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ORIGINAL ARTICLE

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# Polymorphic tandem repeat sequences of the thymidylate synthase gene correlates with cellular-based sensitivity to fluoropyrimidine antitumor agents

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**Abstract** *Purpose*: Thymidylate synthase (TS) is one of the target molecules for the antitumor effects of fluoropyrimidine drugs. The cellular thymidylate synthase level is one of the determining factors for the antitumor activity of fluoropyrimidines. *TYMS*, which encodes TS, has been reported to possess 28-bp tandem repeat sequences in its 5′-untranslated region, the number of which varies. In addition, single nucleotide polymorphisms have also been shown in a triple repeat sequence. In this study, correlation between the polymorphic tandem repeat sequences of the *TYMS* gene and the antitumor activities of 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine (FUdR) were investigated with 30 established human cell lines derived from solid tumors. *Methods*: A reporter assay system was developed in order to compare the ability of the transactivation mediated by the double (2R) and triple (c- or g-type, 3Rc or 3Rg, respectively) repeat sequences using a human colon cancer cell line, DLD-1. The 50% inhibitory concentration ($IC_{50}$) of cell growth by 5-FU and FUdR was measured with 30 different established cell lines of human solid tumors. Genotypes based on the number of the 28-bp *TYMS* tandem repeat for the above cell lines were determined by electrophoretical analysis of PCR products containing the repeat sequences and nucleotide sequencing. *Results*: The reporter activity mediated by the 3Rg sequence was significantly higher than that by the 2R and 3Rc sequences. Activities mediated by the 2R and 3Rc sequences were comparable. According to the reporter assay, 2R and 3Rc were judged as low TS expression alleles and 3Rg as a high TS expression allele. On the basis of $IC_{50}$ values, cells possessing the 2R/2R and 2R/3R repeat of *TYMS* were significantly more sensitive to FUdR than those with the 3R/3R repeat. Cells possessing 3Rg/3Rg (a high TS expression genotype) were significantly less sensitive to FUdR than cells with 2R/2R, 2R/3Rc, and 3Rc/3Rc (low TS expression genotypes). *Conclusions*: Our results of the reporter assays using 2R, 3Rc, and 3Rg repeat sequences prompted us to classify 3Rg as a high TS expression allele, and 2R and 3Rc as low TS expression alleles. The cells with low TS expression alleles were shown to exhibit significantly higher FUdR sensitivity than the cells with high TS expression alleles for the first time. These results were consistent with numerous previous in vitro and in vivo findings that tumors showing high TS expression were less sensitive to fluoropyrimidines. These results support

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the idea that genotyping the tandem repeat sequences of *TYMS* in the 5′-untranslated region is useful for individualized therapy involving fluoropyrimidine antitumor drugs.

**Keywords** 5-Fluorouracil · Thymidylate synthase gene · Polymorphic tandem repeat sequences · Cellular-based sensitivity · 5′-Untranslated region

## Introduction

5-Fluorouracil (5-FU) is a widely used anticancer drug that is prescribed for the treatment of solid tumors, including gastric, colorectal, breast, and ovarian cancers. One important mode of action of 5-FU is to inhibit thymidylate synthase (TS), which catalyzes the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate using 5,10-methylenetetrahydrofolate as a methyl donor as a rate-limiting step in cancerous tissues [5, 18]. The TS levels in cultured cell lines or in cancerous tissues are correlated with antitumor effects of 5-FU, and cells or tumor tissues expressing high levels of TS are less sensitive to 5-FU as compared to those expressing low levels of TS [2, 7, 28, 29]. Elements supporting basal expression of TS were identified about a decade ago by Kaneda et al. [13, 14]. The *TYMS* exonic and intronic sequences were identified, and it was reported that the *TYMS* gene is TATA box-, CAAT box-, and GC box-less in its 5′-flanking region. Nevertheless, there are three sequences that fit the consensus sequences of the GC box (GGGCGG), all of which seemed to be functional [14].

The *TYMS* gene, encoding TS, contains a variable number of tandem repeat sequences (28 base pairs) in its 5′-untranslated region [9]. Two, three, four, five, and nine repeats are found in Caucasians, Asians, and African populations [9, 15, 19, 21–23]. For example, double (2R) and triple (3R) repeats are mainly found with allele frequencies of 0.18 and 0.80, respectively, in the Japanese [9]. Remarkable ethnic differences in the 3R allele frequency were reported, for example, the 3R allele frequency in American Caucasians was 0.55 [23]. Pullarkat et al. [27] has reported that TS mRNA levels were the highest in tumor and normal tissues with a 3R/3R genotype, second highest in those with a 2R/3R genotype, and the lowest in those with a 2R/2R genotype [27]. It has also been reported that TS protein measurements of surgically resected colorectal cancer tissues with 2R or 3R of *TYMS* revealed that TS protein levels in the tumor tissues were the highest with those with 3R/3R, followed by 2R/3R, and the least in those with a 2R/2R genotype with regard to colon cancer [16, 23] and gastric cancer [14]. Recently, a novel single nucleotide polymorphism (SNP, $g > c$) was found in the repeat sequence [17, 20]. The polymorphic site is located within the USF consensus element in the second repeat of the 3R allele. The substitution of the wild-type g allele (3Rg) with a variant c allele (3Rc) alters USF protein binding ability and results in the remarkable reduction of the transcriptional activation of *TYMS* [20]. These observations suggest defining 2R/2R, 2R/3Rc, and 3Rc/3Rc as low TS-expressing genotypes, 2R/3Rg and 3Rc/3Rg as intermediate TS-expressing genotypes, and 3Rg/3Rg as a high TS-expressing genotype. This data prompted us to examine whether growth inhibition of established cell lines by 5-FU correlated with their *TYMS* tandem repeat genotypes.

In this study, we first evaluated the function of the various *TYMS* tandem repeat sequences by the firefly luciferase reporter assay using a colon cancer cell line, DLD-1. Then, *TYMS* repeat sequences were genotyped from 30 cultured cell lines established from solid tumor tissues and the 50% inhibition of their growth was measured after a 144 h exposure to 5-FU or 5-fluoro 2′-deoxyuridine (FUdR), which is likely to be a precursor of 5-fluoro 2′-deoxyuridine monophosphate (FdUMP), a TS inhibitor.

## Materials and methods

### Cells and culture conditions

Established cell lines from various solid tumor tissues were obtained from the Health Science Research Resource Bank (Osaka, Japan), the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), or the Japanese Collection of Research Bioresources, National Institute of Health Sciences (Tokyo, Japan). Cells were grown at 37°C under a humidified atmosphere of 5% $CO_2$–95% air in a recommended culture medium (either RPMI1640, Minimum Essential Medium or Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum and 100 U/mL penicillin-100 μg/mL streptomycin.

### Cloning of DNA fragments containing double or triple tandem repeats and construction of reporter plasmids

Genomic DNA of the cells was extracted from the sediment, which was obtained after the cells were lysed by sonication and subjected to centrifugation at 10,000 *g* for 15 min. DNA fragments containing the tandem repeats of the *TYMS* 5′-untranslated region were amplified by PCR using forward (TS33) and reverse (TS28) primers and cellular genomic DNA as a template with the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 15 s, with a final extension at 72°C for 7 min. Nucleotide sequences of the TS33 and the TS28 primers were 5′-GCCTCGAGCGGGACGGCCGCGGGAAAA-3′ and 5′-TCCGAGCCGGCCACAGCCAT-3′, respectively [16]. The resultant PCR products were subcloned into the pCRII vector (Invitrogen, Carlsbad, CA, USA). Lengths of amplified 2R and 3R (3Rg and 3Rc) PCR

products were 139 bp and 167 bp, respectively. The pCRII vector was digested with *Nco*I and *Xho*I and inserted into a firefly luciferase reporter vector (pGL3-Basic vector, Promega, Madison, WI, USA) that was cut with the same restriction enzymes. Then, the plasmids were further digested with *Nhe*I and *Bam*HI, followed by insertion into a cytomegalovirus (CMV) promoter-containing mammalian expression vector, pCI (Promega), which had been digested with *Nhe*I and *Bam*HI. Thus, these constructs contained DNA fragments of 2R, 3Rg, and 3Rc repeat sequences between the CMV promoter and the firefly luciferase gene as schematically illustrated in Fig. 1a, b.

## Luciferase assay

Human colon cancer DLD-1 cells were plated in 24-well plates at a density of $3\times10^5$ cells/well and incubated for 20 h. Then, each plasmid (250 ng) and phRL-TK (200 ng, Promega), which was used for normalization of transfection efficiencies, were co-transfected into the DLD-1 cells by a lipofection method using the Plus reagent (Invitrogen) and Lipofectamine (Invitrogen). After the cells were incubated for 48 h at 37°C, luciferase activity was measured by the Dual-Glo Luciferase assay system (Promega) according to the manufacturer's instructions. Transfection efficiencies were standardized by *Renilla* luciferase activity attributed to the co-transfected phRL-TK vector.

## Genotyping of *TYMS* tandem repeats in cultured cells

Genotyping of the established cells was done by PCR amplification according to the same method as cloning the DNA fragments containing *TYMS* tandem repeat sequences. The resulting PCR products were separated by electrophoresis using a 3% NuSieve 3:1 gel (Biowhittaker, Rockland, ME, USA). Lengths of amplified 2R and 3R (3Rg and 3Rc) PCR products were 139 bp and 167 bp, respectively.

## Sequence analysis of *TYMS* tandem repeats.

First, the entire *TYMS* gene was amplified from 200 ng of genomic DNA using 1.25 units of *Z-Taq* (Takara Shuzo, Tokyo, Japan) with 0.2 μM primers (5′-CTGGCGGTTTTTAATCAAGTAGAAAAGCTG-3′ and 5′-CCCCGAAGTGGTTTTGCATCTTTTCCTTGC-3′). The first round PCR conditions for the amplification of the entire *TYMS* were 30 cycles of 98°C for 5 s, 60°C for 5 s, and 72°C for 190 s. Next, exon 1 was amplified by *LA Taq* (2.5 units) (Takara Shuzo) with 0.5 μM primers (5′-TGTGCTGCTGGCTTAGAGAA-3′ and 5′-AGGTTAGGACTAAACGGGGT-3′). The second round PCR for the amplification of exon was performed by using 1 μL of the first PCR products as the template with GC Buffer I (Takara Shuzo). The second round PCR conditions for the amplification of each exon were 94°C for 1 min followed by 35 cycles of

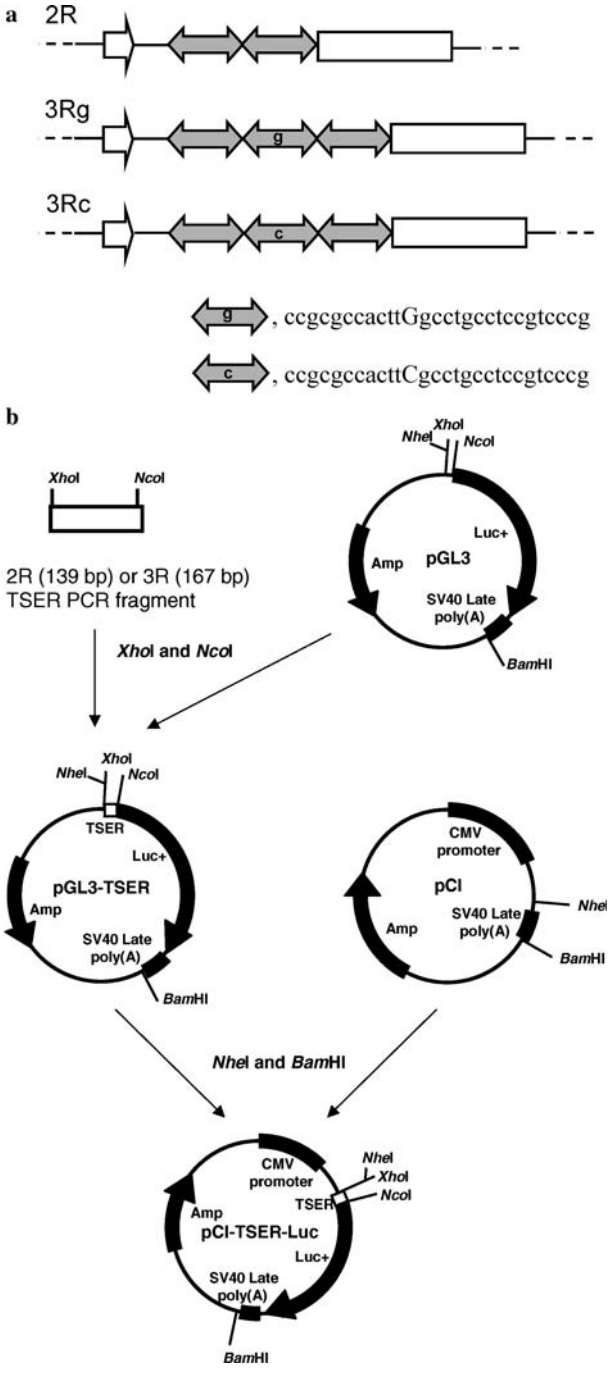


**Fig. 1** Structure of the *TYMS* 5′-untranslated region (5′-UTR) tandem repeats-luciferase fusion constructs. Constructs for the luciferase reporter assay were generated as described in the Materials and methods. Open boxes represent the firefly luciferase gene (**a**). A *gray arrow* with two *arrowheads* represents one 28-bp unit of the *TYMS* 5′-UTR tandem repeat (**a**). The *open arrows* indicate the location of the cytomegalovirus promoters, located upstream of the 28-bp tandem repeats (**a**). In **b** a diagram to illustrate the steps that led to the different repeats and genotypes was shown

94°C for 30 s, 60°C for 30 s, and 72°C for 2 min. Then, the PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and were directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). The tandem repeat polymorphisms were confirmed by repeated sequence analysis of PCR products generated by amplification of new genomic DNA.

### Cellular sensitivities toward 5-FU and FUdR

Cells were plated in 96-well plates at a density of $5\times10^3$ cells/well and incubated for approximately 5 h at 37°C. Either 5-FU or FUdR at various concentrations (0.01-500 μM (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, and 500 μM) or 0.001-50 μM (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 1, 3, 10 and 50 μM), respectively) was added (four wells for one drug concentration) and incubated for 6 days. This drug treatment regimen is based on a reported observation on basic study results that FUdR as well as 5-FU reported to require long exposure to cancer cells for cell-killing action due to its cell-cycle-phase specific effects [11]. Cells were washed with phosphate-buffered saline and stained with 0.4% crystal violet-methanol solution. $IC_{50}$ values (drug concentration inducing 50% cell growth inhibition), which were determined from percent cell growth of the untreated control, were determined from at least three independent experiments. Mean $IC_{50}$ values were used for subsequent analyses and those obtained in each experiment varied within 15%.

### Statistical analyses

Statistical significance of the differences in $IC_{50}$ values between cells with 2R/2R plus 2R/3R versus 3R/3R was analyzed by an unpaired t-test with Welch's correction. One-way ANOVA with Tukey's Multiple Comparison Test evaluated differences in $IC_{50}$ values and linear trends among cells with low, intermediate, and high TS expression genotypes for statistical significance. These statistical analyses were done using a SAS software package (SAS Institute Inc. Cary, NC, USA) and a Prism 3.0 (Graph Pad Software, Inc., San Diego, CA, USA).

### Measurements of cellular TS mRNA levels

Total RNAs from 22 cell lines whose sensitivities to 5-FU and FUdR were determined in terms of growth inhibition were extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). For real time PCR assay, cDNAs were prepared using Multiscribe Reverse Transcriptase from TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). TS mRNA expressions in the cultured cell lines were determined by Prism 7700 real time PCR system (Applied Biosystems) using TS mRNA sequence-specific probes and primers (exon 1/2, Hs00426586_m1 and exon 6/7, Hs00426591_m1) of TaqMan Gene Expression Assays (Applied Biosystems). TS mRNA levels were expressed after normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA determined using TaqMan Endogenous Controls (Applied Biosystems). Results using exon 1/2 and exon 6/7 of TS mRNA sequences were consistent.

## Results

### Analysis of function of 2R, 3Rc, and 3Rg by the luciferase reporter assay

Reporter constructs, which contain the 2R, 3Rc, or 3Rg sequence, were transfected into a colon cancer cell line, DLD-1, which was reported to express high-level TS. This high-level TS suggests that DLD-1 cells possess abundant regulatory factors interacting with the *TYMS* tandem repeat sequences and contain an upstream stimulatory factor, USF-1. As shown in Fig. 2, relative luciferase activity, mediated by the 3Rg repeat, showed the highest of the three different reporter constructs. The one base substitution ($g>c$) in the triple repeat revealed significantly low luciferase activity, which is similar to that mediated by the 2R sequence. The remarkable reduction in the relative luciferase activity by the $g>c$ substitution in the triple repeat was consistent with the recent report that the $g>c$ substitution disturbs the

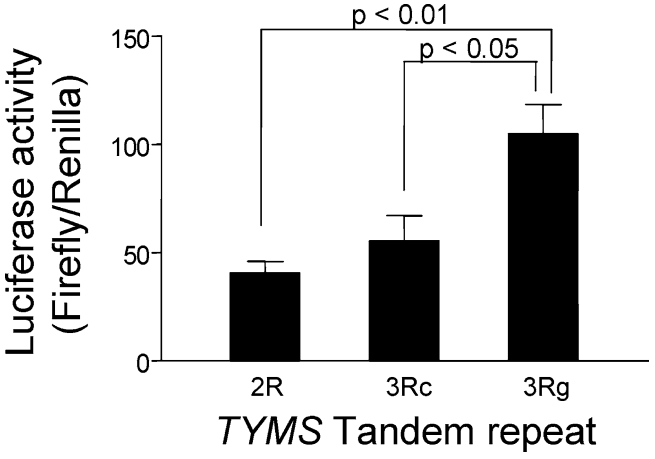


**Fig. 2** Reporter assay results with the firefly luciferase gene constructs containing *TYMS* tandem repeat sequences. The luciferase reporter assay was performed as described in the Materials and methods. The luciferase activity is expressed after normalization of firefly luciferase activity by *Renilla* luciferase activity in order to normalize differences in transfection efficiency

USF-1 site for USF-1 protein binding and thus, abolishes the ability to transactivate the luciferase reporter gene [20]. These results suggest that genotypes 2R/2R, 2R/3Rc, and 3Rc/3Rc may be classified as low TS expression genotypes, 2R/3Rg and 3Rc/3Rg as intermediate TS expression genotypes, and 3Rg/3Rg as high TS expression genotypes.

## *TYMS* genotypes and growth inhibition of cells exposed to 5-FU and FUdR

*TYMS* genotypes of the tandem repeat sequences were performed for the established cell lines whose sensitivities to 5-fluorouracil and FUdR were measured. *TYMS* genotypes observed in this study were assigned according to the number of cell lines and the tissues from which the cell lines were derived: one small cell lung carcinoma, one lung adenocarcinoma, and four colon adenocarcinoma cell lines had 2R/2R; one mucious cystadenoma and one colorectal adenocarcinoma cell line had 2R/3Rc; one squamous cell lung carcinoma and one buccal squamous carcinoma cell line had 2R/3Rg; one tongue squamous carcinoma, one thyroid carcinoma, two small cell lung cancer, one lung cancer, one breast cancer, one gastric cancer, one hepatoma, one ovarian cancer and one teratocarcinoma cell line had 3Rc/3Rc; one glioblastoma, one esophagus squamous carcinoma, one hepatocarcinoma and one colon adenoma cell line had 3Rc/3Rg; one tongue squamous carcinoma, one oral cancer, one giant cell lung cancer, one squamous cell lung carcinoma, one stomach choriocarcinoma and one testicular tumor cell line had 3Rg/3Rg. Subsequently, we examined the growth inhibition of the cells induced by 5-FU or FUdR after exposure for 6 days. Figure 3 shows the $IC_{50}$ values of the cell lines with the *TYMS* double repeat (2R) and triple repeat (3R) after 6 days of exposure to 5-FU and FUdR. The extent of growth inhibition greatly varied depending on the cell lines used, but drug concentrations inducing 50% growth inhibition by 5-FU and FUdR tended to be higher with the cells possessing a 3R/3R genotype than those with at least one 2R allele. The difference with FUdR reached statistical significance ($P = 0.019$, Fig. 3b), but that with 5-FU did not ($P = 0.441$, Fig. 3a). Decomposition of 5-FU by enzymes like dihydropyrimidine dehydrogenase may contribute in larger extent to drug sensitivity. Next, we attempted to analyze the cellular sensitivities to 5-FU and FUdR according to genotypes associated with TS expression: low TS expression genotypes, 2R/2R, 2R/3Rc, 3Rc/3Rc; intermediate TS expression genotypes, 2R/3Rg and 3Rc/3Rg; and high TS expression genotype, 3Rg/3Rg (Fig. 4). This classification revealed a statistically significant difference in $IC_{50}$ values for FUdR between the cell lines with the low and high TS expression genotypes ($P < 0.01$, Fig. 4b) by one-way ANOVA with Tukey's Multiple Comparison Test, although no statistically significant difference was observed with 5-FU as shown in Fig. 4a. A statistically significant trend ($P = 0.0034$) was also observed among $IC_{50}$ values for FUdR among the low, intermediate, and high TS expression genotypes (Fig. 4b).

## Cellular TS mRNA levels in relation to *TYMS* genotypes

Total RNAs from 22 cell lines whose sensitivities to 5-FU and FUdR were determined in terms of growth inhibition were extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Relative TS mRNAs were determined in the following 22 cell lines: one lung adenocarcinoma, and four colon adenocarcinoma cell lines with 2R/2R; one mucious cystadenoma and one colorectal adenocarcinoma cell line had 2R/3Rc; one buccal squamous carcinoma cell line with 2R/3Rg; one tongue squamous carcinoma, one small cell lung cancer, one breast cancer, one gastric cancer, one hepatoma, and one teratocarcinoma cell line with 3Rc/3Rc; one glioblastoma, one esophagus squamous carcinoma, and one colon adenoma cell line had 3Rc/3Rg; one tongue squamous carcinoma, one oral cancer, one giant cell lung cancer, one squamous cell lung carcinoma, and one testicular tumor cell line with 3Rg/3Rg. TS mRNA

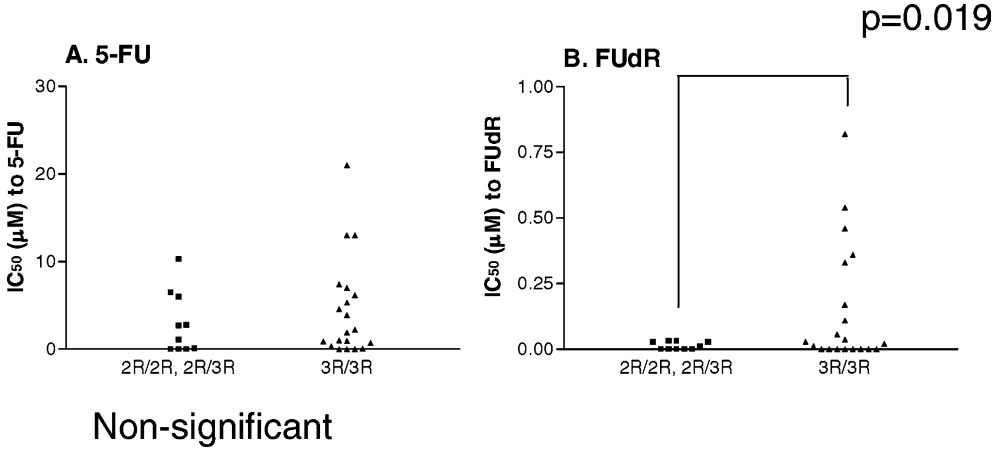


**Fig. 3** $IC_{50}$ values after treatment of 30 different solid tumor-derived cells with 5-FU (**a**) or FUdR (**b**) depending on double (2R) and triple (3R) repeats of the *TYMS* 5′-UTR sequence. Growth inhibition of the cells by 5-FU (**a**) or FUdR (**b**) was determined as described in the Materials and Methods. Comparison of $IC_{50}$ values was done between cells with the 2R/2R plus 2R/3R genotypes and those with a 3R/3R genotype

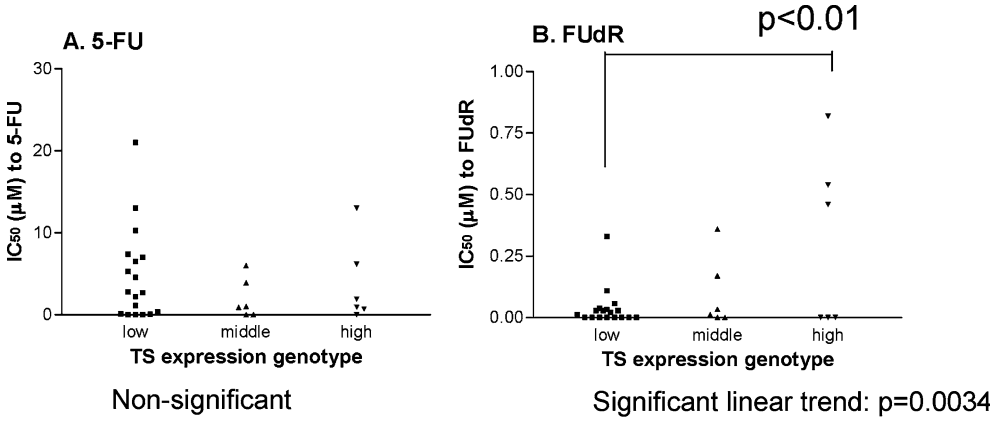


**Fig. 4** $IC_{50}$ values after treatment of 30 different solid tumor-derived cells with 5-FU (**a**) or FUdR (**b**) depending on low (2R/2R, 2R/3Rc, and 3Rc/3Rc), intermediate (2R/3Rg and 3Rc/3Rg) and high (3Rg/3Rg) TS-expression genotypes. Growth inhibition of cells by 5-FU (**a**) or FUdR (**b**) was determined as described in the Fig. 3 legend. One-way ANOVA with Tukey's Multiple Comparison Test analyzed differences of the mean of $IC_{50}$ values for the three-genotype groups and a linear trend among the three genotype categories was evaluated

expressions in the cultured cell lines were determined by Prism 7700 real time PCR system (Applied Biosystems) as described in Materials and Methods. Relative TS mRNA levels were expressed after normalized by GAPDH mRNA. As illustrated in Fig. 5, we failed in obtaining statistically significant difference in relative TS mRNA levels in terms of low (2R/2R, 2R/3Rc, and 3Rc/3Rc), middle (2R/3Rg and 3Rc/3Rg), and high (3Rg/3Rg) TS expression genotypes. No significant difference was observed in terms of 2R/2R + 2R/3R and 3R/3R genotypes, either.

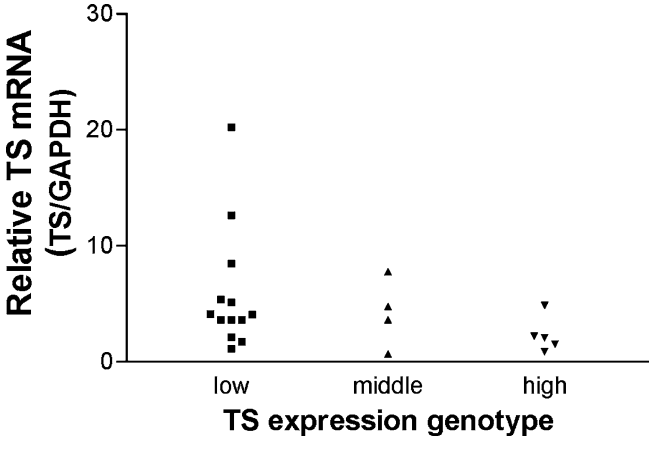


**Fig. 5** Relative TS mRNA levels in 22 cell lines in relation to high, middle, and low TS expression genotypes. Relative TS mRNA levels were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels as described in Materials and methods. No statistical significant trend was observed in the relative TS mRNA levels depending on the three kind of the TS expression genotypes

## Discussion

Thymidylate synthase is one of the determining factors in cancer cell sensitivity to fluoropyrimidine anti-cancer drugs. Several investigators have reported that high-level expression of TS in cancer cells was associated with 5-FU resistance [2, 7, 17]. Ma et al. [24] showed that the TS mRNA level sharply correlated with FUdR sensitivity of human colon cancer cell lines. Fujii et al. [5] reported that high 5-FU sensitivity was observed in surgically resected colorectal carcinoma cells showing low-TS-activity and with high-orotate phosphoribosyltransferase (OPRT)-activity. The low-TS-activity and the high-OPRT-activity cancers showed high cell proliferative activity. Similarly, among cell lines of the National Cancer Institute's Anticancer Drug Screen, cell lines with faster doubling times were significantly more sensitive to 5-FU and FUdR [6]. Conflicting results were, however, reported with clinical outcomes in the fluoropyrimidine-based drug therapy of high- and low-TS expressing cancers. In references [4] and [29], patients with low intratumoral TS expression had a significantly better outcome after 5-FU based chemotherapy than those with high TS expression [4, 29]. In contrast, references [3] and [30] reported that patients with high tumoral TS expression benefited from 5-FU based chemotherapy. Both of these reports commonly documented that higher relapse and death rates were observed with high TS expressors as compared with low TS expressors, which gave low TS expressors significantly better overall survival [3, 30]. These clinical study results may have some relationship with the results reported in references [5] and [6] that cancers expressing low-TS-activity and showing faster doubling times were more sensitive to 5-FU and FUdR [5, 6].

In this study, we first examined effects of *TYMS* tandem repeat sequences on gene expression by a reporter assay using a colon cancer cell line, DLD-1. Our functional characterization of *TYMS* tandem repeat sequences showed that the 3Rg sequence exhibited clearly the highest transactivation. The extent of the transactivation by 3Rc and 2R sequences was signifi-

cantly lower than that mediated by the 3Rg sequence. These results were consistent with the recent results using HeLa cells [17] and human embryonic kidney 293-S cells [20] as recipient cells of reporter constructs. Based on these results, one may define 2R/2R, 2R/3Rc, and 3Rc/3Rc as low TS-expression genotypes, 2R/3Rg and 3Rc/3Rg as intermediate TS-expression genotypes, and 3Rg/3Rg as a high TS-expression genotype. After classification of the 30 established cell lines by the genotypes of the different *TYMS* 5′-UTR tandem repeat sequences, we attempted to correlate the cellular sensitivity to 5-FU or FUdR with the *TYMS* genotypes. We found that the established cancer cells with at least one 2R allele were more sensitive to FUdR, which is the precursor of FdUMP, the ultimate TS inhibitor, as compared with cells with a 3R/3R genotype. According to the classification of these genotypes as low, intermediate, and high TS-expression genotypes, we found that cells with high TS-expression genotypes were the least sensitive to FUdR among the three categories of the TS-expression genotypes. Furthermore, a statistically significant linear trend in FUdR sensitivity was observed among the low, intermediate, and high TS-expression genotypes.

We measured relative TS mRNA levels in 22 cell lines with various *TYMS* genotypes of the 30 cell lines whose drug sensitivities and *TYMS* genotypes were investigated. As a result, no obvious correlation in the TS mRNA levels was observed in the cell lines in terms of low, middle and high TS expression genotypes (Fig. 5). We think that our results are interesting in conjunction with the report of Mandola et al. [20] that the USF-1 sites existing in the 2R and 3R in the *TYMS* gene promoter have been shown to be responsible for greater transcriptional activity from the 3R according to the number of USF-1 sites. Our results may suggest that number of the USF-1 elements is associated with the protein expression, or translation, from TS mRNA.

Clinical studies on the relationship between variable numbers of *TYMS* tandem repeat were documented. Except for a study of oral adjuvant chemotherapy in colorectal cancer [31], patients with a 3R/3R repeat genotype did not respond to the fluoropyrimidine-based chemotherapy, whereas patients with 2R/3R and 2R/2R genotypes responded [1, 10, 32]. Kawakami and Watanabe compared survival after fluoropyrimide chemotherapy, taking the single nucleotide polymorphism in the 3R repeat into consideration, and found that a low TS expression genotype group (2R/2R, 2R/3Rc, and 3Rc/3Rc) responded to the therapy regimen, but that the intermediate and high TS expression genotype group (2R/3Rg, 3Rc/3Rg and 3Rg/3Rg) did not respond to the therapy with statistical significance [17].

In the current study using established cell lines derived from solid tumors, the drug concentration inducing 50% cell growth inhibition was significantly different with FUdR, but not with 5-FU, between cells with 2R/2R + 2R/3R and 3R/3R, or cells with low and high TS expression genotypes. The mode of 5-FU action is complicated as 5-FU undergoes catabolism and anabolism by different metabolic pathways. A major part of the administered 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) [26]. Subsequently, a portion of 5-FU that has escaped degradation by DPD reportedly exhibits cell growth inhibition by its incorporation into RNA. As 5-FU is catabolized by DPD and 5-FU is incorporated into RNA by a pathway other than a pathway involving TS, a significant correlation between drug sensitivity and *TYMS* genotypes might be obtained for FUdR, a precursor of the ultimate TS inhibitor, but not 5-FU. Clinical studies on differences in survival between cases with high and low TS expression genotypes might suggest that 5-FU exerts antitumor effects mainly via a metabolic pathway of 5-FU involving TS inhibition.

Our in vitro analyses showed a statistically significant correlation between tandem repeat sequences of the *TYMS* gene and cell growth inhibition by FUdR. Thus, antitumor effects by fluoropyrimidine were related to *TYMS* genotypes both in the current in vitro study using 30 solid tumor-derived cell lines and in several clinical studies. The importance of DPD is documented by a number of research groups [6, 8, 25, 33]. Therefore, analyses of *TYMS* and *DPYD* (a gene encoding DPD) genotypes will be useful before the administration of fluoropyrimidine-based chemotherapy for individualized cancer chemotherapy.

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## References

1. Chen J, Hunter DJ, Stampfer MJ, Kyte C, Chan W, Wetmur JG, Mosig R, Selhub J, Ma J (2003) Polymorphism in the thymidylate synthase promoter enhancer region modifies the risk and survival of colorectal cancer. Cancer Epidemiol Biomarkers Prev 12:958–962
2. Chu E, Drake JC, Koeller DM, Zinn S, Jamis-Dow CA, Yeh GC, Allegra CJ (1991) Induction of thymidylate synthase associated with multidrug resistance in human breast and colon cancer cell lines. Mol Pharmacol 39:136–143
3. Edler D, Glimelius B, Hallstrom M, Jakobsen A, Johnston PG, Magnusson I, Ragnhammar P, Blomgren H (2002) Thymidylate synthase expression in colorectal cancer: a prognostic and predictive marker of benefit from adjuvant fluorouracil-based chemotherapy. J Clin Oncol 20:1721–1728
4. Edler D, Hallstrom M, Johnston PG, Magnusson I, Ragnhammar P, Blomgren H (2000) Thymidylate synthase expression: an independent prognostic factor for local recurrence, distant metastasis, disease-free and overall survival in rectal cancer. Clin Cancer Res 6:1378–1384
5. Fujii R, Seshimo A, Kameoka S (2003) Relationships between the expression of thymidylate synthase, dihydropyrimidine dehydrogenase, and orotate phosphoribosyltransferase and cell proliferative activity and 5-fluorouracil sensitivity in colorectal carcinoma. Int J Oncol 8:72–78

6. Grem JL, Danenberg KD, Behan K, Parr A, Young L, Danenberg PV, Nguyen D, Drake J, Monks A, Allegra CJ (2001) Thymidine kinase, thymidylate synthase, and dihydropyrimidine dehydrogenase profiles of cell lines of the National Cancer Institute's Anticancer Drug Screen. Clin Cancer Res 7:999–1009
7. Habara K, Ajiki T, Kamigaki T, Nakamura T, Kuroda Y (2001) High expression of thymidylate synthase leads to resistance to 5-fluorouracil in biliary tract carcinoma in vitro. Jpn J Cancer Res 92:1127–1132
8. Higashiyama M, Kodama K, Yokouchi H, Takami K, Fukushima M, Minamigawa K, Takano T, Kobayashi H (2001) Thymidylate synthase and dihydropyrimidine dehydrogenase activities in non-small cell lung cancer tissues: relationship with in vitro sensitivity to 5-fluorouracil. Lung Cancer 34:407–416
9. Horie N, Aiba H, Oguro K, Hojo H, Takeishi K (1995) Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5′-terminal regulatory region of the human gene for thymidylate synthase. Cell Structure and Function 20:191–197
10. Iacopetta B, Grieu F, Joseph D, Elsaleh H (2001) A polymorphism in the enhancer region of the thymidylate synthase promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. Brit J Cancer 85:827–830
11. Inaba M, Mitsuhashi J, Ozawa S (1990) Kinetic analysis of 5-fluorouracil action against various cancer cells. Jpn J Cancer Res 81:1039–1044
12. Ishida Y, Kawakami K, Tanaka Y, Kanehira E, Omura K, Watanabe G (2002) Association of thymidylate synthase gene polymorphism with its mRNA and protein expression and with prognosis in gastric cancer. Anticancer Res 22:2805–2810
13. Kaneda S, Nalbantoglu J, Takeishi K, Shimizu K, Gotoh O, Seno T, Ayusawa D (1990) Structural and functional analysis of the human thymidylate synthase gene. J Biol Chem 265:20277–20284
14. Kaneda S, Takeishi K, Ayusawa D, Shimizu K, Seno T, Altman S (1987) Role in translation of a triple tandemly repeated sequence in the 5′-untranslated region of human thymidylate synthase mRNA. Nucl Acids Res 15:1259–1270
15. Kawakami K, Ishida Y, Danenberg KD, Omura K, Watanabe G, Danenberg PV (2002) Functional polymorphism of the thymidylate synthase gene in colorectal cancer accompanied by frequent loss of heterozygosity. Jpn J Cancer Res 93:1221–1229
16. Kawakami K, Salonga D, Park JM, Danenberg KD, Uetake H, Brabender J, Omura K, Watanabe G, Danenberg PV (2001) Different lengths of a polymorphic repeat sequence in the thymidylate synthase gene affect translational efficiency but not its gene expression. Clin Cancer Res 7:4096–4101
17. Kawakami K, Watanabe G (2003) Identification and functional analysis of single nucleotide polymorphism in the tandem repeat sequence of thymidylate synthase gene. Cancer Res 63:6004–6007
18. Kubota T, Fujita S, Kodaira S, Yamamoto T, Josui K, Arisawa Y, Suto A, Ishibiki K, Abe O, Mabuchi K, Fuse M (1991) Antitumor activity of fluoropyrimidines and thymidylate synthase inhibition. Jpn J Cancer Res 82:476–482
19. Luo HR, Lu XM, Yao YG, Horie N, Takeishi K, Jorde LB, Zang YP (2002) Length polymorphism of thymidylate synthase regulatory region in Chinese populations and evolution of the novel alleles. Biochem Genet 40:41–51
20. Mandola MV, Stoehlmacher J, Muller-Weeks S, Cesarone G, Yu MC, Lenz HJ, Ladner RD (2003) A novel single nucleotide polymorphism within the 5′ tandem repeat polymorphism of the thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. Cancer Res 63:2898–2904
21. Marsh S, Collie-Deguid ES, Li T, Liu X, McLeod HL (1999) Ethnic variation in the thymidylate synthase enhancer region polymorphism among Caucasian and Asian populations. Genomics 58:310–312
22. Marsh S, Ameyaw MM, Githanga J, Indalo A, Ofori-Adjei D, McLeod HL (2000) Novel thymidylate synthase enhancer region alleles in African populations. Hum Mutation 16:528–533
23. Marsh S, McLeod HL (2001) Thymidylate synthase pharmacogenetics in colorectal cancer. Clin Colorectal Cancer 1:175–178
24. Ma T, Zhu ZG, Ji YB, Zhang Y, Yu YY, Liu BY, Yin HR, Lin YZ (2004) Correlation of thymidylate synthase, thymidylate phosphorylase and dihydropyrimidine dehydrogenase with sensitivity of gastrointestinal cancer cells to 5-fluorouracil and 5-fluoro-2′-deoxyuridine. World J Gastroenterol 10:172–176
25. Mori K, Hasegawa M, Nishida M, Toma H, Fukuda M, Kubota T, Nagasue N, Yamana H, Hirakawa-YS Chung K, Ikeda T, Takasaki K, Oka M, Kameyama M, Toi M, Fujii H, Kitamura M, Murai M, Sasaki H, Ozono S, Makuuchi H, Shimada Y, Onishi Y, Aoyagi S, Mizutani K, Ogawa M, Nakao A, Kinoshita H, Tono T, Imamoto H, Nakashima Y, Manabe T (2000) Expression levels of thymidine phosphorylase and dihydropyrimidine dehydrogenase in various human tumor tissues. Int J Oncol 17:33–38
26. Myers CE (1981) The pharmacology of the fluoropyrimidines. Pharmacol Rev 33:1–15
27. Pullarkat ST, Stoehlmacher J, Ghaderi V, Xiong Y-P, Ingels SA, Sherrod A, Warren R, Tsao-Wei D, Groshen S, Lenz H-J (2001) Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. Pharmacogenomics J 1:65–70
28. Saga Y, Suzuki M, Mizukami H, Kohno T, Takei Y, Fukushima M, Ozawa K (2003) Overexpression of thymidylate synthase mediates desensitization for 5-fluorouracil of tumor cells. Int J Cancer 106:324–326
29. Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV (2000) Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res 6:1322–1327
30. Takenoue T, Nagawa H, Matsuda K, Fujii S, Nita ME, Hatano K, Kitayama J, Tsuruo T, Muto T (2000) Relation between thymidylate synthase expression and survival in colon carcinoma, and determination of appropriate application of 5-fluorouracil by immunohistochemical method. Ann Surgical Oncol 7:193–198
31. Tsuji T, Hidaka S, Sawai T, Nakagoe T, Yano H, Haseba M, Komatsu H, Shindou H, Fukuoka H, Yoshinaga M, Shibasaki S, Nanashima A, Yamaguchi H, Yasutake T, Tagawa Y (2003) 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. Clin Cancer Res 9:3700–3704
32. Villafranca E, Okruzhnov Y, Dominguez MA, Garcia-Foncillas J, Azinovic I, Martinez E, Illarramendi JJ, Arias F, Monge RM, Salgado E, Angeletti S, Brugarolas A (2001) Polymorphisms of the repeated sequences in the enhancer region of the thymidylate synthase gene promoter may predict downstaging after preoperative chemoradiation in rectal cancer. J Clin Oncol 19:1779–1786
33. Yoshinare K, Kubota T, Watanabe M, Wada N, Nishibori H, Hasegawa H, Kitajima M, Takechi T, Fukushima M (2003) Gene expression in colorectal cancer and in vitro chemosensitivity to 5-fluorouracil: a study of 88 surgical specimens. Cancer Sci 94:633–638