Polymorphism in the Thymidylate Synthase Promoter Enhancer Region Is Not an Efficacious Marker for Tumor Sensitivity to 5-Fluorouracil-based Oral Adjuvant Chemotherapy in Colorectal Cancer

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ABSTRACT

Thymidylate synthase (TS) is the target enzyme of 5-fluoropyrimidines. The TS gene promoter enhancer region (TSER) possesses tandem, repeated, regulatory sequences that are polymorphic in humans. This polymorphism has been reported to influence TS expression in vitro and in vivo. In this study, we assessed whether or not the TSER genotype is an efficacious marker for tumor sensitivity to 5-fluorouracil (5-FU)-based oral adjuvant chemotherapy for colorectal cancer. One hundred and thirty-five Japanese patients who received curative resection and 5-FU-based oral adjuvant chemotherapy were studied. TSER genotypes of the tumors were analyzed by PCR. The numbers of repeated sequences of representative bands were determined by direct sequence. The genotypes of two-/two-repeats (TSER 2/2), two-/three-repeats (TSER 2/3), three-/three-repeats (TSER 3/3), and three-/five-repeats (TSER 3/5) were found in 11 (8.1%), 32 (23.7%), 85 (63.0%), and 7 (5.2%) tumors, respectively. Patients were classified into two groups: TSER 2/2 or 2/3 group; and the TSER 3/3 group. The relationship between the TSER genotype group and disease-free intervals was analyzed by univariate and multivariate analyses. Five-year disease-free survivals of the TSER 2/2 or 2/3 group and the TSER 3/3 group were 77% and 75%, respectively (P = 0.89). Multivariate analysis revealed that stage was the only independent prognostic factor and that the TSER genotype did not have a prognostic significance (hazard ratio for TSER 3/3, 0.91; P = 0.84). In conclusion, TSER genotype is not an efficacious marker for tumor sensitivity to 5-FU-based oral adjuvant chemotherapy for Japanese colorectal cancer patients after curative resection.

INTRODUCTION

5-FU,2 a classical TS inhibitor, plays a central role in chemotherapy for colorectal cancer. Previous studies have shown that sensitivity to 5-FU-based chemotherapy is associated with the intratumoral level of TS (1, 2). The TS gene is localized on chromosome 18 p11.32 and has a polymorphic, tandem, repeated sequence in its promoter enhancer region (TSER). In a transient expression assay of cancer cells, TS genes with the three-repeat sequence were reported to have greater expression activity than those with the two-repeat sequence (3).

In clinical specimens of gastrointestinal cancer, TS gene expression is significantly higher in tumors with the three-/three-repeat sequence (TSER 3/3) than in those with the two-/two-repeat sequence (TSER 2/2) and two/three-repeat sequence (TSER 2/3) (4). These studies suggest the possibility that TSER polymorphism may be a novel predictor of the efficacy of TS-directed chemotherapy (5). However, to our knowledge, there have not been any investigations that have estimated the significance of the TSER polymorphism in adjuvant TS-directed chemotherapy for colorectal cancers. The aim of this study was to assess whether or not TSER polymorphism can predict the efficacy of 5-FU-based oral adjuvant chemotherapy in patients with colorectal cancer who underwent curative surgery.

MATERIALS AND METHODS

Patients. We studied 135 patients with stage I-III colorectal cancer who underwent “curability A resection” and received postoperative adjuvant chemotherapy from February 1994 to December 1998 at the Division of Surgical Oncology, Department of Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences. The study did not consist of patients with either synchronous or metachronous multiple colorectal cancers. The American Joint Committee on Cancer classification and stage grouping was used to classify

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2 The abbreviations used are: 5-FU, 5-fluorouracil; TS, thymidylate synthase; TSER, thymidylate synthase promoter enhancer region; LOH, loss of heterozygosity; CI, confidence interval.
tumors (6). Curability A resection was postoperatively defined according to the Japanese Classification of Colorectal Carci-
noma (7). Each tumor was histopathologically classified in accordance with the WHO criteria presented by Jass and Sobin (8). The 135 patients included 16 patients with stage I cancer, 61 patients with stage II cancer, and 58 patients with stage III cancer. Thirty-two tumors were classified as well-differentiated adenocarcinomas, 98 tumors were classified as moderately differentiat-ed adenocarcinomas, 3 tumors were classified as poorly differentiat-ed adenocarcinomas, 1 tumor was classified as a mucinous carcinoma, and 1 tumor was classified as a signet ring cell carcinoma. None of the patients was given preoperative chemotherapy or radiotherapy. All patients received oral adjuvant chemotherapy that started 3 weeks after curative surgery and lasted for 12 months [83 patients received Carmofur, 300 mg, 3×/day; 52 patients received UFT (a mixture composed of a fixed 1:4 molar ratio of tegafur and uracil), 400 mg, 2×/day]. The mean age of patients was 62 years (range, 29–78 years). Sixty-nine of the patients were male, and 66 were female. Eighty-one tumors were localized to the colon, and 54 tumors were in the rectum. All patients underwent standard follow-up examinations, including laboratory testing every 3 months. Chest roentgenograms, computed tomography, and abdominal ultrasonography were performed every 6 months. Colonoscopies were performed annually. The median follow-up period was 48 months (minimum follow-up, 4.2 months). Written informed consent was obtained from all patients.

**PCR and Direct Sequencing for TSER Polymorphism.** Genomic DNA was extracted from tumor tissues stored at −80°C using a standard protocol. PCR was performed using TSER-specific primers. Primer sequences and reaction conditions were as reported previously (4). After amplification, the PCR product was electrophoresed on a 2.8% NuSieve agarose gel (Biocompare, Inc., Burlingame, CA) containing ethidium bromide. The number of repeats in representative bands was determined by direct sequencing (9). We determined the genotype as TSER 2/2 or 3/3 (homozygous) when only one band was observed. When two bands were observed, the genotype was determined by direct sequencing (9). We determined the genotype as TSER 2/2 or 3/3 (homozygous) when only one band was observed. When two bands were observed, the genotype was decided as TSER 2/3 or 3/5 (heterozygous) without consideration for differences in band intensities.

**Statistical Analysis.** Statistical analyses were performed using the computer program STATISTICA (StatSoft, Tulsa, OK). Two variables with continuous data, age and maximum tumor diameter, were classified into two groups, based on the median of each variable (66 years and 4.5 cm, respectively). Categorical data were analyzed by χ² or Fisher’s exact test. Analysis of survival was performed using the Kaplan-Meier method, and differences between survival curves were tested for significance using the log-rank test. Multivariate analysis was performed with Cox’s proportional hazard regression model to assess the effects of different variables on patient survival. All tests were two-tailed, and P < 0.05 was considered to be statistically significant.

**RESULTS**

**Frequency of the TSER Polymorphism.** The frequencies of TSER genotypes were as follows: TSER 2/2, 11 tumors (8.1%); TSER 2/3, 32 tumors (23.7%); TSER 3/3, 85 tumors (63.0%); and TSER 3/5, 7 tumors (5.2%). Fig. 1 shows representative samples of the TSER genotypes in colorectal cancers. Among the 32 tumors with TSER 2/3, 7 tumors (21.9%) showed an obvious difference in intensity between two bands. These tumors were thought to have LOH at the TSER polymorphic locus, but we judged the genotype as TSER 2/3.

**Survival Analysis and TSER Polymorphism.** We excluded patients with TSER 3/5 from the survival analysis because a significant difference in TS expression has been reported between tumors with TSER 2/2 and 2/3 and tumors with TSER 3/3 (4). Patients were classified into two groups: the TSER 2/2 and 2/3 group; and the TSER 3/3 group. There was not a significant correlation between clinicopathological features and the TSER genotype groups (Table 1). Fig. 2 shows the disease-free survival of patients with colorectal cancer, according to TSER genotype groups. Five-year disease-free survivals of the TSER 2/2 and 2/3 group and the TSER 3/3 group were 77% and 75%, respectively (P = 0.89, log-rank test). In multivariate Cox’s regression analysis, stage was the only independent prognostic factor, and the hazard ratio for the TSER 3/3 group was 0.91 (95% CI, 0.37–2.27; Table 2).

**DISCUSSION**

Based on prospective studies (10, 11), Japanese clinical oncologists often administer oral 5-FU-based adjuvant chemo-
terapy to colorectal cancer patients after curative resection. Patients prefer oral chemotherapy to i.v. chemotherapy (12). In the interim report of the National Surgical Adjuvant Breast and Bowel Project C-06, there were no differences in toxicity grade and spectrum between i.v. and oral adjuvant chemotherapies for stage II-III colorectal cancer (13). Therefore, it is important to research an efficacious marker of tumor sensitivity to 5-FU-based oral adjuvant chemotherapy for colorectal cancers.

In preoperative chemoradiation therapy for rectal cancer and chemotherapy for metastatic colorectal cancer, the possibilit-y of TSER genotype as a response marker to 5-FU-based chemotherapy has been reported. Villafranca et al. (14) analyzed the TSER genotype in pretreatment biopsy specimens from 65 rectal cancer patients who received preoperative radi-
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...therapy is significantly higher in tumors with a probability of downstaging by preoperative chemoradiation and 5-FU-based chemotherapy. They reported that the 2/2 or 2/3 genotypes when compared with those with the 3/3 genotype. The study was performed in 24 patients with metastatic colorectal cancer who were treated with a bolus injection of 5-FU (15). In this study, we estimated the potential of using the TS genotype and mRNA expression level, but tumors with TSER 2/2 and 2/3 had significantly lower TS enzymatic activity than tumors with TSER 3/3 (16). Thus, the classification into two groups (TSER 2/2 and 2/3 group and TSER 3/3 group) in this study is consistent with previous studies in the literature.

LOH on 18p11 is a problem in TSER genotyping using tumor tissue (17). DNA extracted from frozen tumor materials was contaminated with DNA from normal stromal cells in this study. We judged the genotype as heterozygous (e.g., TSER 2/3) and disregarded allelic imbalances when two bands were observed. Thus the TSER genotypes in this study are thought to agree with those of patients' normal tissue. The frequency of LOH on 18p in colorectal cancer is reported to be 40% (18). In this study, 22% of tumors with TSER 2/3 were suspected to have LOH on 18p11. It is not known whether TS expression levels change in tumor cells with or without LOH on 18p11.

Care must be taken to recognize that ethnic variation exists in the TSER genotype. In the Caucasian and Southwest Asian populations, the frequencies of TSER 2/2, 2/3, and 3/3 are reported to be 16–19%, 43–44%, and 38–40%, respectively (19). In this study, the frequencies of TSER 2/2, 2/3, and 3/3 were 8%, 24%, and 63%, respectively. Therefore, the allele frequency of three repeats tends to be higher in Japanese than in the Caucasian and Southwest Asian population. In general, 5-year disease-free survival rates in colorectal cancer patients were 8%, 24%, and 63%, respectively. Therefore, the allele frequency of three repeats tends to be higher in Japanese than in other ethnic populations. This similarity of the overall 5-year disease-free survival supports the lack of a prognostic significance for the TSER genotype. However, the clinical significance of the TSER genotype must be estimated independently in other populations because of ethnic variation in the allele frequencies.

Previous studies have described that low TS expression in metastatic colorectal cancers is a predictor of clinical response to 5-FU-based therapy (1, 2). On the other hand, in curatively resected colorectal cancers, tumors with low TS expression have no survival benefit with 5-FU-based adjuvant chemotherapy.
Table 2 Prognostic variables for survival in Cox regression analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tbody>
<tr>
<td></td>
<td>HR* (95% CI)</td>
<td>P</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;66</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>≥66</td>
<td>0.28 (0.09–0.81)</td>
<td>0.02</td>
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<tr>
<td>Gender</td>
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<td></td>
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<td>Female</td>
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</tr>
<tr>
<td>Male</td>
<td>2.16 (0.91–5.1)</td>
<td>0.08</td>
</tr>
<tr>
<td>Tumor location</td>
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<tr>
<td>Colon</td>
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<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>0.88 (0.37–2.09)</td>
<td>0.78</td>
</tr>
<tr>
<td>Maximum tumor diameter (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4.5</td>
<td>1</td>
<td></td>
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<tr>
<td>≥4.5</td>
<td>0.62 (0.27–1.41)</td>
<td>0.25</td>
</tr>
<tr>
<td>Tumor grade</td>
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<td></td>
</tr>
<tr>
<td>Well</td>
<td>1</td>
<td></td>
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<tr>
<td>Mod, poor, muc, sig</td>
<td>2.14 (0.50–9.18)</td>
<td>0.31</td>
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<tr>
<td>Venous invasion</td>
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<td>Absent</td>
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<tr>
<td>Present</td>
<td>2.53 (0.99–6.47)</td>
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<tr>
<td>Stage</td>
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<tr>
<td>I, II</td>
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<tr>
<td>III</td>
<td>5.10 (2.00–12.99)</td>
<td>0.01</td>
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<tr>
<td>TSER genotype</td>
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<td></td>
</tr>
<tr>
<td>2/2, 2/3</td>
<td>1</td>
<td></td>
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<tr>
<td>3/3</td>
<td>1.07 (0.44–2.59)</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>0.91 (0.37–2.27)</td>
<td>0.84</td>
</tr>
</tbody>
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*HR, hazard ratio; well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma; muc, mucinous carcinoma; sig, signet ring cell carcinoma.

In conclusion, TSER genotype is not a significant efficacious marker in oral adjuvant chemotherapy for Japanese colorectal cancer patients. Future studies combining several factors, such as TS, thymidine phosphorylase, and dihydropyrimidine dehydrogenase, must be planned to find a beneficial marker in adjuvant chemotherapy for colorectal cancer.

REFERENCES


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