Identification and Functional Analysis of Single Nucleotide Polymorphism in the Tandem Repeat Sequence of Thymidylate Synthase Gene

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ABSTRACT

The variable number of tandem repeat (VNTR) of thymidylate synthase (TS) gene, mainly 2 repeat (2R) and 3 repeat (3R), is one of the genetic variations that can potentially predict the effectiveness of 5-fluorouracil-based chemotherapy. In this study we identified an additional single nucleotide polymorphism (SNP) in the VNTR of TS, followed by functional and clinical analysis of the SNP. Two-hundred fifty eight tumor samples were obtained from patients with primary colorectal adenocarcinoma. We observed three different patterns of electrophoresis by analysis of the VNTR with 2R/3R heterozygote. The sequencing results revealed a SNP, G/C polymorphism, within the 28-bp repeat component of TS VNTR. Each polymorphic allele was assigned as 2G, 2C, 3G, or 3C according to the combination of SNP and VNTR. Functional analysis showed that the plasmid construct with 3G sequence had three to four times greater efficiency of translation than other polymorphic sequences. 3R allele in colorectal cancer was subdivided into around half by the SNP, indicating its commonness among Japanese. TS genotypes of the patients with colorectal cancer were classified into high expression type (2R/3G, 3C/3G, and 3G/3G) and low expression type (2R/2R, 2R/3C, and 3C/3C). The patients who received oral fluoropyrimidines survived longer than the patients with no treatment in the group of low expression type. No benefit of oral fluoropyrimidines was observed in the group of high expression type. These results suggest that the double polymorphism in the TS tandem repeat sequence, the SNP and the VNTR, may provide a potential for more effective prediction of the clinical outcome of 5-fluorouracil-based chemotherapy.

INTRODUCTION

Recent advances in understanding of the genetic variations that influence drug metabolism, toxicity, and effectiveness are about to be introduced in cancer chemotherapy (1). Tailored chemotherapy based on such genetic variations has a great potential to improve cancer treatment. Polymorphism in the tandem repeat sequence of the TS gene is one of the genetic variations that can potentially predict the effectiveness of 5-FU. TS catalyzes the reductive methylation of dUMP by 5,10-methylenetetrahydrofolate to form dTMP, which is a critical reaction for cell proliferation. Accordingly, TS has been an important target of anticancer drugs including 5-FU, Raltitrexed, Pemetrexed, Nolatrexed, and other agents under development (2, 3). The TS gene contains VNTR, mainly 2R and 3R, in its 5'-UTR and is associated with TS expression (4, 5). Several clinical studies have shown the relationship between TS VNTR genotype and the effectiveness of 5-FU-based chemotherapy, although the studies had relatively small numbers of subjects, and validation by further clinical analysis was needed (6–9).

We have reported that the TS VNTR is related with TS protein expression (5) through affecting translational activity of TS mRNA (10), and TS alleles show frequent loss of heterozygosity (11). During the analysis of TS VNTR in which a heteroduplex product between 2R- and 3R-derived PCR product was separated by high-resolution gel, we observed different electrophoresis patterns of the heteroduplex. This observation strongly suggested the presence of an additional polymorphism in the area of TS VNTR. In this study we identified a novel SNP in the VNTR sequence of the TS, followed by functional analysis of the SNP. In addition, the clinical significance of comprehensive genotype of the VNTR and the SNP was additionally investigated.

MATERIALS AND METHODS

Patients and DNA Isolation. A total of 258 tumor samples were obtained by surgical resection from 258 patients with primary colorectal adenocarcinoma. The patients were all Japanese, and comprised 153 males and 105 females, ranging in age from 33 to 93 years, with a mean age of 66.2 years. Approximately 2 g of the surgically removed tissues were frozen immediately in liquid nitrogen and stored at −80°C until DNA isolation. Genomic DNA was isolated either by the standard method of proteinase K digestion and phenol-chloroform extraction or by a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. The remaining section of the sample was fixed with formalin and used for additional histological examination to confirm the diagnosis. All of the histological examinations were performed after staining with H&E. Approval for this project was obtained from the Kanazawa University School of Medicine Ethics Committee.

PCR and Heteroduplex Analysis. PCR with the template of genomic DNA was performed for TS genotyping of VNTR under the conditions described previously (5). The amplified DNA fragments were analyzed by electrophoresis on a 4% agarose gel for 3.5 h at 0.5 V/cm. The gel was stained with ethidium bromide. For heteroduplex analysis, PCR was performed using a primer labeled with fluorescein 5'-isothiocyanate and analyzed by Sambrook gel (Elsatron scientific, Cham, Switzerland) as described previously (11).

Cloning and Sequencing of PCR Product. To determine the sequence of PCR products, the fragments were cloned 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).

Plasmid Construct. Plasmid constructs of Firefly luciferase reporter gene fused downstream to TS 5'-UTR were created by the methods described previously (10). Briefly, the TS 5'-UTR sequence was obtained by PCR using forward primer TS33 GCCCTCGAGCAGCGCCG GCGGGAAAA and reverse primer TS28 TCCGAGCCGCCCACAGCAT. The 5'-UTR sequence was inserted into the pGL3-Basic Vector (Promega), followed by additional insertion into pCI (Promega) for constitutitional expression in transfected cells.

Luciferase Assay and RNA Protection Assay. HEK293 cell was obtained from Health Science Research Resources Bank (Osaka, Japan). Transfection of plasmids into HEK293 cell followed by luciferase assay and RNA protection assay was performed by the methods described previously (10). Briefly, each plasmid was transfected into HEK293 cells in conjunction with control plasmid pRL-CMV (Promega). The cells were harvested after 60 h and separated into two batches. One batch was used for cell lysis and the other for RNA isolation. Cells were lysed, and luciferase activity was measured by the Dual-Luciferase assay system (Promega). RNA was isolated by the single-step guanidine isothiocyanate method and subjected to the RNase protection assay. Translational activity of the construct was assessed by Firefly luciferase activity.
divided by the quantity of Firefly luciferase RNA, and assay results were normalized by the activity or RNA content of the control, Renilla luciferase.

**PCR-RFLP Analysis.** PCR was performed using forward primer TS25 AGGCGCGCGGAAGGGGTCCT and reverse primer TS18 TCCGAGCCGCCACAGGCAT. The PCR conditions were the same as those for TS VNTR. The PCR product was digested with Hae III followed by electrophoresis in 4% agarose gel and ethidium bromide stain. Analysis was performed at least twice to confirm the genotype.

**Statistical Analysis.** The relationships between TS genotype and clinicopathological variables were analyzed by χ² analysis or ANOVA. The cumulative survival rate was obtained by the Kaplan-Meier method, and statistical significance was analyzed by the log-rank test. P < 0.05 was considered to indicate significance.

**RESULTS**

**Identification of a SNP in TS VNTR.** In a previous report, we identified heteroduplex formation between 2R- and 3R-derived PCR products of TS VNTR (11). The heteroduplex products showed three different electrophoresis patterns: Lanes 1 and 2, close to 3R band (type A); Lanes 5 and 6, distant from 3R band (type C); Lanes 3 and 4, the middle of type A and C (type B).

**In Vitro Functional Analysis of SNP and VNTR of TS.** We created a plasmid construct in which TS 5'-UTR of 2G, 2C, 3G, or 3C were fused upstream to firefly luciferase gene to assess the functional difference among the polymorphic sequences. The plasmids were

![Fig. 1. Polymorphic electrophoresis pattern of heteroduplex product in Spreadex gel. PCR product from a sample of 2R/3R genotype forms a heteroduplex that appears in the electrophoresis at positions of longer than 3R band. The heteroduplex product showed 3 different electrophoresis patterns: Lanes 1 and 2, close to 3R band (type A); Lanes 5 and 6, distant from 3R band (type C); Lanes 3 and 4, the middle of type A and C (type B).](image)

![Fig. 2. SNP in the VNTR of TS. A, SNP, G/C polymorphism marked by *, was identified in the 28-bp repeat component of TS VNTR. The last nucleotide, guanine, indicated by a box in the repeat component was substituted with cytidine only if the component was closest to the ATG start codon. B, the series of boxes indicates the combination of the 28-bp repeat components in the VNTR sequence. Closed box is translated region. Four different patterns according to SNP and VNTR was identified and assigned to 2G, 2C, 3G, and 3C.](image)

![Fig. 3. Reporter assay using TS 5'-UTR-luciferase fusion constructs. Sequence structures of constructs are displayed at left. Open arrow with cytomegalovirus indicates cytomegalovirus promoter sequence. Open bar with 2G, 2C, 3G, or 3C indicates the TS 5'-UTR with each polymorphic sequence, respectively. Firefly luciferase activity of each construct, which was normalized by RNA quantity determined by RNase protection assay, is shown on the right. The ratio between luciferase activity and RNA content, which is expressed as Luc/RNA in the figure, indicates the translational efficiency. The translational efficiency of the construct with 3G sequence was three to four times higher than those of the other constructs. The results are based on four independent experiments and the value of the construct with 2G sequence was set as one in each experiment.](image)
transfected into HeLa cell and constitutively expressed by cytomegalo virus promoter. Translational efficiency of the constructs was identical among 2G, 2C, and 3C. However, the 3G sequence showed three to four times greater efficiency of translation than the other sequences (Fig. 3). These results suggest that the SNP affects translatability of TS mRNA, leading to functional subgroups in the 3R allele of TS VNTR.

Frequency of SNP and VNTR of TS in Colorectal Cancer. For better understanding of the clinical significance of the SNP, we analyzed the frequency of comprehensive genotypes of SNP and VNTR of TS in colorectal cancer. The PCR-RFLP method was developed for typing of the SNP in the TS 3R sequence (Fig. 4). SNP typing was not performed for 2R allele, because there was no functional difference between 2G and 2C sequences. TS genotypes including repeat numbers of >3 were excluded for the SNP typing because of their infrequency. The allele type of TS tandem repeat sequence was classified into 2R, 3G, and 3C according to the result of PCR-RFLP analysis. The allele frequency is summarized in Table 1. 3R sequence in the VNTR was subdivided into around half by the SNP, indicating its commonness among Japanese. The allele of 3G was significantly less frequent in females than in male when considering the 3R allele. In addition, the genotype of 3G/3G was significantly less frequent in females when 174 cases of 3R/3R genotype were considered for the analysis (Table 2). There was no significant association of other clinicopathological features including patient age, histological type, and clinical stage with allele or genotype frequency (data not shown).

Comprehensive Genotype of TS Predicts the Benefit from Oral FP in Patients with Colorectal Cancer. To assess the clinical role of the SNP we compared the survival of the patients who received postoperative oral FP to those with no adjuvant treatment under stratification of the patients by TS genotype. Survival analysis was performed with 111 patients who were in Dukes’ stage B or C., underwent curative surgery, and were available with postoperative clinical information including adjuvant treatment and long-term follow-up. The patients whose TS genotypes contained repeat number of >3 were excluded from the analysis. 5-FU, UFT (a combination of Tegafur and Uracil), Doxifluridine, or Carmofur were administered postoperatively to 9, 36, 9, and 10 patients, respectively. The other 47 patients received no adjuvant treatment. The median duration of administration was 99 weeks (range, approximately 13–200), and the median follow-up time was 59 months (range, approximately 11–158). The survival rate of the patients with postoperative oral FP was marginally better than that of the patients with no adjuvant treatment when all of the patients were analyzed (P = 0.052; Fig. 5A). The TS allele of 3G was considered a high expression allele and 2R or 3C as low expression alleles according to the in vitro functional analysis. On the basis of this consideration, TS genotypes of 2R/3G, 3C/3G, or 3G/3G were considered high expression (H) type and 2R/2R, 2R/3C or 3C/3C low expression (L) type. In the L-type group, patients who received oral FP survived longer than the patients with no adjuvant treatment (Fig. 5B). On the other hand, oral FP had no significant effect on the survival rate of the patients in the H-type group (Fig. 5C). The survival benefit from oral FP was not clear when the patients were stratified by TS VNTR only (data not shown).

**Table 1.** Allele frequency of TS polymorphism in colorectal cancer

<table>
<thead>
<tr>
<th>Allele frequency of TS polymorphism in colorectal cancer</th>
<th>2R</th>
<th>3G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>88</td>
<td>215</td>
</tr>
<tr>
<td>Male</td>
<td>53</td>
<td>140</td>
</tr>
<tr>
<td>Female</td>
<td>35</td>
<td>75</td>
</tr>
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</table>

*Numbers within parenthesis indicate %.

**Table 2.** Genotype frequency of TS polymorphism in colorectal cancer

<table>
<thead>
<tr>
<th>Genotype frequency of TS polymorphism in colorectal cancer</th>
<th>2R/2R</th>
<th>2R/3G</th>
<th>2R/3C</th>
<th>3G/3G</th>
<th>3G/3C</th>
<th>3C/3C</th>
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<tbody>
<tr>
<td>Total</td>
<td>10</td>
<td>27</td>
<td>41</td>
<td>51</td>
<td>86</td>
<td>37</td>
</tr>
<tr>
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<td>23</td>
<td>39</td>
<td>44</td>
<td>21</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>9</td>
<td>18</td>
<td>12</td>
<td>42</td>
<td>16</td>
</tr>
</tbody>
</table>

*P < 0.05 when considering 3R/3R group.

**DISCUSSION**

In this study we identified a functional SNP in the VNTR of TS. TS VNTR has been considered a novel predictor of clinical outcome of 5-FU-based chemotherapy. Recent clinical studies have demonstrated that the patients with 3R/3R homozygote obtain less benefit from 5-FU-based chemotherapy compared with patients who possess 2R allele (6–9). In contrast, one study reported no association between type of TS VNTR and effectiveness of fluorouracil and folinic acid (12). These controversial results might be partly attributable to the SNP in the 3R allele because the SNP arises a functionally different subgroup in the 3R allele. A double polymorphism in the TS tandem repeat sequence, SNP and VNTR, may provide a potentially more effective prediction of the clinical outcome with 5-FU-based chemotherapy. The role of this double polymorphism in the TS gene should be validated by a well-controlled large-scale clinical study. Although the number of subjects was small and the results were preliminary, we observed a significant role of the TS double polymorphism for prediction of clinical outcome with oral FP in the current study. Interestingly, TS VNTR polymorphism itself was insufficient for the prediction. The results support the notion that both SNP and VNTR of TS should be considered to use the genotype information of TS for tailored chemotherapy.

In this study, the 3G allele in TS double polymorphism was less frequent in Japanese females with colorectal cancer. This result is intriguing because a previous report demonstrated gender difference in the benefit from 5-FU-based adjuvant chemotherapy among colorectal cancer patients (13). One interesting explanation arose from the associations among TS double polymorphism, efficacy with 5-FU-
based chemotherapy, and patient gender. That is, the TS 3G allele is less frequent in females and may be related with poor response to 5-FU-based chemotherapy, resulting in a better response to the chemotherapy in females than in males. Although this notion is very interesting, other factor(s) may be responsible for the association of gender with the efficacy of 5-FU-based chemotherapy. Therefore, the relationship between TS 3G allele and the efficacy of 5-FU may simply be a coincidence arising from the gender difference in the allele distribution. In addition, it is necessary to analyze the frequency of TS double polymorphism with other ethnic populations, because a gender difference in the benefit from chemotherapy was demonstrated in Caucasians and the TS VNTR is known to have considerable ethnic variation (14). Recent studies suggest that genetic and epigenetic differences of colorectal cancer exist between males and females (15–18). Elucidation of the involvement of TS genotype in the gender difference may be interesting and important aims for additional study.

In conclusion, we identified a functional SNP in the area of TS VNTR and presented the double polymorphism as a potential predictor of the efficacy with 5-FU-based chemotherapy. Pharmacogenomics is of clinical importance and can improve the effectiveness of antineoplastic agents for cancer treatment. Additional studies both on the bench and at bedside are needed to clarify the role of the TS double polymorphism in cancer treatment.

REFERENCES

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