Thymidylate Synthase Expression Correlates Closely with *E2F1* Expression in Colon Cancer[1]

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ABSTRACT

Thymidylate synthase (*TS*) is thought to be one of the target genes that the *E2F1* transcription factor binds to and regulates. However, the relationship between the expressions of *TS* and *E2F1* in primary colon cancer specimens remains unclear. The aim of this study was to define the relation of *TS* and *E2F1* gene expressions in tumor samples from 23 colon cancer patients. *TS* and *E2F1* gene expressions were measured by TaqMan reverse transcription-PCR assay using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard and expressed as a *TS*:*GAPDH* or *E2F1*:*GAPDH* mRNA ratio. A close relationship was found between *TS* gene expression and *E2F1* gene expression ($r^2 = 0.598$, $P < 0.001$) in 23 tumor samples analyzed. Surprisingly, a high correlation between *TS* gene expression and *E2F1* gene expression was observed even in advanced tumors from stage IV colon cancer patients. These results suggest that transcription of the *TS* gene may be regulated by *E2F1* in primary colon cancer specimens and that this gene-regulatory pathway from *E2F1* to *TS* may be highly conserved during malignant progression. Four of the 23 patients showed *TS* overexpression with increased *E2F1* expression. These results suggest that the ability of a tumor to increase *TS* expression may possibly be due to an overexpression of *E2F1*. Although the number of patients was relatively small, our study provides new insights into the molecular mechanisms underlying the regulation of *TS* expression in colon cancers.

INTRODUCTION

TS[3] is a key enzyme in the synthesis of DNA and is the target enzyme of 5-FU, the most widely used chemotherapeutic agent for colon cancer (1–4). Previous investigations have demonstrated that the expression of *TS* mRNA or protein predicts overall survival for colon cancer patients and correlates with resistance to 5-FU (5, 6). Berger *et al.* and Swain *et al.* have shown that acquired resistance to 5-FU is caused by overproduction of TS as a result of gene amplification (7–9). In a study of human breast and colon cancer cell lines exposed to 5-FU, Chu *et al.* (10) noted that resistance correlated with increased levels of *TS* that resulted from a transcriptional or posttranscriptional regulatory event. Thus, mechanisms by which tumor cells may increase *TS* expression have been discussed in terms of the development of 5-FU resistance.

Recently, DeGregori *et al.* (11) have shown the genes encoding S phase-acting proteins, including TS, to be induced by the *E2F1* transcription factor. Moreover, cells overexpressing *E2F1*, in a study of human fibrosarcoma cell lines, were reportedly resistant to 5-FU, with an up-regulation of *TS* expression (12). Thus, the possibility that high *TS* expression in tumors may be the result of *E2F1* overexpression has been suggested. To date, however, the relationship between *TS* and *E2F1* expressions has not actually been studied in surgical specimens of primary colon cancer.

In this study, we investigated the intratumoral expression of both the *TS* and *E2F1* genes in 23 colon cancers using the TaqMan RT-PCR assay and compared the results obtained. We found that *TS* expression correlates closely with *E2F1* expression in colon cancer specimens. Moreover, this correlation was observed in all tumors, regardless of clinical stage. We discuss herein the significance of these observations from the clinical perspective.

MATERIALS AND METHODS

Sample and RNA Preparation. Colon tumors were obtained intraoperatively from 23 colon cancer patients at the Nihon University School of Medicine (Tokyo, Japan). There were 13 men and 10 women, with a median age of 65.0 years and an age range of 43–83 years. A portion of each specimen was used for routine histopathological examinations. The tumors were classified according to the American Joint Committee on Cancer (13). None of these patients had received chemotherapy prior to the operation. All samples were immediately frozen in liquid nitrogen and stored at −80°C until further use. Total RNA was isolated using RNeasy (Qiagen Inc., Chats-

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[3] The abbreviations used are: TS, thymidylate synthase; 5-FU, 5-fluorouracil; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TAMRA, 6-carboxy-tetramethylrhodamine; FAM, 6-carboxyfluorescein.

worth, CA), and DNase treatment was performed using a MessageClean Kit (GenHunter Corp., Brookline, MA), following the manufacturer's instructions.

TaqMan RT-PCR Assay. The TaqMan 5′ nuclease fluorigenic quantitative PCR assay that we used is a well-established method of analyzing gene expression in a wide range of samples (14, 15). Fifty microliters of reaction mixture for RT-PCR were prepared in a single tube: 30 ng of the extracted total RNA; 1× TaqMan EZ buffer [50 mM Bicine, 115 mM potassium acetate, 0.01 mM EDTA, 60 nM Passive Reference 1, 8% glycerol (pH 8.2)]; 3 mM $MgCl_2$; 300 μM dATP, dGTP, and dCTP; 600 μM dUTP; 0.2 μM forward primer; 0.2 μM reverse primer; 0.1 μM TaqMan probe; 5 units of rTth DNA Polymerase; and 0.5 unit of AmpErase UNG (the enzymes and the buffer containing the passive reference were from Perkin-Elmer Corp.). The conditions of one-step RT-PCR were: 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, and then 40 cycles of amplification for 20 s at 95°C and 1 min at 62°C. Triplicate PCR amplifications were carried out for each sample.

Primers and TaqMan Probes. Primers and the TaqMan probe for TS and E2F1 were designed in accordance with Perkin-Elmer Corp. guidelines. Primers and the TaqMan probe for GAPDH (TaqMan GAPDH control reagent kit) were also purchased. The probes were labeled with a reporter dye [FAM or JOE (2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein)], situated at the 5′ end of the oligonucleotide, and a quencher dye (TAMRA), located at the 3′ end. The sequences of primers and probes used were: TS-forward: 5′-CCAGAGATCGGGAGACATGG-3′ (bases 744–763 of the TS coding sequence; Ref. 16); TS-reverse: 5′-TACGTGAGCAGGGCGTAGCT-3′ (bases 789–809 of the TS coding sequence; Ref. 16); TS probe: 5′-FAM-CCTCGGTGTGCCTTTCAACATCGC-TAMRA-3′ (bases 765–788 of the TS coding sequence; Ref. 16); E2F1-forward: 5′-GAGGTGCTGAAGGTGCAGAAG-3′ (bases 607–627 of the E2F1 coding sequence; Ref. 17); E2F1-reverse: 5′-TTGGCAATGAGCTGGATGC-3′ (bases 662–680 of the E2F1 coding sequence; Ref. 17); E2F1 probe: 5′-FAM-CGCATCTATGACATCACCAACGTCCTTG-TAMRA-3′ (bases 631–658 of the E2F1 coding sequence; Ref. 17); GAPDH-forward: 5′-GAAGGTGAAGGTCGGAGT-3′ (bases 1457–1474 of the GAPDH genomic sequence; Ref. 18); GAPDH-reverse: 5′-GAAGATGGTGATGGGATTTC-3′ (bases 3403–3412 of the GAPDH genomic sequence; Ref. 18); GAPDH probe: 5′-JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA-3′ (bases 3374–3393 of the GAPDH genomic sequence; Ref. 18). AmpliTaq DNA polymerase extended the primer and displaced the TaqMan probe through its 5′-3′ exonuclease activity. When the probe is intact the emission spectrum of the reporter is suppressed by the quencher. The nuclease degradation of the hybridization probe releases the reporter, resulting in increased fluorescence emission. The use of a sequence detector (ABI Prism 7700) allows measurement of the amplified product in direct proportion to the increase in fluorescence emission, continuously, during the PCR amplification. The amplification plot is examined early in the reaction, at a point that represents the logarithmic phase of product accumulation. The point representing the detection threshold of the increase in the fluorescent signal associated with the exponential growth of the PCR product for the sequence detector is defined as the cycle threshold (C_T). C_T values are predictive of the quantity of input target (Refs. 14 and 15; *i.e.*, when the PCR conditions are the same, the larger the initial template concentration, the lower the C_T).

Statistical Analysis. The standard curve was created automatically by the ABI Prism 7700 detection system by plotting the C_T against each input amount (containing 100, 50, 10, 5, 1, or 0.1 ng) of control total RNA (total starting RNA) supplied in TaqMan EZ RT-PCR Kit (Perkin-Elmer Corp.). The coefficient of linear regression (r) for each standard curve was calculated. When the C_T value of a sample was substituted into the formula for each standard curve, the relative concentration of *TS*, *E2F1*, or GAPDH could be calculated. To normalize for differences in the amount of total RNA added to each reaction, GAPDH was selected as an endogenous RNA control. The normalized concentration of *TS* or *E2F1*, an arbitrary number that can be used to compare the relative amounts of *TS* or *E2F1* in different samples, was determined by dividing the concentration of *TS* or *E2F1* by the concentration of GAPDH.

Data are presented as mean ± SD. Statistical comparisons between groups of samples were made by ANOVA with the Mann-Whitney test. Differences were considered to be statistically significant when the P was <0.05. Pearson's correlation coefficient analysis was used to evaluate the relation between *TS* and *E2F1* expression levels.

RESULTS

Calibration Curves. For relative quantification of *TS* or *E2F1*, a standard curve was constructed using six different dilutions of control human RNA. Fig. 1 presents the C_T values plotted *versus* the input amount (total starting RNA) to produce standard curves for *TS*, *E2F1*, and *GAPDH* quantitation. The standard curves that were generated to span three logs showed linearity over the entire quantitation range, and provided accurate measurements over a very large range of starting target quantities. Equations were derived from the lines of the standard curves. When the C_T value of a sample was substituted into the formula for each standard curve, the relative concentration of *TS*, *E2F1*,or *GAPDH* could be calculated.

Expression of TS and E2F1 gene. *TS* and *E2F1* expressions were investigated in 23 tumor samples obtained from colon cancer patients: eight in stage II, eight in stage III and seven in stage IV. The normalized concentrations of *TS* and *E2F1* were determined by dividing the concentration of *TS* or *E2F1* by the concentration of *GAPDH*, as the endogenous control. Table 1 shows the clinicopathological factors correlating with *TS* and *E2F1* expression. The mean levels of *TS* and *E2F1* mRNAs in the colon tumors of all 23 patients were 0.98 and 1.52, respectively. The levels of *TS* or *E2F1* expression for men tended to be higher than those for women; the mean respective levels of *TS* and *E2F1* for 13 men were 1.32 and 2.11, *versus* 0.54 and 0.76 for the 10 women. The sex difference was significant ($P < 0.05$) for *TS* expression, but not for *E2F1* expression. The median age of the 23 patients in this study was 65 years. The mean *TS* mRNA level for those younger than the median age was 1.04 *versus* 0.94 for those older than 65 years, the mean *E2F1* mRNA levels 2.30 *versus* 1.10. *TS* and *E2F1* expressions did not differ among stages; the mean *TS* mRNA

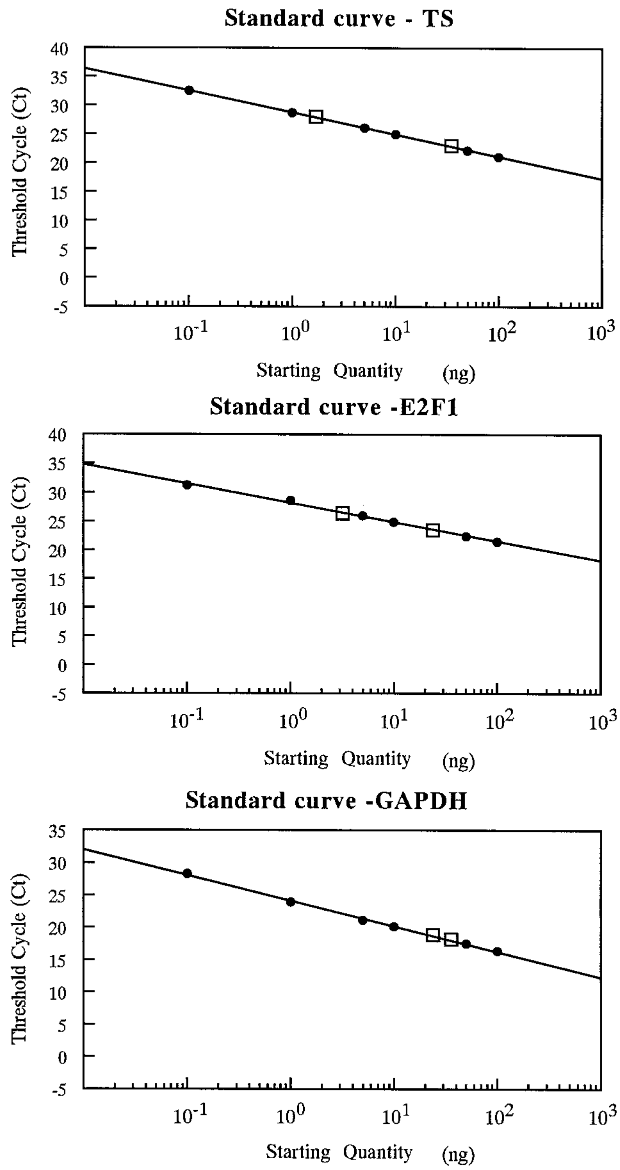

Fig. 1 Standard curves for TS (*top*), E2F1 (*middle*), and GAPDH (*bottom*). The calibration curve of the scatter plot represents log ng of total starting (input) RNA as x and C_T as y. Each *dot* represents the result of triplicate PCR amplifications for each dilution. The □ represents the results of PCR amplification of samples containing unknown quantities of target RNAs. The three formulas for log ng of TS, E2F1, and GAPDH are: TS, $y = -3.81x + 28.74$ ($r = 1.000$); E2F1, $y = -3.31x + 28.18$ ($r = 0.998$); and GAPDH, $y = -3.94x + 24.12$ ($r = 0.999$).

levels for stage II, III, and IV patients were 0.94, 0.87, and 1.16, respectively, whereas the corresponding *E2F1* mRNA levels were 1.88, 1.36, and 1.30. Fig. 2 shows the significant relationship between *TS* and *E2F1* expressions ($r^2 = 0.598$, $P < 0.001$) in the 23 tumor samples. Interestingly, at each stage, the level of *TS* expression correlated closely with that of *E2F1* expression: stages II ($r^2 = 0.846$, $P < 0.001$), III ($r^2 = 0.868$, $P = 0.002$), and IV ($r^2 = 0.988$, $P < 0.001$).

Table 1 Associations of clinical characteristics with *TS* and *E2F1* expressions

	No. of patients	TS/GAPDH mean ± SD	*P*	E2F1/GAPDH mean ± SD	*P*
All patients	23	0.98 ± 1.20		1.52 ± 2.10	
Sex					
Male	13	1.32 ± 1.48	0.048[a]	2.11 ± 2.55	NS[b]
Female	10	0.54 ± 0.48		0.76 ± 1.00	
Age					
≤65	9	1.04 ± 0.81	NS	2.30 ± 2.94	NS
>65	14	0.94 ± 1.44		1.10 ± 1.50	
Stage[c]					
II	8	0.94 ± 0.72		1.88 ± 2.56	
III	8	0.87 ± 0.61	NS	1.36 ± 1.99	NS
IV	7	1.16 ± 2.25		1.30 ± 2.10	

[a] Mann-Whitney *U* test.
[b] NS, not significant.
[c] American Joint Committee on Cancer staging system (11).

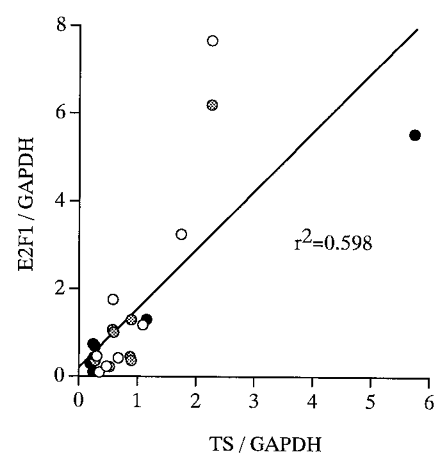

Fig. 2 Relation of *TS* to *E2F1* expression in colon cancer specimens. ○, stage II tumor; *gray circle*, stage III tumor; ●, stage IV tumor.

DISCUSSION

Expression of the *TS* gene has been studied as a prognostic factor and a mechanism of drug resistance for various tumors, including colon cancer (5, 6). Recently, transcription factor *E2F1* was suggested to regulate *TS* gene expression (11, 12). However, the relationship between the expression of *TS* and *E2F1* has not actually been investigated using clinical colon cancer specimens. Moreover, the relationship between a *E2F1* expression of a tumor and its biological behavior remains unclear. In this study, we examined the levels of *TS* and *E2F1* expression in 23 surgical colon cancer specimens using with the TaqMan RT-PCR assay and compared the levels obtained.

As to clinicopathological variables, no significant relation was found in the current study between the expression of *E2F1* and three important clinicopathological variables: age, sex, and staging (Table 1). With the exception of sex, similar results were obtained for *TS* expression. Previous studies found *TS* expression to be associated with clinical stage in colon (5, 6) and gastric cancer (19). However, there were no differences in *TS* expression among stages in this study. These results may be explained as follows. First, the sample size was small, and levels of *TS* and *E2F1* expression were low in most (Fig. 2). Second, we evaluated *TS* expression using a new detection system, the TaqMan RT-PCR assay. There is a possibility that results using this new system may not be totally consistent with those using previous methods. Many samples will need to be analyzed using the TaqMan RT-PCR assay, to allow comparison of the results with those of previous studies (2, 5, 6).

This study is the first to compare *TS* and *E2F1* expressions in the same primary colon cancer specimen. Surprisingly, a significant relationship between *TS* and *E2F1* expressions ($r^2 = 0.598$, $P < 0.001$), as shown in Fig. 2, was found despite the number of patients being relatively small. Our results reinforce previous studies indicating that *E2F1* is a transcription factor regulating *TS* expression (11, 12). We also made several novel observations. As to clinical stage, surprisingly, a high correlation between *TS* mRNA and *E2F1* mRNA expressions was observed in stages II ($r^2 = 0.846$, $P < 0.001$) and III ($r^2 = 0.868$, $P = 0.002$), and even in stage IV ($r^2 = 0.988$, $P < 0.001$), colon cancer. It is noteworthy that this correlation was observed regardless of clinical stage, even in advanced tumors from stage IV colon cancer patients. These results suggest that the gene-regulatory pathway from *E2F1* to *TS* may be highly conserved during malignant progression.

Only four (two stage II, one stage III, and one stage IV) of the 23 tumors showed *TS* overexpression (Fig. 2), with increased *E2F1* expression, although it was difficult to determine a cutoff level between high and low *TS* expression. These results suggest that tumors with high *TS* expression in these cases may be secondary to *E2F1* overexpression. Similarly, DeGregori *et al.* (11) and Banerjee *et al.* (12) have shown that *E2F1*-overexpressing cells had increased *TS* levels, which is consistent with our results. Because elevated *TS* mRNA in colon cancer correlates with a poor response to 5-FU treatment (5), tumors with a high level of *TS* expression would be predicted to be 5-FU resistant. On the other hand, Banerjee *et al.* (12) have also shown cells overexpressing *E2F1* to be more sensitive to etoposide and doxorubicin (*i.e.,* topo II inhibitors) and SN38 (the active metabolite of irinotecan; *i.e.,* a topo I inhibitor), despite being resistant to 5-FU. Therefore, tumors with a high level of *E2F1* expression, as in this study, may be more sensitive to topo I and topo II inhibitors. The levels of both *E2F1* and *TS* mRNAs in tumors are thus potential indicators of which anticancer agents are likely to be effective for colon cancer patients. The ability to predict response and outcome based on *E2F1* and *TS* expression in the primary tumor would provide useful information for many clinicians in planning chemotherapy.

We speculate that one mechanism by which tumor cells increase *TS* expression may be overexpression of *E2F1,* even in primary colon cancers. Because many of the genes encoding S phase-acting proteins (including DNA polymerase α, proliferating cell nuclear antigen, ribonucleotide reductase, and TS) are reportedly induced by *E2F1* (11), tumors with high *E2F1* expression, as demonstrated herein, may be predictive of the overexpression of not only *TS* but also many other genes participating in the progression of cells from the G_1 to the S phase of the cell cycle. Although the ability of a tumor to overexpress *TS* may represent an important protective mechanism in response to 5-FU, as has previously been discussed (1, 4, 5), the possibility that many genes associated with *E2F1* contribute to the mechanism of 5-FU resistance in tumors with a high level of *TS* expression cannot be ruled out. Additional studies are needed to define the molecular mechanism underlying the development of resistance to chemotherapy in colon cancer patients.

In conclusion, we have demonstrated that the level of *TS* mRNA expression correlates closely with the level of *E2F1* mRNA expression; that is, *E2F1* regulation of *TS* expression was demonstrated in colon cancer specimens. These results suggest that the ability of a tumor to overexpress *TS* may be due to enhanced expression of *E2F1*. Although the number of patients was relatively small, our study provides new insights into the molecular mechanisms underlying the regulation of *TS* expression in colon cancers.

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