive genotyping of the VNTR and SNP in the TS 5'UTR. Analyses were performed at least twice to confirm the genotype.

**LOH analysis.** LOH of the TS locus was determined in three distinct ways depending on the TS genotype observed in the normal tissue. The G/C SNP in the 2R allele was not taken into consideration for LOH analyses. Samples that were 2R/3G or 2R/3C were analyzed by PCR followed by separation on Spreadex gel (Elchrom Scientific, Cham, Switzerland). Samples that were 2R/2R, 3G/3G or 3C/3C were evaluated for LOH using the microsatellite marker D18S59, as described previously (18). Samples that were 3G/3C were analyzed using the PCR-PERFLP method, as described above for the SNP genotyping method with the 2R allele. PCR-PERFLP avoids interference due to heteroduplex formation, thereby allowing the exact allele ratio to be determined. The 3G allele produced a 76 bp fragment and the 3C allele a 104 bp fragment with PCR-PERFLP, as visualized by Typhoon fluoroimaging. The image was analyzed using ImageQuant software and the relative ratio between 3G and 3C alleles in tumor DNA was normalized using the ratio measured in the corresponding normal tissue DNA sample. LOH was defined as either the complete absence of one allele, or a decrease in intensity of one allele by at least 50%. LOH of 18q was analyzed using the microsatellite markers D18S58, D18S61 and D18S64. Forward primers were labeled with fluorescein 5'-isothiocyanate and the same method as for microsatellite marker D18S59 was used.

**Statistical analysis.** Relationships between variables were analyzed by Chi-square analysis or the Scheffe post-hoc test used following ANOVA. The cumulative survival rate was

estimated using the Kaplan-Meier method and statistical significance was assessed by the log-rank test. Cox regression modelling was used for multivariate analysis. P-values less than 0.05 were considered significant.

## Results

**Genotype analysis in normal tissue samples.** We previously investigated and compared the TS genotypes between matched normal and tumor tissues from 151 patients with colorectal cancer. The results suggest that frequent LOH of the TS locus detected in the tumors affect the functional TS genotyping (18). Therefore, in the present study, TS genotyping was carried out on DNA from normal tissue rather than from tumor samples in order to avoid possible artifacts from LOH. The VNTR genotype distribution amongst the 246 cases was: 2R/2R (n=9), 2R/3R (n=70), 3R/3R (n=162) and 3R/5R (n=5). Because the 5R allele is rare and analysis of the G/C SNP in the repeat component is difficult, the 5 cases with 3R/5R genotype were excluded. The remaining 241 cases of 2R/2R, 2R/3R and 3R/3R VNTR genotype cases were then screened for the G/C SNP in both the 2R and 3R alleles (Fig. 1). The combined VNTR and SNP genotype frequencies were: 2G/2C (n=1), 2C/2C (n=8), 2G/3G (n=3), 2G/3C (n=3), 2C/3G (n=25), 2C/3C (n=39), 3G/3G (n=48), 3G/3C (n=81) and 3C/3C (n=33). The previously reported 2R allele with a G→C SNP located in the first tandem repeat (17) was not found in our subjects. This should have resulted in a 121 bp fragment following PCR-PERFLP and gel electrophoresis (Fig. 1B). The 2G allele was quite rare in our population and showed no significant associations with any clinicopathological variable or with clinical course (data not shown). Therefore, SNP information for the 2R allele was not considered in further analysis. The distribution of clinicopathological features according to TS VNTR/SNP genotype are shown in Table I. As reported previously (14), the 3G allele was less frequent in females (P=0.04, chi-square test) (male G=115, female G=62, male C=79, female C=68). No significant associations were apparent between the normal tissue TS genotype and any other clinicopathological feature.

**LOH and the residual TS allele in colorectal cancer.** The appropriate method for LOH analysis was selected according to the genotype found in the normal tissue, as shown in Fig. 2. The genotype frequency according to LOH status is also shown in Fig. 2. A novel method for LOH analysis of the 3G/3C genotype was employed in this study and involved the use of PCR-PERFLP to avoid interference with heteroduplex product (Fig. 3). Fifteen cases could not be evaluated for LOH status because both the TS genotype and the D18S59 microsatellite marker were homozygous. Therefore, complete TS VNTR/SNP genotype information together with tumor LOH status was available for 226 patients. The frequencies were: 2R/2R (n=3), 2R/3G (n=11), 2R/3C (n=20), 3G/3G (n=13), 3G/3C (n=34), 3C/3C (n=14), 2R/loss (n=26), 3G/loss (n=58) and 3C/loss (n=47). The overall frequency of LOH for the TS locus was 58.0% (131/226). Lost alleles were evenly distributed between 2R (n=23), 3G (n=58) and 3C (n=50). Associations between LOH status and clinicopathological features are shown in Table II. The absence of LOH for TS was significantly associated with proximal tumor location

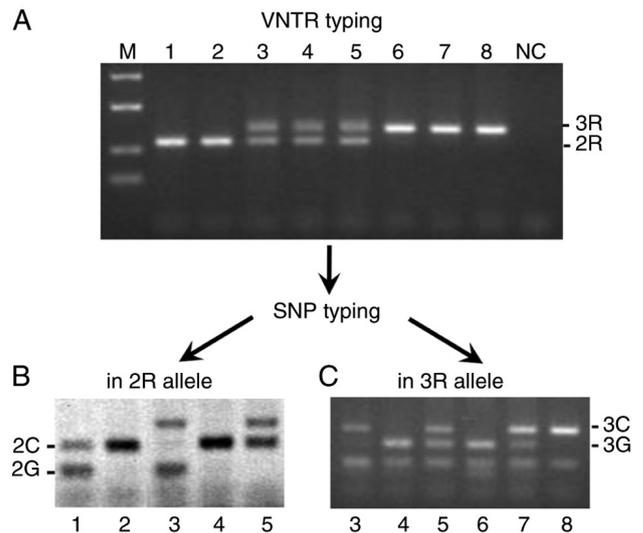


Figure 1. TS VNTR and SNP analysis. (A) VNTR analysis using PCR amplification and separation of products on a 3% agarose gel. (B) SNP analysis in the 2R allele using PERFLP followed by separation on a 3% agarose gel and scanning with a fluorimager. (C) SNP analysis in the 3R allele by RFLP and separation on a 3% agarose gel. The DNA fragments stained using ethidium bromide are displayed as white pixels on a black background, whereas those labeled by fluorescein are displayed as black pixels on a white background. The numbers on these panels indicate the same samples being analyzed. The genotypes are as follows: 1, 2G/2C; 2, 2C/2C; 3, 2G/3C; 4, 2C/3G; 5, 2C/3C; 6, 3G/3G; 7, 3G/3C; and 8, 3C/3C. M indicates the size marker (50, 100, 200 and 300 bp) and NC indicates that there was no template control. TS, thymidylate synthase gene; VNTR, variable number tandem repeat; SNP, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism.

and with mucinous histology. No other statistically significant associations were observed.

**Patient prognosis and TS LOH status.** A number of studies have reported that LOH at a given gene locus is associated with poor prognosis. We therefore analyzed the prognostic significance of TS LOH status before examining the prognostic role of the TS genotype. This was performed for 153 patients with clinical stage II or III disease who underwent curative surgery and where clinical information including long term follow-up and use of adjuvant therapy was available. Of these patients, 90 (59%) showed TS LOH and had significantly shorter survival (P=0.0005) compared to patients with no LOH (n=63; Fig. 4). We also compared the frequency of LOH between the tumors at clinical stage II and III by Chi-square test. We found no statistical difference in frequency of LOH between the two groups of tumors (P=0.22). Multivariate analysis with the parameters listed in Table III demonstrated that TS LOH status, receipt of adjuvant chemotherapy and clinical stage were independent prognostic factors in this patient cohort.

**TS LOH is often accompanied by 18q LOH.** TS is located on 18p11.32. Earlier studies reported that 18q LOH was associated with poor prognosis in CRC (20,21). Thus, we analyzed the relation between TS LOH and 18q LOH in 41 randomly selected samples comprising 15 cases with no TS LOH and 26 cases with TS LOH. Three microsatellite markers (D18S58, D18S61, D18S64) located at 18q22.3, 18q22.2 and 18q21.32, respectively, were used to determine 18q LOH. Fig. 5 shows the TS LOH

Table I. Thymidylate synthase genotype and clinicopathological features.

Parameter	2R/3R			3R/3R		
	2R/3R	2R/3G	2R/3C	3G/3G	3G/3C	3C/3C
Total	9	28	42	48	81	33
Sex						
Male	5	18	23	37	41	19
Female	4	10	19	11	40	14
Age (years)						
Mean	63.3	70.4	63.7	66.3	65.5	66.3
SD	15.8	11.1	11.2	13.2	11.1	13.5
Stage						
I	1	4	6	3	7	3
II	5	5	17	18	32	10
III	3	13	17	14	27	13
IV	0	6	2	13	15	7
Tumor site						
Proximal	2	9	12	19	32	9
Distal	7	19	30	29	49	24
Pathology						
Tub1	7	8	17	21	39	13
Tub2	1	17	21	23	36	16
Muc	1	0	1	2	1	2
Por	0	3	3	2	5	2

Muc, mucinous adenocarcinoma; Por, poorly differentiated adenocarcinoma; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma.

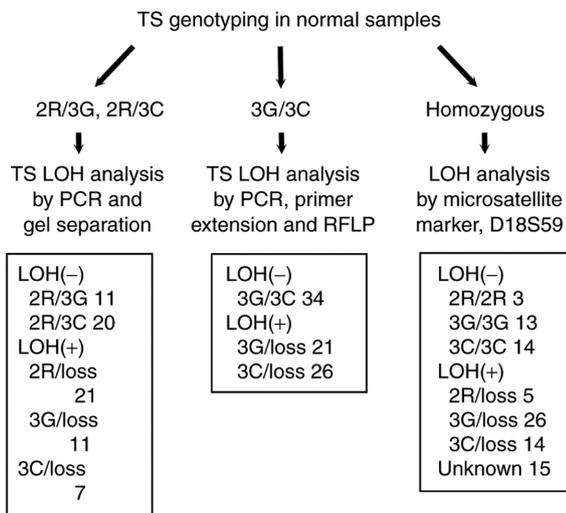


Figure 2. Flow chart of LOH analysis and the frequency of TS genotypes. The LOH status of tumors was analyzed according to the TS genotype in normal sample. The TS genotype frequency in relation to LOH status is shown in the box. LOH, loss of heterozygosity; TS, thymidylate synthase gene; RFLP, restriction fragment length polymorphism.

status as well as that of each 18q microsatellite marker. Whenever LOH was observed at the TS gene locus, it was also consistently present at other 18q loci. On the other hand, three tumors (from

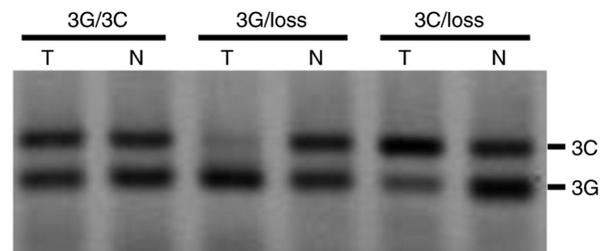


Figure 3. Representative cases of LOH analysis in 3G/3C genotype samples. The PCR-primer extension restriction fragment length polymorphism method detailed in the materials and methods was used to avoid interference by the heteroduplex product. The image of DNA fragments labeled by fluorescein are displayed as black pixels on a white background. Thymidylate synthase genotype and LOH status is indicated on the top of matched T and N lanes. LOH, loss of heterozygosity; T, tumor; N, normal.

patients no. 4, 13 and 16 shown in Fig. 5) showed LOH at one or more 18q microsatellite markers in the absence of LOH at TS. In these tumors, LOH was not observed for all three markers, indicating the chromosomal loss occurred in a relatively small area of 18q. These results suggest that LOH at TS and 18q are simultaneous events in most CRC, although a few tumors have small areas of LOH at 18q without allelic loss at the TS locus.

*TS genotype as a prognostic and predictive factor in tumors with LOH.* Since TS LOH was a strong prognostic factor (Fig. 4)

Table II. Loss of heterozygosity of the thymidylate synthase locus and clinicopathological features.

Parameter	No LOH	LOH	P-value
Total	95	131	
Sex			0.63
Male	55	80	
Female	40	51	
Age (years)			0.16
Mean	64.6	66.9	
SD	12.9	11.3	
Stage			0.61
I	11	12	
II	30	52	
III	39	46	
IV	15	21	
Tumor site			<0.0001
Proximal	48	30	
Distal	47	101	
Pathology			0.038
Tub1	42	55	
Tub2	39	69	
Muc	6	1	
Por	8	6	

Muc, mucinous adenocarcinoma; Por, poorly differentiated adenocarcinoma; SD, standard deviation; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma.

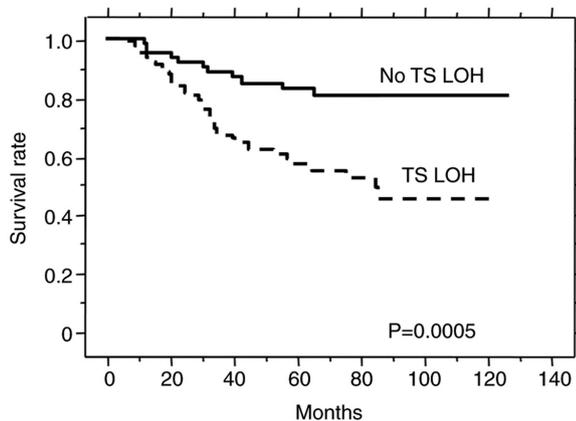


Figure 4. Overall survival of patients with curatively resected colorectal cancer according to their TS LOH status. TS, thymidylate synthase gene; LOH, loss of heterozygosity.

and also influences the TS genotype observed in tumors, we explored the role of TS genotype separately in patient groups stratified according to their LOH status. In patients without TS LOH (n=63), the tumor genotype is identical to that found in normal tissue. These patients were classified into 6 groups: 2R/2R (n=2), 2R/3G (n=6), 2R/3C (n=13), 3G/3G (n=10), 3G/3C (n=23) and 3C/3C (n=9). The relatively small number

Table III. Multivariate analysis of prognostic factors.

Variable	Hazard ratio	95% CI	P-value
TS LOH			
Yes vs. no	3.01	1.36-6.64	0.0065
Adjuvant chemotherapy			
Yes vs. no	0.53	0.28-0.98	0.045
Sex			
Male vs. female	1.22	0.66-2.26	0.53
Age			
≥66 vs. <66	1.15	0.62-2.16	0.66
Stage			
II vs. III	0.53	0.29-0.96	0.039
Tumor site			
Proximal vs. distal	0.63	0.28-1.43	0.27
Pathology			
Tub2 vs. Tub1	1.27	0.368-2.38	0.45
Muc vs. Tub1	2.42	0.50-11.7	0.27
Por vs. Tub1	0.53	0.068-4.21	0.55

CI, confidence interval; LOH, loss of heterozygosity; M, mucinous adenocarcinoma; Por, poorly differentiated adenocarcinoma; TS, thymidylate synthase; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma.

of cases for each genotype prevented analysis of the prognostic value of these groups. When the genotypes were grouped into L-type (2R/2R, 2R/3C, 3C/3C; n=24) and H-type (2R/3G, 3G/3C, 3G/3G; n=39) according to criteria from our previous report (14), no prognostic significance was observed in the overall patient group, in patients treated by surgery alone, or in patients treated with adjuvant chemotherapy (data not shown).

In patients with TS LOH (n=90), the tumor TS genotypes were: 2R/loss (n=20), 3G/loss (n=39) and 3C/loss (n=31). No prognostic significance was observed for these genotypes in the overall group of patients with TS LOH (Fig. 6A) or in patients who received adjuvant chemotherapy (Fig. 6C). However, the 3C/loss genotype was associated with significantly shorter survival in patients treated by surgery alone (Fig. 6B). These results suggest that patients with the 3C/loss genotype have poor prognosis when treated by surgery alone, but may benefit from chemotherapy as observed by the similar survival rate to patients with other genotypes. Indeed, patients with the 3C/loss genotype who received adjuvant chemotherapy survived significantly longer than those treated by surgery alone (Fig. 6D). Thus, the 3C/loss genotype appears to be a prognostic marker for poor outcome, as well as a predictive marker for good response to 5-FU-based chemotherapy.

## Discussion

In this report we genotyped TS for VNTR status and for SNPs located within the 2R and 3R alleles. We have evaluated the TS locus for LOH in CRC. In agreement with our previous observations, LOH was quite frequent regardless of the TS



to the 3G allele (14). Low expression of TS mRNA (6) and of the TS protein (8) in CRC have both been associated with good response to 5-FU-based chemotherapy. The current result showing the TS 3C/loss genotype is a marker for good response to 5-FU-based adjuvant chemotherapy (Fig. 6D) is therefore consistent with our previous *in vitro* observations and with the results of Salonga *et al* (6) and Soong *et al* (8). Although we cannot explain why this genotype was associated with poor prognosis (Fig. 6B), the result concurs with a previous study showing that low TS expression is a marker of worse prognosis in patients treated by surgery alone (8).

Due to the retrospective nature of this study and the potential for biases, further analyses are required to validate the results, particularly for the TS genotype groups in cases with LOH. The 2G allele was rare and no 2R allele with the G→C SNP in the first tandem repeat was found in our patient cohort, suggesting that SNP typing of the 2R allele can be omitted in further studies of the Japanese population. However, there is considerable ethnic variation in the allele frequency for 2R and the incidence is higher in Western populations (22). Therefore, additional analysis of the SNP in the 2R allele may be required for Caucasian populations.

The LOH status of TS was closely associated with 18q LOH status, with the latter being reported previously as a prognostic factor in CRC (23,24). The mechanism by which 18q LOH is linked to poor prognosis of CRC patients is not known, although the loss of several tumor suppressor genes in this region including *DCC*, *SMAD4* and *SMAD22* has been implicated. The current study sheds light on loss of the whole of chromosome 18 as a prognostic factor. LOH of 18p and 18q should be analyzed simultaneously to investigate whether the minimally lost regions on 18q or the whole allelic loss of chromosome 18 have stronger prognostic significance.

TS LOH is a significant prognostic factor in CRC. Furthermore, the 3C/loss genotype appears to be prognostic in patients treated by surgery alone and predictive in patients who receive 5-FU-based adjuvant chemotherapy. Since TS LOH status influences the TS genotype of tumors and also has a significant prognostic role, TS LOH should be incorporated into all future studies of TS genotype, particularly in relation to its predictive value. Stratification of CRC patients into subgroups defined by TS LOH status is therefore essential in obtaining clear evidence for a clinical role of the TS genotype.

### Acknowledgements

The authors would like to thank Dr. Barry Iacopetta (School of Surgery, University of Western Australia) for the critical reading of the manuscript and for providing valuable suggestions.

### Funding

No funding was received.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

MKo and KK conceived and designed the current study. MKo, HB, MKa and KK collected clinical samples. MKo, HB, MKa, HT and KK performed the experiments and analyzed the data. MKo, TM and KK drafted the manuscript. MKo and TM confirmed the authenticity of all the raw data. All authors read and approved the final version of manuscript.

### Ethics approval and consent to participate

The current study was performed in accordance with the Declaration of Helsinki. Since tissues used in this study were obtained from the patients diagnosed between 1999 and 2010, written informed consent was available for most but not all patients. However in accordance with Japanese ethical guidelines and law, the study protocol was reviewed and approved by the Kanazawa University Human Genome/Gene Analysis Research Ethics Committee (approval nos. 181 and 264). Following instruction by the Ethics Committee at approval, all patients were publicly provided with an opportunity to opt-out from registration to the current study. None declined. All samples were anonymized before analysis was performed to guarantee the protection of privacy.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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