Thymidylate Synthase Gene Polymorphism Predicts Toxicity in Colorectal Cancer Patients Receiving 5-Fluorouracil-based Chemotherapy

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ABSTRACT

Purpose: The target enzyme for 5-fluorouracil (5-FU) is thymidylate synthase (TS). The TYMS gene encoding this enzyme is polymorphic, having either double (2R) or triple (3R) repeats of a 28-bp sequence in the promoter region and a 6-bp variation in the 3′-untranslated region (3′-UTR). TS expression predicts response to 5-FU-based chemotherapy, and the expression seems to be determined by the TYMS gene promoter. The aim of this study was to investigate the utility of determining these two TYMS gene polymorphisms to predict the toxicity and efficacy of 5-FU treatment in patients with colorectal cancer.

Experimental Design: The determination of TYMS genotypes was performed in tumor and normal tissues by PCR amplification from 90 patients with colorectal cancer who were treated with adjuvant or palliative 5-FU-based chemotherapy. Associations between polymorphisms in the TYMS promoter and in the 3′-UTR gene and clinical outcome of these 90 patients treated with 5-FU-based chemotherapy were evaluated individually. The linkage between TYMS promoter and TYMS 3′-UTR region polymorphisms was evaluated and a haplotype analysis was performed.

RESULTS: Individuals who were homozygous for the double repeat in the TYMS promoter region had more severe side effects to 5-FU. Patients with a 2R/2R, a 2R/3R, or a 3R/3R genotype had a grade 3 or 4 toxicity rate of 43, 18, and 3% respectively (P < 0.01). The TYMS promoter and TYMS 3′-UTR polymorphisms were in linkage disequilibrium, and the haplotype 2R/ins 6-bp was significantly associated with a high risk of severe side effects to 5-FU. The TYMS promoter and TYMS 3′-UTR polymorphisms were not associated with a response to 5-FU and survival of patients who received palliative 5-FU-based chemotherapy.

Conclusions: This study demonstrated that TYMS genotyping could be of help in predicting toxicity to 5-FU-based chemotherapy. TYMS genotyping might make it possible to individualize treatment for patients with colorectal cancer.

INTRODUCTION

Pharmacogenetics is becoming an increasingly important field in the study of cancer chemotherapy treatment. Genetic factors could alter drug metabolism and activity and could predict drug toxicity and/or efficacy. Determination of polymorphisms in xenobiotic metabolizing enzymes before the administration of chemotherapy could offer new strategies for optimizing the therapy of individual patients. Currently, 5-fluorouracil (5-FU) is the most frequently used chemotherapy agent in the treatment of colorectal cancer both in adjuvant and in palliative therapy. Thymidylate synthase (TS) is an enzyme that catalyzes the transformation of dUMP, which is essential for DNA replication. TS is considered to be the main intracellular target of fluoropyrimidines (1). An active metabolite of fluorodeoxyuridineylate (5-FdUMP), prevents DNA synthesis by forming stable complexes with TS and folate as a cofactor, thus blocking the conversion of dUMP to dTMP. Information regarding TS tumor tissue levels may be important because a low levels of tumor TS expression in colorectal patients receiving 5-FU-based chemotherapy was related to clinical responsiveness as well as to longer survival (2–8). The TYMS promoter enhancer region is polymorphic, and one of the polymorphisms influences the translation efficiency of TYMS mRNA (9–11). TYMS promoter comprises a 28-bp sequence, usually presented as a double-tandem repeat (2R) or a triple-tandem repeat (3R), and it was demonstrated that homozygous 3R/3R cells over-expressed TYMS mRNA compared with homozygous 2R/2R cells (10, 11). More recently, a single nucleotide polymorphism G>C has been described at the twelfth nucleotide of the second repeat of the 3R allele, leading to a tri-allelic locus (2R, 3RG, and 3RC). This polymorphism changes a critical residue in the USF E-box consensus element. The 3RC allele showed transcriptional activity that was similar to that of the 2R allele (12). Furthermore, a genetic polymorphism in the 3′-untranslated region (3′-UTR) of the TYMS gene has been identified, usually presented as a deletion of 6 bp at position 1494 (13). Preliminary data by Lenz et al. (14) demon-
strated a significant association between the 6-bp deletion and low TYMS mRNA expression in colorectal tumor tissue. The aim of this study was to determine whether the different polymorphisms in the TYMS promoter and the TYMS 3′-UTR region may be predictive of toxicity, tumor response, and survival in patients with colorectal cancer receiving 5-FU-based chemotherapy.

PATIENTS AND METHODS

Patients. From May 1995 to May 2001, 90 patients [47 men and 43 women (mean age, 65 years; range, 42–83 years)], who underwent primary colorectal cancer resection and received 5-FU-based chemotherapy were retrospectively included. This study was conducted in one medical center, and informed consent was obtained from all of the patients for using their tissues for research purpose. Anatomic locations of primary tumors were proximal in 24 cases, distal in 47 cases, and rectal in 19 cases. The distribution of tumors according to the tumor-node-metastasis (TNM) classification system were 14 stage II, 25 stage III, and 51 stage IV. The sites of synchronous metatasis were liver in 45 cases, peritoneum in 8 cases, lung in 5 cases, lymph nodes in 2 cases, and bone in 1 case. Four patients with rectal cancer underwent preoperative or postoperative radiotherapy.

Thirty-four patients received adjuvant 5-FU-based chemotherapy (11 stage II, 21 stage III, and 2 stage IV). The adjuvant treatments were as follows: a monthly schedule of leucovorin and 5-FU bolus (Mayo Clinic regimen; n = 10), a semimonthly regimen of 5-FU and leucovorin (LV5FU2 regimen; n = 22), a semimonthly regimen of 5-FU, leucovorin, and oxaliplatin (n = 1), and a semimonthly regimen of 5-FU, leucovorin, and irinotecan (n = 1).

Sixty-five patients received palliative 5-FU-based chemotherapy. Nine patients received prior adjuvant 5-FU-based chemotherapy. Among these nine patients, three received palliative 5-FU-based chemotherapy less 1 year after the end of the adjuvant chemotherapy. The palliative treatments were as follows: a Mayo Clinic regimen (n = 3), a LV5FU2 regimen (n = 29), a semimonthly regimen of 5-FU, leucovorin, and oxaliplatin (n = 26), a semimonthly regimen of 5-FU, leucovorin and, irinotecan (n = 4), and a 5-FU continuous regimen (n = 3).

Toxicity was recorded according to the criteria of the World Health Organization (WHO; ref. 15). Physical examination and a full blood count were performed before each cycle. All of the patients who had received at least one course of chemotherapy were evaluated for toxicity. Neurologic toxicity was excluded from the analysis in patients treated with an oxaliplatin-5-FU combination, because the most substantial adverse reaction associated with the use of oxaliplatin is neurologic. Tumor response was measured after every 2 or 3 months by computed tomography scan, or earlier in case of clinical suspicion of progression. Assessment of objective response was made according to the criteria of the WHO (15). The same chemotherapy regimen was maintained until disease progression or severe toxicity.

Samples and DNA Extraction. Representative samples of tumor and normal tissues obtained immediately after surgical resection and frozen in liquid nitrogen were used as the source of DNA for TYMS genotyping of 72 patients in the study. In another 18 cases, formalin-fixed paraffin-embedded surgical tumor and normal tissues were used. DNA was extracted using the QiaAmp DNA Minikit (Qiagen, Courtabeuf, France).

TYMS Genotyping. The TYMS genotyping was performed in normal and tumor tissues. Genotyping of the TYMS gene can be affected by the loss of heterozygosity (LOH) at 18p in the tumor DNA. The tumor TYMS genotyping was evaluated knowing the allelic status of the tumors.

TYMS Promoter Polymorphisms. PCR amplifications were performed on a GeneAmp PCR system 9700 (Applied Biosystems, Courtabeuf, France), as described previously (16). Amplification products were analyzed by capillary electrophoresis (automatic sequencer, Applied Biosystems 3900). Products of 210 bp, 238 bp, or both of these products, depending on the TYMS promoter genotype, were observed. Patients who were homozygous for the triple repeat (3R/3R) displayed only the larger PCR product, those homozygous for the double repeat (2R/2R) displayed only the smaller PCR product, and heterozygous individuals (2R/3R) showed both the larger and smaller PCR products.

The single nucleotide polymorphism G→C at the 12th nucleotide of the second repeat of 3R allele (rs2853542) was determined by RFLP. Briefly, the previous PCR product of the 2R/3R and 3R/3R patients was digested overnight at 37°C by HaeIII. The digestion product was analyzed on 8% acrylamide gel electrophoresis.

TYMS 3′-Untranslated Region Polymorphisms. PCR amplifications were performed on a GeneAmp PCR system 9700 (Applied Biosystems) in a 15-μL final volume with 100 ng of DNA, 0.5 mmol/L MgCl₂, 10× buffer, 0.2 mmol/L dNTPs, 0.375 μmol of each forward GAC GAA TGC AGA ACA CTT and reverse AAT CTG AGG GAG CTT GC primer, and 0.0375 units of Taq polymerase Cetus (Qiagen). Primers were synthesized by Genset (Paris, France). The PCR cycle was as follows: 15 minutes at 95°C followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension of 6 minutes at 72°C. Amplification products were analyzed on capillary electrophoresis (automatic sequencer, Applied Biosystems 3900). Products of 104 bp, 110 bp, or both of these products, depending on the TYMS genotype, were observed. Patients who were homozygous for the 6-bp deletion in the 3′-UTR of TYMS gene (del 6bp/del 6bp) displayed only the smaller PCR product, those homozygous for the wild-type allele (ins 6bp/ins 6bp) displayed only the larger PCR product, and heterozygous individuals (ins 6bp/del 6bp) showed both the larger and smaller PCR products.

Allelic losses at the TYMS locus were determined by comparing normal and tumor genotyping. The following ratio was calculated α = (IA*IB)/IB, where IA and IB corresponded to the fluorescence intensity of the 238-bp allele and the 210-bp allele in tumor DNA, and IA and IB corresponded to the fluorescence intensity of the 238-bp allele and the 210-bp allele in normal DNA. A ratio ≤0.5 or >2 indicated LOH. Concerning the tumor TYMS promoter genotype, a 2R/3R tumor exhibiting loss of the 2R allele was classified as a 3R/3R tumor, and a 2R/3R tumor exhibiting loss of the 3R allele was classified as a 2R/2R tumor. Similar
determinations were performed concerning the TYMS 3′-UTR polymorphisms.

**Statistical Analysis.** The TYMS promoter and TYMS 3′-UTR polymorphisms were first analyzed separately. The $\chi^2$ test was used to compare the observed genotype distributions with those expected by the Hardy-Weinberg equilibrium. Pairwise linkage disequilibrium between TYMS promoter and TYMS 3′-UTR region polymorphisms was estimated by a log-linear model and the extent of disequilibrium was expressed in terms of $D'$ which is the ratio of the unstandardized coefficient to its maximal/minimal value (17, 18).

The purpose of each analysis was to evaluate the association between the polymorphisms and outcome, i.e., toxicity to chemotherapy, response, and survival. $\chi^2$ or Fischer’s two-tailed exact test was used to determine the relationship between each categorical variable with the TYMS promoter and the TYMS 3′-UTR genotype. Normal tissue genotype was used to test the association between the TYMS promoter and the TYMS 3′-UTR polymorphisms and clinical outcome (the toxicity, response to chemotherapy, and survival). Gene–dose effects were modeled by assigning a value of 0, 1, or 2 to the genotype variable according to the subject’s number of small alleles, i.e., 2R and del 6bp, zero, one, and two small alleles, respectively. Tumor tissue genotype after correction for allelic loss was also used to test the association between the TYMS promoter or the TYMS 3′-UTR polymorphisms, and efficacy of treatment, i.e., response and survival. For the survival analysis, patients who received palliative 5-FU-based chemotherapy less than 1 year after the end of the adjuvant chemotherapy were excluded. Survival was calculated as the time from the start of palliative chemotherapy until progression (i.e., time to progression) or until death from any cause, or last contact if the patient was known to be alive (i.e., overall survival). Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test (19). The median time of survival was used to summarize the survival data.

To better characterize the contribution of the TYMS gene to clinical outcome, a haplotype analysis was performed using a maximum likelihood method recently proposed for haplotype–phenotype association (20). This method allows one to estimate haplotype frequencies as well as covariate-adjusted haplotype effects, expressed as odds ratios (ORs) for a binary phenotype (case–control status) by comparison with a reference haplotype. This method, initially developed for binary and quantitative trait analyses, was here extended to survival trait analysis by use of a parametric Weibull model for describing the association between haplotypes and the survival outcome (21). Linking this Weibull model to the maximum likelihood procedure for haplotype-based association analysis reported by Tregouet et al. (20) allows one to estimate haplotype effects expressed as hazard risk ratios (HRRs) by comparison with a reference haplotype, HRRs that are obtained by exponentiating the $\beta$ parameters associated with the inferred haplotypes. In all analyses, the most frequent haplotype was taken as the reference haplotype (20). All statistical tests were two-sided, and $P < 0.05$ was used to indicate statistical significance.

**RESULTS**

**Distribution of TYMS Polymorphisms**

**TYMS Promoter Polymorphisms.** TYMS promoter genotypes were assessed for 87 of the 90 patients. The distribution of the TYMS promoter genotype was 16% 2R/2R [14 patients; 95% confidence interval (CI), 9–26%], 51% 2R/3R (44 patients; 95% CI, 40–61%), 32% 3R/3R (28 patients; 95% CI, 23–43%), and 1% 3R/4R (1 patient; 95% CI, 0.06–7%) in normal tissue. The above distribution is in close agreement with that predicted by the Hardy-Weinberg equilibrium. Analysis of allelic imbalances was assessed for 42 of the 44 patients with a heterozygous 2R/3R genotype in normal tissue and revealed allelic loss in 48% (20/42; 95% CI, 32–63%) of the tumor DNA samples. Alleles 2R and 3R were lost in 10 cases. These results suggested the absence of selection of allelic losses during colorectal carcinogenesis.

The frequency of the single nucleotide polymorphism G>C at the 12th nucleotide of the second repeat of the 3R allele determined on a subset of 65 patients was 51 and 49% for the G and C allele, respectively. These two previous polymorphisms were combined and the distribution of the different genotypes was as follows: 2R/2R (18%), 2R/3R (32%), 2R/3R (16%), 3R/3R (15%), 3R/3R (5%), and 3R/3R (14%). This distribution did not follow significantly the one predicted by the Hardy-Weinberg equilibrium.

**TYMS 3′-Untranslated Region Polymorphisms.** TYMS 3′-UTR genotypes were assessed for 85 of the 90 patients. The distribution of the TYMS 3′-UTR genotype was 7% del 6bp/del 6bp (6 patients; 95% CI, 3–15%), 53% del 6bp/ins 6bp (45 patients; 95% CI, 42–64%), and 40% ins 6bp/ins 6bp (34 patients; 95% CI, 30–51%). The above distribution is in close agreement with that predicted by the Hardy-Weinberg equilibrium. Analysis of allelic imbalances was assessed for 35 of the 45 patients with a heterozygous del 6bp/ins 6bp genotype in normal tissue and revealed allelic loss in 54% (19 of 35; 95% CI, 37–71%) of heterozygous del 6bp/ins 6bp genotype tumor samples. Alleles del 6bp and ins 6bp were lost in 11 and 8 cases, respectively.

The number of double heterozygous for both TYMS promoter and TYMS 3′-UTR polymorphisms was 22.

**Analysis of Linkage Disequilibrium Measure between TYMS Promoter and TYMS 3′-UTR Polymorphisms.** The TYMS promoter and TYMS 3′-UTR polymorphisms were in linkage disequilibrium. The $D'$ value calculated by the estimation maximization algorithm was −0.76 (i.e., allele 2R is preferentially associated with allele ins 6bp). The corresponding $P$ value was inferior to 10$^{-9}$.

**Analysis of Toxicity**

The different regimen of 5-FU-based chemotherapy showed similar rates of grade 3 and 4 toxicity (i.e., 16% for 5-FU regimen alone and 18% for bitherapy regimen).

Except for three patients who had to stop adjuvant chemotherapy for toxicity, the duration of adjuvant 5-FU-based chemotherapy was 6 months. Palliative 5-FU-based chemotherapy was administered for a median duration of treatment of 22 weeks [2–84]. Grade 3 or 4 toxicities per patient are summarized in Table 1. Grade 3 peripheral neuropathy occurred in 10%
of patients (9 patients); and this side effect was excluded from the analysis because all of these patients were treated with 5-FU-oxaliplatin. Maximal toxicity per patient (neuropathy excluded) was WHO grade 3 and 4 in 13% (12 patients) and 3% (3 patients) of patients, respectively. No toxic deaths were observed in this study. The most prevalent nonhematologic toxicities reported were digestive, with 8% (seven patients) of patients reporting grade 3 or 4 diarrhea and 4% (four patients) experiencing grade 3 nausea or vomiting.

Analysis of the association between TYMS promoter genotype and toxicity was performed for 86 patients. One patient with 3R/4R TYMS promoter genotype and three patients with not assessable TYMS promoter genotypes were excluded from the analysis. Analysis of the association between TYMS 3’-UTR genotype and toxicity was performed for 85 patients. Five patients with not assessable TYMS 3’-UTR genotypes were excluded from the analysis.

A significant inverse relation was observed between the number of 28-bp tandem repeats in the promoter region of the TYMS gene and the severity of toxicity. In 43% (6 of 14) of patients with normal tissue 2R/2R genotype a toxicity grade 3 or 4 was observed compared with 18% (8 of 44) in the 2R/3R group (2R/3RC, 4 patients; 2R/3RG, 4 patients), and 4% (1 of 28) in the 3R/3R group (3RC/3RG, 1 patient; P < 0.02). When we analyzed the results according to the G>C polymorphism at the 12th nucleotide of the second repeat of the 3R allele, there was no evidence for a role of this single nucleotide polymorphism in the occurrence of grade 3 or 4 toxicity. No association was observed between TYMS 3’-UTR polymorphisms and the severity of toxicity (Fig. 1B).

Table 1. Association between TYMS haplotypes and the risk of toxicity grade 3–4

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3R ins 6bp</td>
<td>0.10</td>
<td>—*</td>
<td></td>
</tr>
<tr>
<td>3R del 6bp</td>
<td>0.23</td>
<td>2.7 (0.6–11.9)</td>
<td>0.18</td>
</tr>
<tr>
<td>2R ins 6bp</td>
<td>0.63</td>
<td>8.1 (1.6–41.8)</td>
<td>0.012</td>
</tr>
<tr>
<td>2R del 6bp</td>
<td>0.036</td>
<td>3.2 (0.2–46.2)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* Reference haplotype.

The number of variant 2R alleles was associated with an increasing OR of toxicity grade 3 or 4 (P trend = 0.002). An OR of 20 (95% CI, 1.5–282) was observed for patients with the homozygous genotype carrying two 2R alleles and an OR of 6 (95% CI, 0.70–54) for patients with the heterozygous genotype as compared with the reference group of patients homozygous for the 3R allele. To address a possible effect of the different types of 5-FU-based chemotherapy regimens (i.e., 5-FU regimen alone versus bitherapy regimen) on the toxicity risk associated with the 2R/2R genotype, we performed a Mantel-Haenzel test. No significant heterogeneity was observed between the two groups.

These results were slightly modified after adjustment for the TYMS 3’-UTR polymorphism.

Because the TYMS promoter (3R > 2R) and the TYMS 3’-UTR (ins 6bp > del 6bp) polymorphisms were in linkage disequilibrium, a haplotype analysis was performed using a maximum likelihood method recently proposed for haplotype-phenotype association. The different haplotype frequencies were estimated among patients who developed grade 3 or 4 toxicities and those who did not develop such toxicities. A significant haploptic effect was observed (P < 0.01). The different ORs are given in Table 2. Only the haplotype 2R/ins 6bp was significantly associated with a high risk of severe side effects to 5-FU.

Analysis of Efficacy of Palliative 5-FU-based Chemotherapy

Among 65 patients who received palliative 5-FU-based chemotherapy in this analysis, we excluded the 3 patients who received palliative 5-FU-based chemotherapy less than 1 year after the end of the adjuvant 5-FU-based chemotherapy.

Response to Treatment. Of the 62 eligible patients, 58 were assessable for response. The overall response to 5-FU-
Predicting Toxicity in 5-FU-based Chemotherapy

In our study, a genotyping approach has been proposed. PCR amplification of the TYMS promoter and the 3'-untranslated region, as an indirect method for determination of the TS expression levels, could be a good alternative to the classical assay for TS-activity determination (high-performance liquid chromatography).

**DISCUSSION**

In this present study, we investigated whether the determination of two common polymorphisms of the TYMS gene in colorectal cancer patients who received 5-FU-based chemotherapy, could be used to predict the toxicity to 5-FU-based chemotherapy and for a subgroup of metastatic patients, tumor response and survival. Our analysis suggests that relevant polymorphisms of the TYMS gene can result in differences of defined clinical end points among colorectal cancer patients who received 5-FU-based chemotherapy.

In several tumor cell lines, TS activity levels were found to be predictive of response to fluoropyrimidines as well as newer folate-based TS inhibitors (22–24). Furthermore, clinical studies suggest that TS expression could predict overall outcome and response to 5-FU in colorectal cancer patients. In the adjuvant setting, the TS expression is one of the most promising predictors of disease-free survival and overall survival in colorectal cancer patients (27, 28). In the setting of advanced metastatic disease, either high levels of TYMS mRNA or TS protein measured from the metastatic disease site predict poor response to fluoropyrimidine-based therapy (4, 8). Therefore, a better understanding of the mechanism of regulation in TS expression is of substantial importance for a more effective clinical cancer chemotherapy strategy with TS inhibitor. An important mechanism of expression control may come from genetic polymorphisms of the TYMS gene. A tandem-repeat sequence has been identified in the promoter of the TYMS gene (10). It was shown to be polymorphic, containing either two or three 28-bp repeats; the three 28-bp repeat (3R) is associated with higher expression of TS than the two 28-bp repeat (2R; refs. 10, 11, and 29). More recently, a common polymorphism in the 3'-UTR of the TYMS gene has been identified, usually presented as a 6-bp deletion at bp 1494 (13). It has been reported that the 6-bp deleted allele is associated with decreased TS mRNA level in colorectal tumors leading to the hypothesis that this polymorphism could play a role in mRNA stability and translation (14). Patients harboring an ins 6bp/ins 6bp genotype demonstrated a more than 4-fold increase in expression of TYMS mRNA when compared with a homozygous deletion genotype. The heterozygous genotype showed an intermediate expression level.

In our study, a genotyping approach has been proposed. PCR amplification of the TYMS promoter and the 3'-untranslated region, as an indirect method for determination of the TS expression levels, could be a good alternative to the classical assay for TS-activity determination (high-performance liquid chromatography).

**Table 3** Tumor promoter TYMS polymorphism and response to chemotherapy

<table>
<thead>
<tr>
<th>TYMS promoter polymorphisms, n (%)</th>
<th>2R/2R</th>
<th>2R/3R</th>
<th>3R/3R</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial or complete response</td>
<td>5/13 (39)</td>
<td>4/15 (27)</td>
<td>11/25 (44)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>8/13 (61)</td>
<td>11/15 (73)</td>
<td>14/25 (56)</td>
<td>&gt;0.7</td>
</tr>
</tbody>
</table>

NOTE. n = 53 patients; four patients were not assessable for tumor TYMS promoter genotype and one patient with 3R/4R TYMS promoter genotype was excluded.

**Table 4** Tumor TYMS 3'-UTR polymorphism and response to chemotherapy.

<table>
<thead>
<tr>
<th>TYMS 3'-UTR polymorphisms, n (%)</th>
<th>del 6bp/ del 6bp</th>
<th>ins 6bp/ del 6bp</th>
<th>ins 6bp</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial or complete response</td>
<td>4/9 (44)</td>
<td>2/7 (29)</td>
<td>14/30 (47)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5/9 (56)</td>
<td>5/7 (61)</td>
<td>16/30 (53)</td>
<td>&gt;0.8</td>
</tr>
</tbody>
</table>

NOTE. n = 46 patients; 12 patients not assessable for tumor TS 3'-UTR genotype were excluded.
chromatography, quantitative reverse transcription PCR, immunohistochemistry). The two 28-bp repeat and the 6-bp deletion allele frequencies of the TYMS gene were 67% (58 of 87) and 60% (51 of 85), respectively. These frequencies were similar to that observed in previous reports for Caucasian individuals (13, 30, 31). We demonstrated that among colorectal cancer patients who received 5-FU-based chemotherapy, those possessing the 2R variant allele showed a significantly higher risk of severe toxicity to chemotherapy. The risk of toxicity increased significantly with the number of 2R alleles. This result suggests that low TYMS mRNA expression levels in normal tissue are associated with a higher risk of the cytotoxic effects of 5-FU. The decreasing TYMS mRNA expression in normal tissue of patients with the 2R/2R genotype did not protect the normal cells against damage by 5-FU-based chemotherapy because of the high efficacy of TS inhibition. The resulting increase in normal cell death rate leads to severe toxicity. The correlation observed in the present study between the TYMS promoter genotype and the 5-FU-based chemotherapy toxicity might be obscured by the fact that patients were treated with different 5-FU-based chemotherapy regimens. Analysis by type of 5-FU-based chemotherapy show that the rate of toxicity is dependent on the number of patient with a favorable TYMS promoter genotype included in each group rather than the specific 5-FU-based chemotherapy, and no significant heterogeneity concerning the toxicity risk associated with the 2R/2R genotype was observed according to the different 5-FU-based chemotherapy regimens. Only one previous report has studied the association between TYMS promoter genotype and toxicity to 5-FU. In a series of 52 patients with metastatic colorectal cancer treated only with 5-FU, Pullarkart et al. (32) reported a significant inverse association between the number of the 28-bp tandem repeats in the 5′-promoter region of the TYMS gene and the severity of toxicity to 5-FU. In 63% (5 of 8) of patients with 2R/2R genotypes, a toxicity grade 3 was observed, compared with 27% (6 of 22) in the group 3R/3R, and 32% (6 of 19) in the 2R/3R group. Our results are similar to these and confirm the TYMS tandem repeat promoter polymorphism germinol genotyping relevance for 5-FU tolerance prediction in a larger series of patients with colorectal cancer. However the role of genetic polymorphisms of other enzymes, such as dihydropyrimidine dehydrogenase (DPYD) involved in 5-FU catabolism, should be explored to improve the prediction of 5-FU toxicity (33).

The TYMS gene is located on the short arm of chromosome 18, which has been shown deleted in 40% of colorectal cancer (34). If the link between TYMS polymorphism and TS level is relevant, the TS level in tumors with loss of the small

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**Fig. 2** Kaplan-Meier estimates of survival according to TYMS promoter polymorphism for patients who received palliative 5-FU-based chemotherapy. A, time to progression (n = 55 patients; P = 0.08). B, overall survival (n = 56 patients; P = 0.08). Solid line, 2R/2R; dashes, 2R/3R; dotted line, 3R/3R.

**Fig. 3** Kaplan-Meier estimates of survival according to TYMS 3′-UTR polymorphism for patients who received palliative 5-FU-based chemotherapy. A, time to progression (n = 48 patients; P = 0.7). B, overall survival (n = 49 patients; P = 0.04). Solid line, del 6bp/del 6bp; dashes, ins 6bp/del 6bp; dotted line, ins 6bp/ins 6bp.

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**Table 5** Association between TYMS haplotypes and the risk of progression

<table>
<thead>
<tr>
<th>Haplotypic HRR (95% CI)</th>
<th>P</th>
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<tr>
<td>2R/3R del 6bp/ins 6bp</td>
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<tr>
<td>2R ins 6bp</td>
<td>0.400</td>
</tr>
<tr>
<td>3R del 6bp</td>
<td>0.336</td>
</tr>
<tr>
<td>3R ins 6bp</td>
<td>0.264</td>
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* Reference haplotype.

**Table 6** Association between TYMS haplotypes and the risk of death

<table>
<thead>
<tr>
<th>Haplotypic HRR (95% CI)</th>
<th>P</th>
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<tr>
<td>2R ins 6bp</td>
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<tr>
<td>3R del 6bp</td>
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<tr>
<td>3R ins 6bp</td>
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</table>

* Reference haplotype.
allele should be higher than in those with loss of the larger one in heterozygous patients. Kawakami et al. (35) demonstrated that TS protein expression in tumor was dependent on allelic status. 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 that with the 3R/loss genotype. A recent study by Uchida et al. (36), comparing normal and tumor tissue, revealed that the response rates of 2R/loss carriers to fluoropyrimidines is significantly superior when compared with individuals whose tumor harbors a 3R/loss genotype. The analysis of LOH should be relevant when studying the TS polymorphism. In our study, tumor genotyping takes into account the fact that LOH is observed in a subgroup of the specimen. The tumor genotyping was not relevant for response prediction to 5-FU-based chemotherapy in our series of colorectal cancers; neither was TYMS polymorphism genotyping from normal tissue relevant for response and survival prediction to 5-FU-based chemotherapy (data not shown). Three studies analyzed correlation between tumor response to 5-FU-based chemotherapy and TYMS genotype distribution (31, 32, 37). In those studies, the difference in TYMS promoter genotype between tumor and normal tissue due to LOH was not considered. Two of them suggested that TYMS genotypes might be related to 5-FU-based chemotherapy responsiveness with a higher response rate in patients with 2R/2R genotype. The remaining one of those three studies showed that the TYMS promoter genotype was not associated with responsiveness to 5-FU-based combination with irinotecan or oxaliplatin in metastatic colorectal cancer. Moreover, in that study, the 6-bp deletion in the 3'-UTR of TYMS gene was associated with less favorable outcome, which is similar to our results. This is in contrast to the gene expression data presented by Lenz et al. (14) because most studies demonstrated an association between low TS levels and favorable clinical outcome (4, 38–41).

Additional studies are needed to identify the regulatory factors by which this polymorphism alters TS expression, which could help to interpret these preliminary conflicting data. For example, the most recent data by Mandola et al. (12) suggest that the impact of a 3R genotype on TS transcriptional activation may ultimately be related to the presence or the absence of the USF binding sites and the G>C single nucleotide polymorphism in the second repeat of 3R alleles. The 3RC allele showed transcriptional activity that was similar to that of the 2R allele. Our analyses on this polymorphism did not reveal any relation to toxicity. Indeed to the contrary of expected results, there is no evidence for a more severe toxic effect of 5-FU for patients with 3RC/3RC or 2R/3RC as compared with 2R/3RG, 3RG/3RG. We do not include this polymorphism in our haplotype analysis for two reasons: first, because the distribution of the resulting combined tri-allelic genotypes did not follow the Hardy-Weinberg equilibrium, the inferred haplotypes could not be consistently deduced; second, because the number of possible haplotypes increases from four to six, the power of our study would be insufficient to observe a significant haplotypic effect.

Moreover, our results are in accord with those reporting that TS activity measured in metastasis, and not TYMS promoter polymorphism, was the only significant predictor of treatment efficacy (16). Previous studies indicated that metastases from colorectal tumor do not necessarily contain similar levels of TS enzyme compared with TS levels in the primary tumors (6, 42–44). We observed a trend in favor of a better overall survival for patients with 2R/2R genotype, but the difference did not reach the threshold of significance. This could be due to the small size of our series. However, our present results suggest that the TYMS promoter genotype cannot serve as a substitute for TS activity to predict 5-FU-based chemotherapy responsiveness. Considering the tumoral evolution from initial tumor to distant metastasis, it is not surprising that measurement at the target level is the most relevant approach. Protein or RNA studies for metastatic disease also require a sample of target tissue, because of the inability of the primary tumor to predict expression in the secondary tumor (45, 46). In contrast, germinal DNA analyses probably provide better prediction of function than does tumor DNA analysis and appear to be a promising approach for predicting toxicity. Another explanation is that different 5-FU-based chemotherapy regimens received by patients may explain the lack of association between TYMS genotype and the response to chemotherapy.

Most studies have focused on the single TYMS promoter polymorphism. The TYMS 3'-UTR polymorphism affects TYMS mRNA expression, as does the TYMS promoter polymorphism. These polymorphisms were tested individually for association with the toxicity and efficacy of 5-FU treatment in patients with colorectal cancer, and only the TYMS promoter polymorphism was shown to be associated with a high risk of severe side effects to 5-FU. Using the genotypic information at each site is not fully efficient because it does not use the whole genotypic information available within a gene. Because haplotypes may have a particular significance with regard to functionality or as markers for unknown functional variants, it appears more and more evident that, to better characterize the role of a candidate gene, the full haplotypic information should be exploited (47, 48). We here extend this work by performing a haplotype analysis, which enables us to assess the consequences on the phenotype of the copresence of several variants on the same gene. For this purpose, a new maximum likelihood method reported by Tregouet et al. (20) was used for estimating simultaneously haplotype frequencies and haplotype–phenotype effects. Detailed haplotype analysis additionally revealed a significant haplotypic effect. The haplotype 2R/ins 6bp was significantly associated with a high risk of severe side effects to 5-FU-based chemotherapy. This finding illustrates the complexity of the pharmacogenetic analysis of drug effects and the necessity to explore in detail the polymorphisms of drug target genes.

In conclusion, both single locus and haplotype analyses suggest that the TYMS promoter genotype seems to be a significant predictor factor of 5-FU-related toxicity. Patients with the 2R/2R genotype were 20 times more likely to have grade 3–4 toxicity compared with those with 3R/3R. These findings could lead to individualization of treatment for patients with colorectal cancer requiring 5-FU-based chemotherapy as well as identification of patients who require monitoring or adjustment of the 5-FU dose.
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