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Clinical report

Retrospective study on thymidylate synthase as a predictor of outcome and sensitivity to adjuvant chemotherapy in patients with curatively resected colorectal cancer

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We carried out a retrospective evaluation of thymidylate synthase (TS) expression in tumor tissue, and its relation to outcome and response to treatment. The treatment consisted of chemotherapy with tegafur and uracil (UFT). The study group comprised 245 patients with curatively resected Dukes' stage B or C colorectal cancer who were postoperatively enrolled in a controlled study and assigned to receive UFT or no adjuvant chemotherapy. TS expression in tumor tissue was evaluated immunohistochemically with the use of recombinant human TS-specific antibody. Results were as follows. There was no relation between TS expression and the rate of 5-year disease-free survival. Similar results were obtained in both colonic and rectal tumors. The rate of 5-year disease-free survival was significantly higher in the UFT group than in the group receiving no adjuvant chemotherapy ($p=0.0055$). The difference in survival became more marked among patients whose tumors had diffuse TS expression ($p=0.0027$). There was no difference in survival between the treatment groups among patients whose tumors had focal TS expression. We conclude that, although unrelated to outcome, TS activity may be useful in predicting the response to adjuvant chemotherapy with UFT in patients with curatively resected Dukes' stage B or C colorectal cancer. [© 2002 Lippincott Williams & Wilkins.]

Key words: Adjuvant chemotherapy and UFT, colorectal cancer, retrospective study, thymidylate synthase.

Introduction

Thymidylate synthase (TS) is the rate-limiting enzyme governing the pathway of *de novo* DNA synthesis, making it an important target enzyme for chemotherapy. 5-Fluorouracil (5-FU) and its analogs act mainly by inhibiting TS activity via the activated metabolite F-dUMP.[1–3] The level of TS expression influences sensitivity to chemotherapy.[4,5] Recently, TS expression in tumors has attracted considerable attention because of its potential role as a prognostic factor.[6–11] However, the relation of TS protein expression in colorectal tissue to outcome and response to 5-FU-based postoperative adjuvant therapy remains a matter of debate.

From March 1991 through April 1994 the Study Group of Tokai Adjuvant Chemotherapy for Colorectal Cancer (TAC-CR) performed a controlled trial evaluating the response to postoperative treatment with tegafur and uracil (UFT) in patients who had curatively resected Dukes' stage B or C colorectal cancer. The rate of disease-free survival was significantly higher in the UFT group than control. In particular, UFT was significantly more effective in suppressing local recurrence in patients with rectal cancer.[12] In the present study, we retrospectively examined TS expression in tumor tissue obtained from patients enrolled in the TAC-CR controlled trial, and assessed the relation of TS expression to outcome and the response to postoperative treatment with UFT.

Patients and methods

Patients

Attempts were made to obtain histopathological specimens from 289 patients registered in the TAC-CR trial who were considered eligible for this study. Suitable specimens could not be obtained from 39 patients. Tumors from 250 patients (86.5%) were studied immunohistochemically with the use of

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recombinant human TS-specific antibody (RTSSA; Taiho Pharmaceutical, Tokyo, Japan). Five tumors could not be assessed because of damaged specimens or other reasons. Tumors from a total of 245 patients (84.8%) were therefore evaluated. For postoperative adjuvant chemotherapy, patients registered in the TAC-CR trial were given UFT, a preparation combining uracil and tegafur in a molar ratio of 4:1, at an oral dose equivalent to 400 mg/day of tegafur. Treatment with UFT was continued for 2 years after surgery.

Immunohistochemistry

Surgically resected specimens were fixed in 10% formalin and embedded in paraffin. The tissue sections were cut at 3–4 μm and underwent immunohistochemical staining using the avidin–biotin–peroxidase complex (ABC) system (Vectastatin Elite ABC kit; Vector, Burlingame, CA). Antigen retrieval was done by microwave treatment. The sections were then treated with 0.3% H_2O_2 in absolute methanol for 20 min at room temperature in order to inhibit endogenous peroxidase activity. After background staining was blocked for 30 min at room temperature with 10% normal goat serum, the sections were incubated overnight at 4°C with primary antibody, recombinant human thymidylate synthase-specific antibody (RTSSA, 1:1000; Taiho Pharmaceutical).[13–15] After washing with PBS, the sections were allowed to react with the biotinylated secondary antibody and avidin–biotin–peroxidase, respectively, for 30 and 60 min at room temperature, respectively. Finally, antigen–antibody reactions were detected using diaminobenzindine (DAB) as a chromgen. Counter-staining was done with hematoxylin.[15–17]

Evaluation of immunohistochemical labeling

TS expression of each specimen was evaluated on the basis of staining intensity and staining extent. Staining extent was expressed as the ratio of positively stained tumor cells to all tumor cells (%). A staining extent of less than 25% was classified as TS focal and that of 25% or more was classified as TS diffuse (Figure 1).

Staining intensity was assessed by comparing the staining intensity of the main part of the tumor with that of the normal colonic mucosa in the same section or from the same patient (the general staining intensity of the normal mucosa was used as a reference for specimens including no normal mucosa). The intensity was then evaluated according to the following four-grade scale: (–) no staining of tumor; (±) staining intensity of tumor weaker than that of normal mucosa; (+) staining intensity of tumor similar to that of normal mucosa; (++) staining intensity of tumor stronger than that of normal mucosa (Figure 1). TS low was defined as a staining intensity of (−), (±) or (+), and TS high was defined as (++).

All specimens were evaluated independently by two pathologists. Any disagreement in evaluation was resolved by consensus.

Results

Evaluation of TS immunostaining

TS extent was TS diffuse in 156 tumors (63.7%). and TS focal in 89 (36.3%). TS intensity was TS high in 88 tumors (35.9%) and TS low in 157 (64.1%).

Clinical characteristics

The clinical characteristics of the 245 patients whose tumors were evaluated for TS expression were compared with those of the 289 patients registered in the original TAC-CR study. There were no differences between these groups in tumor site (colon or rectum), Dukes' classification or the proportion of patients who postoperatively received adjuvant therapy (Table 1).

The clinical characteristics of patients with TS diffuse tumors and those with TS focal tumors were similar with respect to tumor site, Dukes' classification and whether or not they received postoperative adjuvant chemotherapy (Table 2). Although TS intensity was slightly but not significantly higher in colonic tumors, there was no difference in clinical characteristics between the TS low and TS high groups, similar to the results for TS extent. Since there was no consistent relation between TS extent and TS intensity, these characteristics were considered distinct variables.

TS expression and outcome

The relation between TS expression and 5-year disease-free survival (DFS) was examined. There

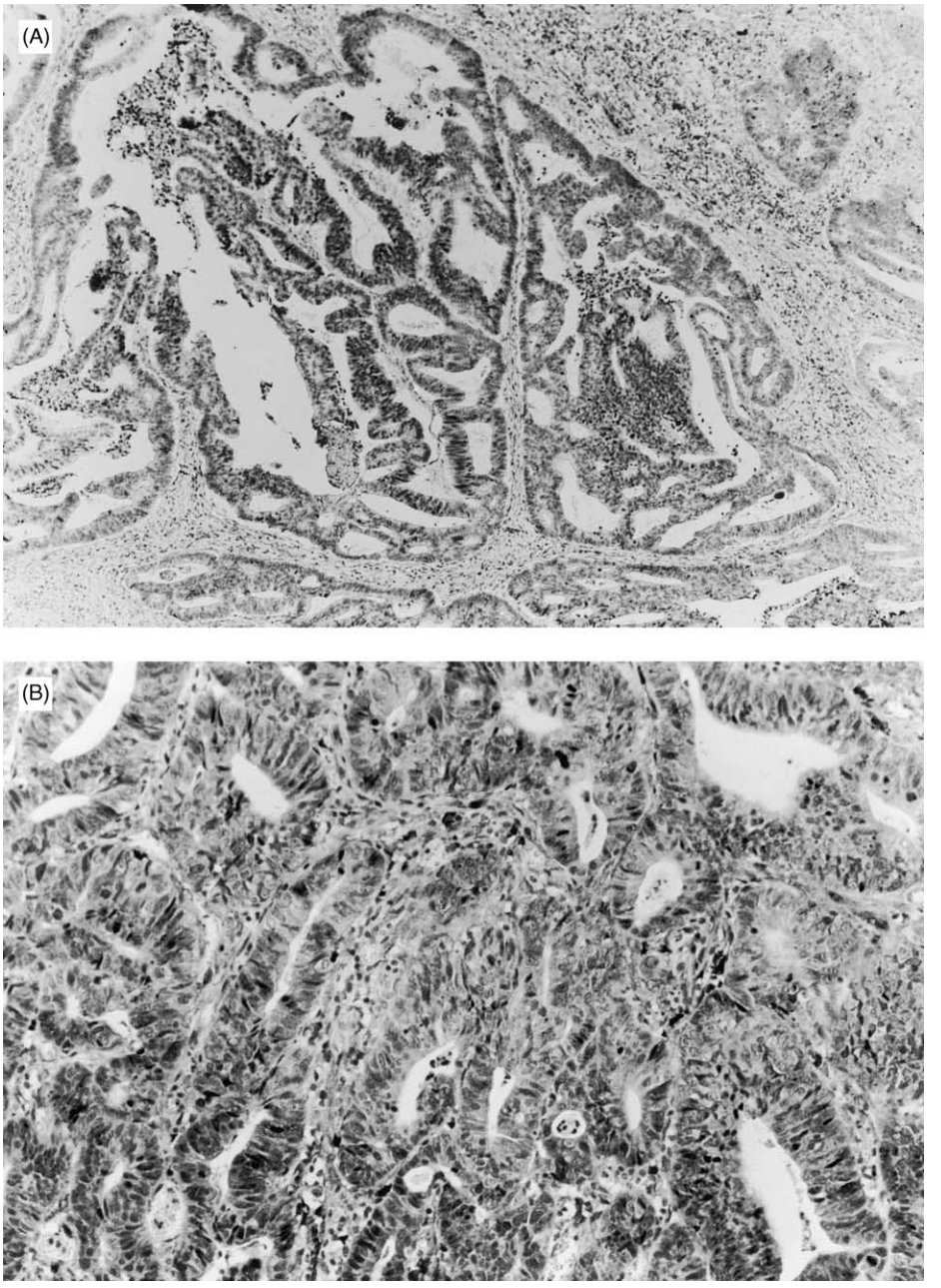

Figure 1. Representative examples of immunohistochemical staining using the recombinant human TS-specific antibody (RTSSA). (A) Staining extent 10–20% of tumor cells positive and staining intensity (+): rectal tumor (× 16). (B) Staining extent 80–90% of tumor cells positive and staining intensity (+ +): colon tumor (× 40).

was no difference in DFS between the TS focal and TS diffuse groups (Figure 2) or between the TS high and TS low groups. DFS according to TS expression was also analyzed for different tumor sites (colon versus rectum), but no differences were found. The Cox proportional-hazards model was used to analyze the effects of four covariates on survival: tumor site (colon or rectum), whether or not the patient received postoperative adjuvant chemotherapy, Dukes' classification and TS extent. The analysis identified tumor site ($p=0.0009$) and postoperative adjuvant therapy ($p=0.0074$) as independent predictors of survival. TS extent was unrelated to survival. Similar results were obtained when TS intensity was substituted as a covariate for TS extent.

Table 1. Differences in clinical characteristics between the original TAC-CR study and the present retrospective study

	Original TAC-CR study	Present study	χ^2-test
Tumor site			
colon	160	137	$p=0.9671$
rectum	129	108	
Dukes' classification			
B	133	115	$p=0.9006$
C	156	130	
Treatment group			
controls	145	122	$p=1.0000$
UFT group	144	123	

Table 2. Patient characteristics according to TS extent

	TS focal	TS diffuse	χ^2-test
Tumor site			
colon	50	87	$p=0.9430$
rectum	39	69	
Dukes' classification			
B	42	73	$p=0.9415$
C	47	83	
Treatment group			
controls	45	78	$p=0.9227$
UFT group	44	78	

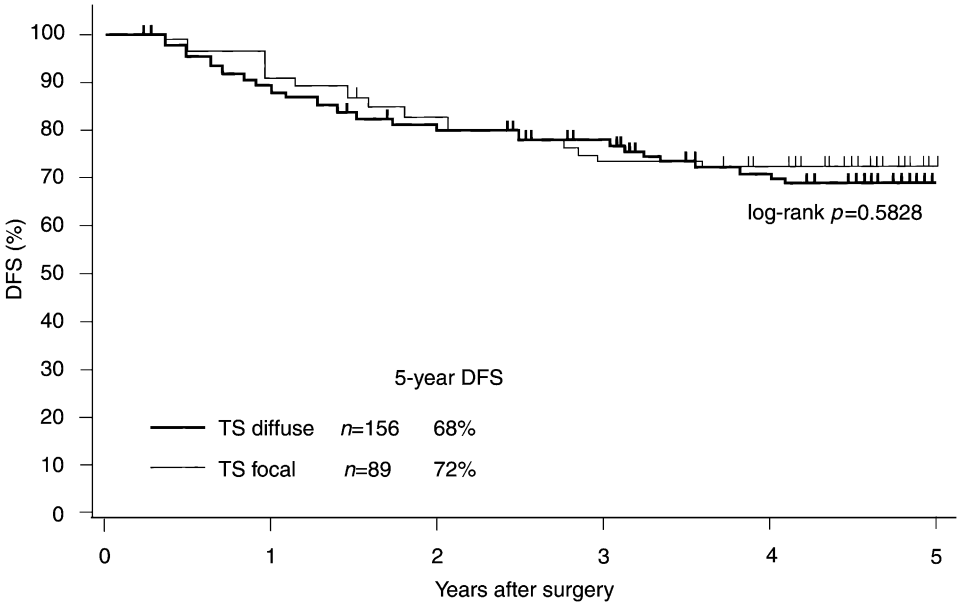

Figure 2. Five-year DFS: TS diffuse versus TS focal. Stratification according to TS staining showed no significant difference ($p=0.5828$).

TS expression and response to postoperative adjuvant chemotherapy

The value of TS expression as a predictor of response to postoperative adjuvant chemotherapy was assessed. The 245 patients whose tumors could be evaluated for TS expression were divided into a UFT group ($n=122$) and a non-treated control group ($n=123$). In the overall study group, 5-year DFS was significantly better in the UFT group than control

(p=0.0055), consistent with the results of the original study. Survival was also assessed separately in patients with TS diffuse tumors and those with TS focal tumors. Five-year DFS among patients with TS diffuse tumors was significantly better in the UFT group than in the control group (p=0.0027) and the difference between these groups was slightly greater than when the overall study group was evaluated. In patients with TS focal tumors, however, there was no difference in survival, in contrast to the outcome in the overall study group (Figure 3). Survival was also separately evaluated for colonic cancer and rectal cancer. In colonic cancer, there was no difference between the treatment groups when the overall study group was analyzed. In patients with TS diffuse colonic tumors, there was a trend toward better survival in the UFT group, but the difference between the groups was not significant (Figure 4). In rectal cancer, survival differed significantly between the treatment groups when the overall study group was evaluated (p=0.0037). In patients with TS diffuse rectal tumors, the difference in 5-year DFS between the treatment groups was slightly greater than that obtained when data from all patients with rectal cancer were analyzed (p=0.0076). In patients who had TS focal rectal tumors, there was no difference in survival between the treatment groups, in contrast to the results obtained when all patients with rectal cancer were included in the analysis (Figure 5. As for TS intensity, TS low tumors were associated with significantly better 5-year DFS in the UFT group than in the control group (p=0.0027), similar to the results obtained for the overall study group. Although there was no significant difference between the treatment groups among patients with TS high tumors, the profiles of the survival plots were similar to those in the overall study group and in patients with TS low tumors.

Discussion

The relationship between TS expression and outcome in patients with cancer has been studied with the use of various types of antibodies, such as monoclonal antibodies (TS106),[8,10,13,14] polyclonal antibodies[11] and RTSSA.[9,15,18,19] Diverse results have been obtained. RTSSA, which we used in this study, was previously used by Yamachika *et al.* to evaluate TS expression in 86 patients with curatively resected colorectal cancer[15] and by Kuniyasu *et al.*[18] in 134 patients with resected gastric cancer who received postoperative chemotherapy with fluorouracil derivatives or no chemotherapy. Although the outcome was poor in patients whose tumors had high TS expression, chemotherapy was shown to be effective. However, Yamachika *et al.*[15] defined high TS expression as a 10% or higher rate of positively stained cells (low expression versus high expression=81 versus 19%), considerably lower than our criterion of 25% or higher. This difference in the

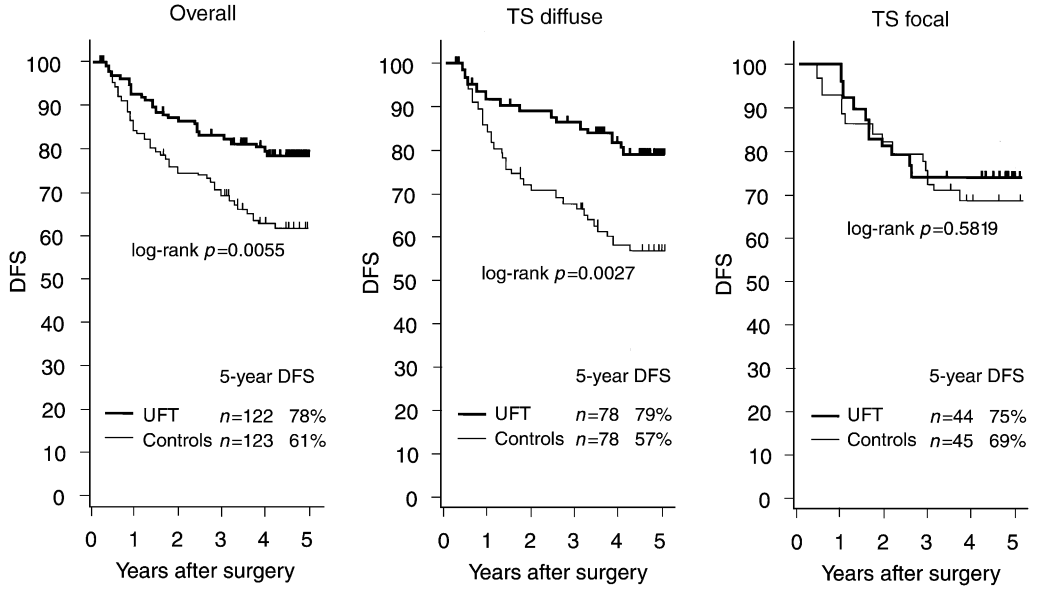

Figure 3. Five-year DFS according to TS expression. The TS diffuse UFT group had significantly better outcome (p=0.0027).

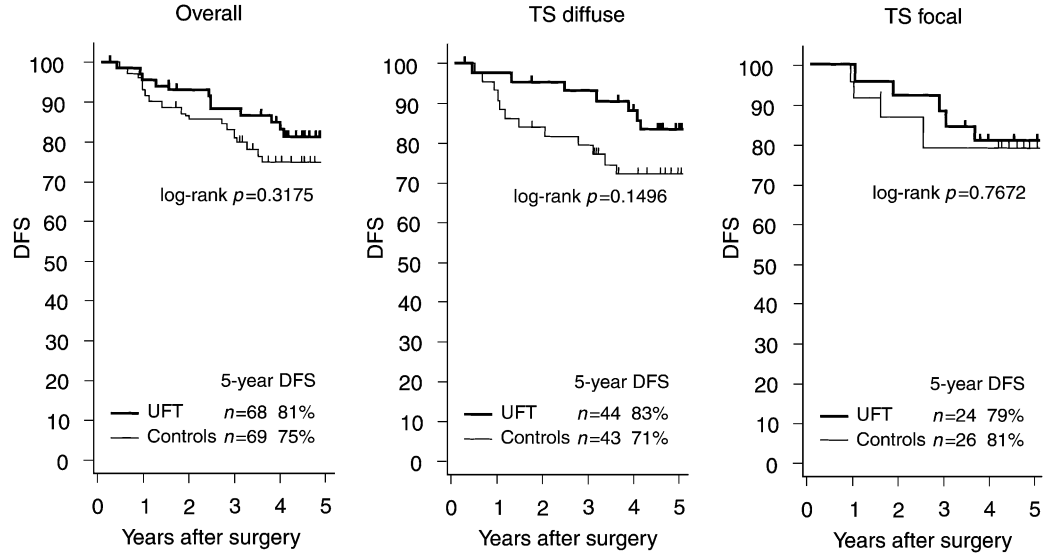

Figure 4. Five-year DFS according to TS expression in colon cancer.

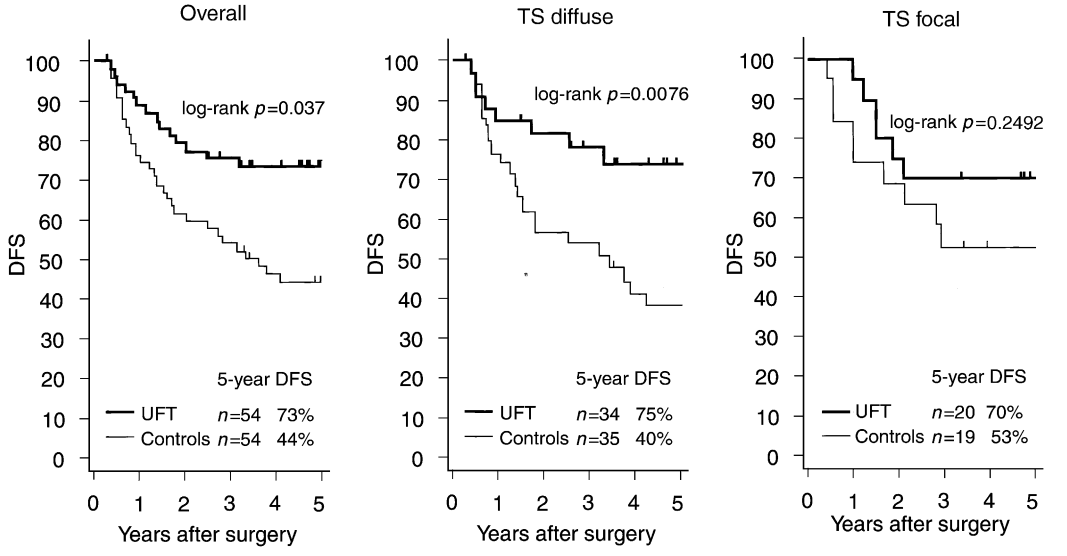

Figure 5. Five-year DFS according to TS expression in rectal cancer.

borderline value for high and low TS expression was most likely related to the omission by Yamachika *et al.*[15] of antigen inactivation by microwave treatment during immunohistochemical staining. Similar to our criterion, Kuniyasu *et al.*[18] used 25% as the cut-off value for high and low TS expression, but obtained a low expression rate of 58% and a high expression rate of 42%. These values differ considerably from the rates of 36.3% for low expression and 64.7% for high expression in our series of colorectal tumors, suggesting that TS expression depends on the organ harboring the primary tumor (data not shown). Suzuki *et al.*[19] also used RTSSA to study TS expression in 66 women with stage IIIb cancer of the uterine cervix who received radiotherapy. That study reported a poor outcome in patients with TS high or TS diffuse tumors, but defined TS diffuse as a positive staining rate of 10% or higher and defined TS high as merely the presence of positive staining. These criteria contrast sharply with those used in our study. The monoclonal antibody TS106 has been used in studies of colorectal cancer, gastric cancer,

head and neck cancer, and breast cancer. Although these investigations similarly show that tumors expressing TS are associated with a poor prognosis, the relation between TS expression and sensitivity to chemotherapy remains controversial. However, the results of these studies cannot be directly compared due to the lack of uniform criteria for the immunohistochemical evaluation of TS expression. Further studies are required to establish more objective criteria defining factors related to prognosis and chemotherapeutic sensitivity, and to develop new types of antibodies.

The principal objective of our study was to determine whether TS expression in colorectal cancer tissue could be used to predict outcome or sensitivity to postoperative adjuvant chemotherapy with UFT. The study group comprised patients registered in the TAC-CR study, who were randomly assigned to receive postoperative chemotherapy or no adjuvant treatment. Among the 289 eligible subjects, paraffin-embedded blocks of tumor tissue were able to be evaluated in 245 (84.8%). This value is substantially higher than rate of 36.7% (294 of 801) reported by Johnston *et al.*[10] Moreover, the clinical characteristics of subjects whose tumors could be assessed for TS expression also corroborate the high sampling rate and indicate that the subjects were representative of patients registered in the TAC-CR study.

Our study found no discernible relation between TS expression and outcome, in contrast to previous reports that high TS expression indicates a poor prognosis. This discrepancy can be attributed to the fact that in our study both tumors with high and low TS expression were treated by UFT, and the former were more sensitive to UFT treatment. When survival was evaluated according to TS extent in the untreated control group, the rate of 5-year DFS was 57% among patients with TS diffuse tumors (n=78), as compared with 69% in those with TS focal tumors (n=45). Overall survival in the control group was thus poorer in patients with TS diffuse tumors. However, the rate of 5-year DFS among patients with TS diffuse tumors who received UFT was 79% (n=78). This marked suppression of recurrence by postoperative adjuvant chemotherapy may have improved survival among all patients with TS diffuse tumors, resulting in survival similar to that in patients with TS focal tumors.

As for the relation between TS expression and postoperative adjuvant therapy, the response to UFT according to low and high TS intensity was similar to that in the overall study group, indicating that TS intensity is not a predictor of response. TS extent, however, was found to be related to response. Although there was no therapeutic response to UFT in patients with TS focal colonic or rectal tumors, patients with TS diffuse rectal tumors showed significantly improved survival in response to UFT. A trend toward improved survival was also seen in patients with TS diffuse colonic tumors. These findings strongly suggest that TS extent can be used to predict the response to UFT.

Prospective clinical trials are required to validate the usefulness of TS extent in predicting the therapeutic response to postoperative adjuvant chemotherapy with UFT. This drug has recently received considerable attention and has been newly classified a dihydropyrimidine dehydrogenase (DPD) inhibitory fluoropyrimidine derivative (DIF).[20,21] Identification of other factors involved in the metabolism of UFT, a representative DIF that activates 5-FU in tumor cells, is likely to further enhance the ability to predict the therapeutic response to chemotherapy, but must await future studies.

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