Genomic Copy Number Changes Affecting the Thymidylate Synthase (TYMS) Gene in Cancer: A Model for Patient Classification to Aid Fluoropyrimidine Therapy

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Abstract
Thymidylate synthase (TS) is an important target for 5-fluorouracil (5FU)–based therapy. The TS polymorphic 5′-untranslated region tandem repeat sequence is under investigation to guide 5FU treatment, yet current protocols omit consideration of copy number changes at the TS locus. We surveyed the TS tandem repeat sequence and found copy number changes in gastrointestinal cancers. Ten of 12 informative cases had loss of heterozygosity (LOH), whereas two others and an additional cell line had a novel TS genotype, allelic imbalance at the TS locus due to polysomy. Experimentally, we studied a diploid colorectal cancer line heterozygous at TS to mimic three common TS genotypes of cancers. Using genetic engineering, we deleted the short tandem repeat allele (two repeats) and then produced a TS+/- line that had a reduced TS protein expression and was hypersensitive to 5FU and 5-fluoro-2′-deoxuryridine in vitro as compared with syngeneic control lines. We linked this sensitivity directly to the reduced TS expression by introducing exogenous TS mRNA into the TS+/- line (i.e., increased TS copies). Our model predicts that the 5FU sensitivity of a tumor is modified by aneuploidy producing copy number changes of TS alleles by one or more of the following: LOH, amplification, and, as presented here, copy number changes due to polysomy. The data suggest that TS copy number in a patient’s tumor may be a dominating variable affecting 5FU responsiveness. (Cancer Res 2006; 66(19): 9369-73)

Introduction
A primary mode of action of 5-fluorouracil (5FU) is to inhibit thymidylate synthase (TS) through the formation of a covalent ternary complex with tetrahydrofolate and 5-flouro-2′-dUMP (FdUMP). Considerable published evidence suggests that interindividual variation in cellular susceptibility to TS inhibition results from variation in the TS expression level of a patient’s tumor (1). In general, the low TS expression status of a tumor is thought to predict 5FU responsiveness, whereas high TS expression provides resistance (1).

Two high-frequency alleles of TS, differing in the length of a 28-bp tandem repeat sequence in the 5′-untranslated region (1, 2), were linked to TS expression and TS enzymatic activity in tumors, however, these associations were correlative rather than experimental (3–6). In the non-Japanese population, three main genotypes exist: (a) homozygous for two tandem repeats (2R/2R), (b) homozygous for three tandem repeats (3R/3R), and (c) heterozygous (2R/3R). A number of clinical studies found an increase in TS mRNA and protein in patients with three repeats as compared with two. Thus, the TS-tandem repeat (TS-TR) was proposed to predict 5FU responsiveness. Mechanistically, Kawakami et al. (7, 8) found the TS-TR to regulate the efficiency of translation rather than gene transcription. In contrast, Pullarkat et al. (9) and Uchida et al. (10) reported the 3R allele to associate with higher TS mRNA levels in the tumors.

Most clinical studies have analyzed TS genotype in the germ line (constitutional DNA) even though shortcomings exist (8, 10, 11). First, germ line analysis does not often reflect the tumor genotype, especially in chromosomally unstable tumors. Second, prior studies did not classify tumors with microsatellite instability (MSI), in which neither loss of heterozygosity (LOH) nor polysomy at TS are likely to occur. Third, the possibility of TS gene amplification (12), which may occur only after 5FU treatment, has been statistically associated with recurrence and is not considered. Finally, although some reports noted LOH at TS, they did not consider the possibility that copy number increases in individual alleles can coexist with the deletion affecting the other allele (13).

We assayed for copy number ratios of 3R and 2R repeats in a panel of pancreatic cancers matched with normal tissue and in gastrointestinal cancer cell lines using TS allele–specific capillary electrophoresis. To determine the effects of copy number changes on 5FU sensitivity, we constructed syngeneic cell lines mimicking three diverse TS genotypes: (a) retention of one allele (i.e., one copy), (b) two retained alleles (diploid scenario), and (c) TS overexpression (i.e., potentially modeling an increased TS copy number).

Materials and Methods
Capillary electrophoresis analysis of TS-TR (TS-TR assay). Genomic DNA was previously isolated from xenografted pancreatic tumor tissue, paired normal tissue, or cancer cell lines (from American Type Culture Collection, Manassas, VA, or generously donated by Dr. B. Vogelstein, Johns Hopkins University). Amplification of TS-TR was done in 20 μl using 1 μl of DMSO and 1.5 units of Taq Gold polymerase (Applied Biosystems, Foster City, CA). Primers were 5′-FAM-GTGCGGCTCCGCTTCC3′ (TS-F) and 5′-GAGCCGCCACAGGCATG-3′ (TS-R2). One microliter of amplification product was added to a mixture of dithromamide and ROX GS500 size standard (Perkin-Elmer, Wellesley, MA), denatured at 95°C for 2 minutes, and visualized on an ABI 3100 Genetic Analyzer (Applied Biosystems). To quantify the extent of allelic imbalance, mixtures of known 3R/3R and 2R/C0 lines were analyzed using the BioScan (GenomeLab System, Agilent Technologies). The ratio of 3R/C0 was calculated as the ratio of the area under each copy number's peak and visualized on an ABI 3100 Genetic Analyzer (Applied Biosystems).
2R DNA were tested to optimize the number of amplification cycles. The average 3R/2R ratio of 15 germ line heterozygotes (2R/3R) was 1.13 (range, 0.78-1.45). All samples with allelic imbalance were analyzed by at least two independent reactions.

**Targeted disruption of one TS allele in RKO cells.** One TS allele was targeted following the method of Kohli et al. (14). In brief, the targeting construct deleted exon 2 of the TS gene (primer sequences are available upon request). Hygromycin-resistant clones were screened by PCR for homologous integration events. After confirmation of a targeted allele, the hygromycin resistance gene was removed from a RKO cell line retaining only one functional TS allele (RKO.TS+/− cells) via Cre-mediated excision.

**Retention of 3R allele in RKO.TS+/− cells.** Genomic DNA was isolated from RKO.TS+/− cells using the QIAampDNA Mini Kit (Qiagen, Valencia, CA). PCR amplification generated products of 2,231 bp (3R) and 2,203 bp (2R) using the primers, TS1 forward, 5’-AGGTCCCGGGTTTCCCTAAAG-3’; TS2 reverse, 5’-TCCAAACACCCCTCCAGAAAC-3’. Primers were a 376-bp product for 3R, a 348 bp product for 2R, and 376 and 348 bp products for heterozygous 3R and 2R, respectively. The RKO.TS+/− genotype was confirmed by sequencing with the primers TS2 forward, 5’-AGGTCCCCGGGTTCCTTAAG-3’; and TS2 reverse, 5’-TGCCTGGCTGTTCCCTTT-C’.

**TS overexpression in RKO.TS+/− cells.** Full-length TS cDNA (generously donated by Dr. Maria Zajac-Kaye, Molecular Therapeutic Program, NCI, NIH, Bethesda, MD) was excised and subcloned into pcDNA 3.1/neo+ (pcDNA.TS; Invitrogen, Carlsbad, CA). We transfected RKO.TS+/− cells with pcDNA.TS using LipofectAMINE (Invitrogen). Stable transfectants were selected in zeocin-supplemented medium (1 mg/mL). Multiple clones (termed RKO.TS.high clones) were selected to account for clonal variability. RKO.TS.high.1 and RKO.TS.high.2 are two individual clones.

**Real-time PCR analysis of TS mRNA, reverse transcription-PCR assay of allele-specific expression, and TS protein expression.** We extracted RNA using the RNeasy Mini Kit (Qiagen). RNA (1 μg) was converted to cDNA for real-time PCR using SuperScript II Reverse Transcriptase (Invitrogen). Real-time PCR was done on the iCycler (Bio-Rad, Hercules, CA) with TS- and glyceraldehyde-3-phosphate dehydrogenase–specific primer sets (available upon request). Standard curves, melting curves, and PCR efficiencies were obtained for each primer set. Reverse transcription-PCR to analyze TS allele–specific expression was done as described (8). For each clone or cell line, equal cell numbers were lysed. Five to 20 μg of protein/well were separated by protein electrophoresis as previously described (Brody et al., in press, Cancer Biology and Therapy).

At least three experiments were done for each data point. Representative data are shown.

**Cell survival assays.** We plated 1,500 to 2,500 cells per well in a 96-well plate. After 1 to 2 days, cells were exposed to either SFU, 5-fluoro-2′-deoxyuridine (FUDR), or 5-fluorouridine (FUR; Sigma, St. Louis, MO). Six days later, cells were lysed, the DNA labeled with 0.5% PicoGreen (Molecular Probes, Eugene, OR), and fluorescence measured to assess changes in cell number.

**Results**

Survey of TS-TR in pancreatic xenografted tumors and matched normal tissue. We analyzed 19 matched pairs of normal and tumor specimens for TS-TR. Seven were homoyogous in both the germ line and tumor specimens (two cases were homoyogous 2R, and five were homoyogous 3R). Twelve were heterozygous (2R/3R) in the germ line/normal specimen (Table 1). Of these 12 cases, 10 had LOH in the corresponding xenografted tumor specimen, as indicated by a loss of a TS allele. Of the 10 cases with LOH, 4 retained the 2R allele (group I, 33%) and 6 retained the 3R allele (group II, 50%) in the tumor. In 2 of the 12 cases that were heterozygous in the germ line, the corresponding tumor contained both 3R and 2R alleles, but at an unequal ratio (group III, 17%; Table 1). In both cases, there were more copies of the 3R allele than 2R (Fig. 1A). Thus, for all 12 of the 2R/3R cases in the germ line, none retained the same genotype in the corresponding tumor (Table 1). We then analyzed an additional 32 xenografted cancers for which a corresponding normal specimen was not available. Of these tumor specimens, 9 (9 of 32, 28%) had only the 2R allele, 13 (13 of 32, 41%) had only the 3R allele, and 8 (8 of 32, 25%) had both.

**Table 1. TS-TR genotype of 12 informative cases of matched normal and tumor specimens (germ line 2R/3R) and gastrointestinal cell lines**

<table>
<thead>
<tr>
<th>Case group no./cell line</th>
<th>Cancer TS-TR genotype</th>
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<tr>
<td>Group I</td>
<td>2R</td>
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<tr>
<td>Group II</td>
<td>3R</td>
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<tr>
<td>Group III</td>
<td>2R/3R with allelic imbalance</td>
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<tr>
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<td>2R/3R</td>
</tr>
<tr>
<td>Hs766T</td>
<td>3R</td>
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</tr>
<tr>
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<td>3R</td>
</tr>
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Figure 1. A, results of capillary electrophoresis from matched normal and tumor specimens demonstrating allelic imbalance in the tumor; x axis is length in bases; y axis is fluorescence intensity. Peaks at 201 and 229 bases are amplification products from 2R and 3R alleles, respectively. B, linearity of the TS-TR assay. Known homoyogous 2R and 3R specimens were mixed at various ratios representing the “theoretical 3R/2R ratio,” and then analyzed by the TS-TR assay. The “actual 3R/2R ratio” represents the peak height of the 3R allele divided by the peak height of the 2R allele. Specimens demonstrating allelic imbalance were compared with the standard curve generated with the known mixtures. The calculated 3R/2R ratios of samples demonstrating allelic imbalance were 3.0, 2.8, 2.7, 2.2, and 2.1.
the 2R and 3R alleles at approximately equal molar ratios. Two (2 of 32, 6%) of the tumor specimens had both the 2R and 3R alleles but with allelic imbalance due to having more copies of the 3R allele than 2R (polysomy). We also analyzed 13 pancreatic and colorectal cancer cell lines for TS-TR (Table 1). Five (5 of 13, 38%) of the cell lines had the 3R allele only, five (5 of 13, 38%) had the 2R allele only, and 2 cell lines (2 of 13, 15%) had both 2R and 3R alleles at approximately equal molar ratios. One cell line (Su86.86; 1 of 13, 8%) had both the 2R and 3R alleles with allelic imbalance indicative of more copies of the 3R allele than 2R (Table 1). Thus, we identified five cases of allelic imbalance at TS (four xenografts, one cell line; Table 1; Fig. 1A), all having an increased number of 3R alleles relative to 2R alleles. To quantify the allelic imbalance, we prepared mixtures of known germ line 2R/2R DNA with 3R/3R DNA at various ratios. These mixtures were analyzed by the TS-TR assay. The results were used to generate a standard curve (Fig. 1B). Specimens having allelic imbalance were compared with the standard curve to determine the ratio of 3R to 2R alleles (Fig. 1B).

Characterization of RKO.TS+/- cells. RKO cells are heterozygous for TS-TR (2R/3R; Table 1). We disrupted one TS allele (14). To determine which allele we disrupted (i.e., 2R or 3R), we amplified genomic DNA from parental RKO cells and RKO.TS+/- cells and then digested with PstI (Fig. 1B). Parental cells were confirmed to be heterozygous for 3R and 2R (Table 1; Fig. 2A). RKO.TS+/- cells retained the 3R allele, indicating the disruption of the 2R allele (Fig. 2A). Sequencing confirmed the retention of the 3R allele, which contained the 3G single nucleotide polymorphism (data not shown; ref. 15).

Analysis of TS expression in genetically engineered cells. We analyzed TS protein levels by immunoblot. RKO.TS+/- cells (TS+/- add back) expressed high amounts of TS as compared with parental RKO cells (parental). Equal amounts of protein were loaded as indicated by the nonspecific bands. All experiments were done at least thrice.

Figure 2. TS genetic and expression analysis of cell culture models. A, PCR amplification of TS-TR region from genomic DNA followed by PstI restriction digestion shows parental RKO cells to be heterozygous 2R/3R and RKO.TS+/- cells have lost a functional 2R allele (functioning as 3R/loss). Sequencing confirmed these data and showed the existence of the 3G single nucleotide polymorphism (data not shown). B, Western blot of TS protein expression. RKO.TS+/- cells (TS+/-) expressed low amounts of TS protein, whereas RKO.TS.high clones (TS+/- add back) expressed high amounts of TS as compared with parental RKO cells (parental). Equal amounts of protein were loaded as indicated by the nonspecific bands. All experiments were done at least thrice.

Figure 3. Fluoropyrimidine sensitivity of RKO.TS+/- as compared with RKO parental cells and RKO.TS.high clones. RKO.TS.high clones are RKO.TS+/- with restored TS expression. Therefore, observed differences between RKO.TS+/- and RKO.TS.high clones are likely due to TS expression differences. A, FU sensitivity; B, FUDR sensitivity; C, FUR sensitivity. At least five experiments were done for each compound. Points, mean; bars, SE (n = 3 for each data point per experiment; ▲, RKO.TS.high.1 cells; ■, RKO.TS.high.2 cells; ●, parental RKO cells; ○, RKO.TS+/- cells).
Protein lysates used in these experiments were extracted at multiple times and confluences of the cell culture to exclude a cell cycle–dependent expression effect or an autoregulatory compensation that might occur over time. Real-time PCR found that RKO.TS+/− cells expressed less TS mRNA than the parental cell line. Reverse transcription-PCR showed no significant differences in allele-specific expression of the 2R and 3R alleles in the parental line (data not shown; ref. 8).

Fluoropyrimidine sensitivity of RKO cell lines. RKO.TS+/− cells were hypersensitive to 5FU and FUDR (Fig. 3 and B), two available chemotherapeutics that are quickly metabolized to FdUMP, and thus, target TS. In order to ensure that any differences in 5FU sensitivity observed were due to TS expression, we overexpressed exogenous TS in the RKO.TS+/− cells to generate RKO.TS.high cells and confirmed overexpression of TS protein in these cells (Fig. 2B). RKO.TS.high clones were nearly 6-fold more resistant than RKO.TS+/− cells; parental RKO cells had intermediate resistance. 5FU and FUDR resistance in the RKO.TS.high cells confirmed that the drug sensitivity of RKO.TS+/− cells was attributable to their TS expression (Figs. 2B and 3A and B). We also tested a related compound, FUR, a fluoropyrimidine favoring RNA-directed toxicity. Cell survival following FUR treatment was equivalent for each of the clones, indicating that the different sensitivities in these cell lines was specific for 5FU and FUDR (Fig. 3C).

Discussion

Clinical studies attempting to predict 5FU-responsiveness, based on TS tandem repeat status of the germ line, should be attentive to the dramatic TS genotypic differences between normal tissue and tumor (11). In our series, all 12 informative cases had allelic imbalance at TS, including 2 cases which had copy number increases. Future studies should reveal the frequency of copy number changes (i.e., increases and decreases) in patients homozygous (2R/2R or 3R/3R) in the germ line. The copy number plasticity of the TS gene in a chromosomally unstable tumor may explain some observations in the 5FU literature. First, the relapse of patients for whom 5FU therapy had produced a clinical response or remission could be explained by copy number increases due to allelic imbalance as described with TS amplification (12). Second, TS copy number changes due to allelic imbalances reported here may help explain TS expression differences previously thought to result from TS-TR status. Finally, TS copy number may explain reports wherein MSI tumors do not respond to 5FU-based therapy as do tumors with chromosomal instability (16). TS copy number changes in MSI tumors should be rare, whereas the majority of chromosomally unstable tumors are likely to have one allele of TS due to LOH (5, 16).

To study TS copy number change in an experimental cell culture model, we constructed a trio of syngeneic cell lines that mimic three distinct TS genotypes of patients’ tumors. As expected, TS-overexpressing clones (a model for tumors with increased TS copy number) were 5FU- and FUDR-resistant as compared with the parental line. Interestingly, the RKO.TS+/− cell line (a model for a single-copy TS due to LOH) was hypersensitive to 5FU and FUDR as compared with the parental RKO line. Etienne et al. found that TS activity was significantly higher in 2R/3R tumors as compared with 2R/2R or 3R/3R in 103 metastatic colorectal cancer patients receiving 5FU. It is possible that the lower TS activity in these groups was due to the high frequency of LOH in the homozygous tumors (17). Uchida et al. reported that colon cancer patients with LOH (either 2R/loss or 3R/loss) had improved survival compared with all other possible genotypes (10). This again is consistent with our model in which LOH would lead to significantly reduced TS expression and increased 5FU sensitivity (Figs. 2 and 3), at least in those cases wherein the remaining allele is not duplicated.

The RKO.TS+/− clone had reduced TS mRNA and protein expression as compared with the parental line (Fig. 2B; data not shown). This was despite the line having retained a functional long (3G) allele, proposed to express the highest amount of TS of all allelic variants (15). TS protein levels were reported to be regulated by a negative feedback loop involving the mRNA (18). Kitchens et al. refuted this model by demonstrating that FdUMP binding to TS did not affect the protein binding to the mRNA and thus its translation, and found also that the mRNA binding site did not prevent the up-regulation in response to drug (19). However, the ability of TS protein to regulate its mRNA in the absence of drug was not addressed in such studies. In our experimental cell model (RKO.TS+/−), TS was incapable of up-regulating its expression to compensate for loss of gene dosage. It is possible that the mRNA expression could be affected by an unexpected alteration, for example, the expression of individual alleles is highly plastic (20). Either possibility could confound attempts to develop reliable clinical tests predictive of 5FU responsiveness. Additional studies of TS transcriptional stability and plasticity should be pursued.

In summary, TS copy number in gastrointestinal cancers does not usually agree with the germ line genotype. The determination of the former may be of some value in predicting the clinical success of 5FU-based therapy, but this may prove to be methodologically difficult because copy numbers need not be uniform among the cells of a metastatic cancer. Future clinical studies should take into account the potential effects on treatment response that may be attributable to variation in TS gene copy number between the patients and among the tumor cells of individual patients.

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