Genotype of thymidylate synthase likely to affect efficacy of adjuvant 5-FU based chemotherapy in colon cancer

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Abstract. Thymidylate synthase [TS, (EC 2.1.1.45)] is the target enzyme in 5-fluorouracil treatment. Recently, the DNA polymorphism of this gene has been found to affect TS protein (pTS) expression. However, no prospective studies have been performed to evaluate the influence of this polymorphism on the clinical efficacy of 5-FU-based adjuvant chemotherapy for colorectal cancer (CRC). In this study, we investigated the genotype of *TS* and immunopathological findings of pTS in 161 colon cancer specimens from patients who were registered in a prospective adjuvant immunochemotherapy clinical trial. The clinical course and prognosis of these patients were checked after the study had been completed. This study comprised 11 (6.8%) cases of 2R/2R, 40 (24.8%) of 2R/3R, and 110 (68.3%) of 3R/3R genotypes. All of the 2R/2R cases were still alive at the time of analysis although this finding was not statistically significant. In this prospective examination on a randomized controlled trial, the patients with colon cancer of the 2R/2R TS genotype may be good responders to 5-FU-based adjuvant chemotherapy. Furthermore, differences in the proportions of the *TS* genotypes can account for the interracial differences in the adverse effects of 5-FU-based chemotherapy.

Introduction

Thymidylate synthase [TS, (EC 2.1.1.45)] catalyzes reductive methylation of deoxyuridine-5'-monophosphate (dUMP) to form deoxythymidine-5'-monophosphate (dTMP), which is a rate-limiting step in *de novo* synthesis of pyrimidine nucleotide. This enzyme is also the target enzyme of 5-fluorouracil (5-FU). 5-Fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), converted from 5-FU, inhibits TS protein (pTS) directly by forming inactive ternary complex, which is thought to be one of the main anticancer mechanism of 5-FU (1). Consequently, high expression of pTS can be expected to result in poor response of the tumor to 5-FU and evaluation of pTS to be a predictor of the efficacy of 5-FU treatments and the possibility of the occurrence of associated adverse events. Several studies have been performed to clarify the relationship between pTS expression and the response to 5-FU-based chemotherapy. In most of them, pTS expression was assessed with immunohistochemistry (IHC) because of its technical ease (2-10). However, IHC by itself contains several potential sources of bias, including type of staining technique and subjective judgements. Furthermore, considerable discrepancies have been observed in the relationship between the level of pTS expression estimated with IHC and the number of FdUMP binding sites (unpublished data), although the former correlates with the latter.

TS gene has polymorphic tandem repeat sequences (TRS) in its non-translating region (NTR), with the number of TRS regulating the translation activity of *TS* mRNA. The less common double repeat (2R) is associated with a 2.6-fold lower pTS expression in HeLaS3 cells than the expression level observed with the triple repeat (3R) (11). A study using surgical specimens has also demonstrated that pTS expression is significantly higher in 3R/3R than in 2R/2R genotype tumor tissues (12). These findings suggest that the difference in *TS* genotype always affects the pTS expression level in the tumor tissue, and may thus be a novel predictor of the efficacy of 5-FU-based chemotherapy. We hypothesized further that *TS* genotype would be superior to pTS expression levels assessed with the IHC because of the former's stability. Adjuvant chemotherapy is usually performed several months after surgery. Consequently, the status of pTS during chemotherapy may be different from that at surgery and this change in the condition of pTS would have a confusing effect on the evaluation. For this reason, we aimed to assess the DNA polymorphism of TS gene, and to gain the first data on the frequency in Japanese of this polymorphism.

We assessed the frequency of the tandem repeat sequence polymorphism in Japanese colon cancer patients who were

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enrolled in a prospective, randomized trial of adjuvant immunochemotherapy. In addition, we investigated their prognosis and pTS status in relation to this polymorphism.

Patients and methods

Clinical trial. Between February 1991 and March 1993, 446 patients were enrolled in a prospective randomized trial, conducted by the 'Study Group of Colon Cancer Immunotherapy with PSK plus 5-FU' (CIP), to compare the efficacy of immunochemotherapy with 5-FU plus Polysaccharide K (PSK) with that of chemotherapy with 5-FU alone as adjuvant therapy for patients with curatively resected colon cancer and with macroscopically positive lymph node metastases (13). The therapy consisted of post-operative continuous 5-FU infusion for 4 weeks (2000 mg/m^2/48 h/week) plus 18 months of oral 5-FU (200 mg/body/day), with or without 18 months of PSK (3 g/body/day). The 5-year disease-free survival rate was 76.8% for the immunochemotherapy group with PSK (n=220) and 74.2% for the control chemotherapy group (n=221), and the corresponding 5-year survival rates were 83.2 and 80.5%.

Prospective study design for determining the correlation between the TS genotype and prognosis for patients with colorectal cancer. Developments and progress made in relation to the molecular basis of 5-FU including its genotype, prompted the CIP study group to design a study to examine the correlation between TS genotype and the prognosis for patients entered in the trial. The CIP Genetic Marker Committee (CIP-GMC) was established as part of the CIP study group in April 1995. A letter was sent by the CIP-GMC to all the participating institutions requesting paraffin-embedded tissue blocks obtained from patients enrolled in the CIP trial. After obtaining clearance from the institutional review board at each of the institutions, 47 out of a total of 93 participating institutions agreed to provide the specimens. As a result, the CIP-GMC obtained 233 paraffin blocks from the 466 registered cases.

After completion of the collection of the paraffin blocks in March 1996, they were cut into 4-μm-thick sections both for cancerous DNA extraction and for the immunohistochemical examination.

cDNA preparation. The 233 paraffin-embedded samples were processed by stepwise deparaffination and RNA was extracted with the acid guanidine method. cDNA was obtained by reverse transcription, and used for the next step. Because of the small quantity of samples available and technical problems, we could obtain enough cDNA samples from 161 of the 233 cases.

Polymerase chain reaction to determine TS genotype. The *TS* genotype of the collected specimens was determined as described elsewhere (12). Briefly, the sequences of the primers used were TS12 5'-GTGGCTCCTGCGThFCCCCC-3' (sense) and TS18 5'-TCCGAGCCGGCCACAGGCAT-3' (antisense). Polymerase chain reaction (PCR) using these primers amplifies the non-translating region of *TS* mRNA, which contains the polymorphic TRS. PCR was performed in a volume of 20 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10% dimethyl sulfoxide, 0.2 mM dNTPs, 0.5 mM of each primer, 0.2 pg of cDNA and 0.5 units of DNA polymerase (Nippon Gene, Toyama, Japan). Thirty cycles of PCR were performed for 40 sec at 94˚C, 1 min at 62˚C and 40 sec at 72˚C with 5 min elongation at 72˚C after the completion of the last cycle. The amplified DNA fragments were analyzed by electrophoresis on a 4% agarose gel to estimate the *TS* genotype.

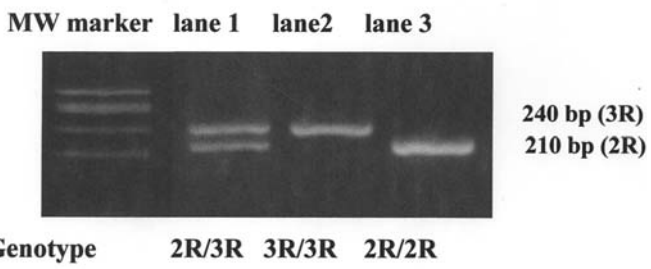

Figure 1. Electrophoresis of the PCR products. Lane 1, the product of a 2R/3R case. It shows long (240 base pairs) and short (210 base pairs) bands, each of which corresponds to 3R and 2R cDNA, respectively. Lane 2 and lane 3, products of 3R/3R and 2R/2R cases. A single band corresponding to either 3R (240 base pairs) or 2R (210 base pairs) was observed.

Immunohistochemical staining and scoring. From August to September 1996, 229 of the 233 paraffin-embedded tissue sections were subjected to IHC staining and analyzed. The results and methodological details have been published (5). One hundred fifty-nine of the 161 cases whose *TS* polymorphisms were successfully analyzed were enrolled in this IHC study and the data were obtained.

Statistical methods. Relationships between TS genotype and clinicopathological features including the therapeutic arms were analyzed by using univariate and multivariate analyses. Five-year overall survival (OS) rates and 5-year disease-free survival (DFS) rates were estimated for the 2R2R and the 2R3R/3R3R groups, and 95% confidence intervals were also calculated. Fisher's exact test was used to compare the rates of the two groups.

Results

TS genotype. TS genotypes were identified in the cancer tissue of 161 cases. There were 11 cases (6.8%) of 2R/2R in the TRS region, 40 cases (24.8%) of 2R/3R, and 110 cases (68.3%) of 3R/3R. Longer repeats than three were not observed in this study. Fig. 1 shows the results of representative electrophoresis of the PCR products. No significant differences among the genotypes were found in terms of age, sex, site of primary tumor, or histological type of tumor (Table I). Table II shows the results of multivariate analysis which disclosed that the variables with statistical significance were Dukes' stage and pathological lymph node metastasis. The relationship between the results of immunohistochemical and TRS analysis depicted in Table III shows no significant correlation.

Table I. The relation between clinicopathological features and TS genotype (%).

Factors	2R2R	2R3R	3R3R	P-value
Clinical				
Abnormal CEA	9.1	20.0	27.3	0.314
Male ratio	72.7	62.5	54.6	0.398
Age <70	63.6	85.0	80.0	0.290
Dukes C	36.4	42.5	50.9	0.484
Treated with PSK	36.4	40.0	52.7	0.274
Pathological				
Differentiated cancer	90.0	100.0	99.9	0.100
Lymph node metastases	36.4	42.5	50.9	0.484

Table II. Multivariate analysis using proportional hazard model for DFS.

Variables	Hazard ratio	95% C. I.	P-value
Sex			
M/F	0.96	0.51-1.83	0.902
Age			
<70/≥70	1.18	0.49-2.87	0.713
Location			
Left/Right	1.27	0.63-2.58	0.509
Dukes			
C/A, B	2.37	1.14-4.92	0.021
ly			
+/-	1.98	0.65-6.07	0.230
v			
+/-	0.84	0.43-1.62	0.598
PSK			
Treated/Not	0.89	0.47-1.68	0.711
Tandem repeat			
23, 33/22	1.37	0.32-5.82	0.672

ly, lymphatic vessel invasion; v, venous vessel invasion; PSK, Polysaccharide K (oral immunotherapeutic agent).

Relationship between TS polymorphism and clinical course. Figs. 2 and 3 show overall survival (OS) and disease-free survival (DFS) for the groups classified by TS genotype. All 2R/2R cases survived during follow-up period, but some patients in both the 2R/3R and 3R/3R groups died. The 5-year OS rates for the 2R2R and 2R3R/3R3R groups were 100% (71.5-100%) and 83.3% (76.4-88.9%), respectively, while the corresponding 5-year DFS rates were 81.8% (48.2-97.7%) and 75.3% (67.6-82.0%). The 95% confidence intervals are shown in parentheses. Although both 5-year OS and DFS rates of the 2R2R group were higher than those of the 2R3R/3R3R

Table III. Relation between TS genotype and pTS expression.

	2R2R	2R3R	3R3R
pTS(+)	3	9	14
pTS(-)	8	29	94
NA		2	2

NA, data not available.

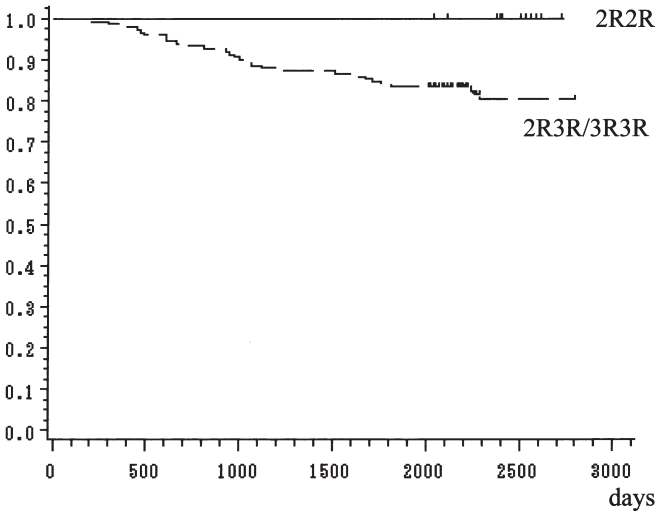

Figure 2. Overall survival for the 2R2R (N=11) and 2R3R/3R3R (N=150) groups.

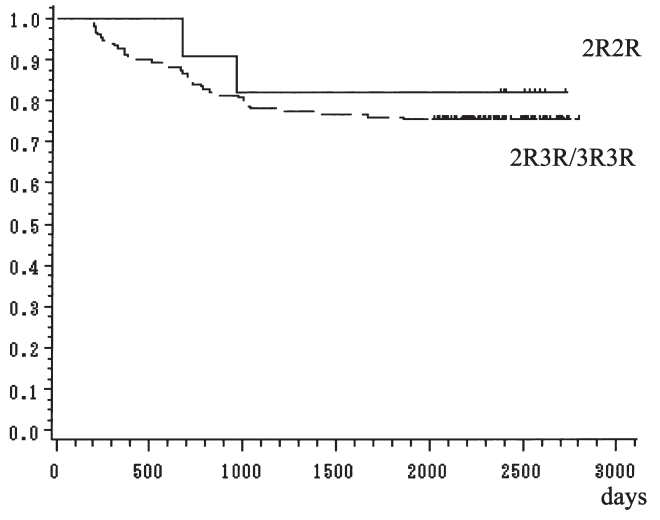

Figure 3. Disease-free survival for the 2R2R (N=11) and 2R3R/3R3R (N=150) groups.

Table IV. Reported frequency of TS genotype in colorectal cancer.

Author/ref.	n	2R2R	2R3R	3R3R	Other	(%) of 2R2R genotypes
Pullarkat (20)	52	11	26	15		21.2
Iacopetta (21)	221	163[a]		58		N/A
Lecomte (22)	87	14	44	28	1	16.1
Kawakami (12)	151	6	92	50	3	4.0
Our results	161	11	40	110		6.8

[a]Total number of 2R2R and 2R3R cases.

group, the differences were not statistically significant, possibly because of the small number of 2R2R cases enrolled in this study.

Discussion

5-FU is a key drug in colorectal cancer chemotherapy. The target enzyme for this agent is thought to be pTS, and many authors have discussed the relationship between chemotherapeutic efficacy and the enzyme activity in the tumor.

Because of its technical ease, immunohistochemistry (IHC) had been mostly used to evaluate pTS activity. Many studies of chemotherapy administered in an adjuvant setting after resection of colorectal cancer have been reported. Johnston *et al* (2), Yamachika *et al* (3), Takenoue *et al* (4) and Sakamoto *et al* (5) performed IHC and reported pTS positive cases showed poor prognosis. However, these findings remain controversial. Sanguedolce *et al* (6), Cascinu *et al* (7), Tomiak *et al* (8), Allegra *et al* (9) and Nanni *et al* (10) performed analyses using almost the same modality for almost the same populations as in the studies by Johnston, Yamachika and Takenoue, but they could not find any significant data. We think there are three reasons for this discrepancy. First, most of the studies were retrospective and some of them made no mention of details of the regimen, so there may be bias in both therapy and patient characteristics that cannot be identified. Second, IHC itself has potential bias, including the staining method, the position of the dividing line between high and low expressions, the method used for heterogeneous stain evaluation and for maintaining consensus among the investigators. Furthermore, a considerable difference has been observed between the level of pTS expression estimated with IHC and the actual number of FdUMP binding sites of pTS. To resolve these problems, some researchers have tried to use quantitative methods. Van Triest *et al* (14) used the FdUMP binding assay, which directly shows the number of active pTS sites. Kornmann *et al* (15) used the semi-quantitative RT-PCR method to evaluate *TS* mRNA. However, both methods need fresh frozen samples and the techniques are more complicated than those for IHC. IHC therefore remains the method most often used to evaluate TS expression, even though the method entails many problems. The third potential source of bias is that TS activity is regulated by many factors because pTS is not only a target enzyme of 5-FU, but also a rate-limiting enzyme for pyrimidine synthesis. The induction of pTS is thought to occur when there is a major increase in the demand for dTMP, which is a product of the reaction catalyzed by pTS. Furtermore, the diversity of intercellular expressions of pTS in cancer may reflect the cell cycle, because pTS is considered to be an S-phase specific enzyme. These problems are thought to make the evaluation of pTS expression by means of IHC ambiguous.

Moreover, 5-FU treatment for carcinoma may also affect the pTS activity itself. Some authors have reported an increase in FdUMP binding sites in cases treated with 5-FU chemotherapy. Van der Wilt (16) reported that pTS levels in tumors from 5-FU treated mice tended to be higher than in those in controls, and this finding has been confirmed by Peters *et al* (17) in a clinical trial. They reported operative specimens obtained after longer chemotherapy showed higher total pTS than the specimens from subjects that received shorter chemotherapy. In addition, Omura *et al* reported the number of FdUMP binding sites of pTS significantly increased after about a 10-day administration of tegafur and uracil for colorectal cancer (18). These findings suggest pTS evaluation using the sample obtained before chemotherapy would be less informative than that obtained after chemotherapy. That is, most of the trials mentioned above might have selected 'inadequate' samples to evaluate the chemotherapy effect. For an accurate evaluation of a patient's TS potential, therefore, we think it is very important which cells are to be assessed and when. For this reason, biological features that are not influenced by the cell's environmental factors, including the cell cycle and prior chemotherapy, may be preferable for this evaluation. The *TS* gene has a polymorphic tandem repeat sequence (TRS) in its non-translating region, and the number of TRSs regulates the translation activity of *TS* mRNA (11,19). While this polymorphism may be a more useful prognostic marker than those used for other methods, including IHC analysis of pTS, few details are available on its frequency, relation to prognosis, and response to chemotherapy. Pullarkat *et al* (20) reported this gene polymorphism was a good marker for the prediction of chemotherapeutic response to and toxicity of 5-FU treatment for advanced colorectal cancer. For the evaluation of adjuvant settings, Iacopetta *et al* (21) and Lecomte *et al* (22) confirmed the usefulness of this marker, but not in a prospective trial and no comparison with IHC data was made.

Our results show a favorable survival and disease-free period after adjuvant chemotherapy in 2R/2R colon cancer cases, although survival benefits were not statistically

significant because of the small size of the 2R/2R subset. This might be the reflection of the fact that our regimen is therapeutically not powerful enough for 2R/3R and 3R/3R cases, but we can at least conclude that cases with the 2R/2R genotype may be good responders to this regimen. However, Jakobsen *et al* (23) mentioned a good response rate for 3R/3R cases in a therapeutic setting. This discrepancy may be accounted for by differences in residual cancer cells and chemotherapy regimen. That is, the balance of pTS production and its inhibition by chemotherapy is extremely important. If 5-FU-based regimens were divided into two groups, that is, one with weak TS inhibition but less toxicity and the other with strong TS inhibition but more toxicity, 2R/2R cases should be treated with the former regimens and 3R/3R cases with the latter. Such a division suggests the possibility of tailor-made chemotherapy for this polymorphism.

Our results also point to the lower prevalence of the 2R allele in Japanese than that reported in other nations. Table IV shows reported prevalences of this polymorphism, including our findings, which may partly explain the interracial differences in therapeutic efficacy and in the occurrence of adverse effects such as diarrhea.

We investigated *TS* gene polymorphism in cancer tissue. The *TS* gene locus is 18p, where LOH is frequently observed in cancer tissue. Thus the status of the *TS* functional genotype may differ in cancer and normal tissue. Theoretically, the risk of adverse events would then be related to the pTS status in normal tissue and the response potential would be linked to its status in cancer. The *TS* genotype of the best candidate for 5-FU-based chemotherapy should be 2R/3R in normal tissue, and 2R/loss in cancer tissue. However, in this study we could not distinguish 2R/loss from 2R/2R or 3R/ loss from 3R/3R. More detailed evaluation using microsatellite markers is therefore essential to determine the influence of functional *TS* genotype on both the favorable and adverse effects of 5-FU.

In conclusion, the polymorphism of thymidylate synthase is likely to help predict the efficacy of adjuvant oral 5-FU chemotherapy, and this valuable feature may clarify the possibility of tailor-made 5-FU-based chemotherapy.

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