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Fluorouracil is well known as a standard drug that is converted to fluorodeoxyuridine monophosphate (FdUMP) and leads to the inhibition of thymidylate synthase (TS) in tumor tissues. The role of this key enzyme is the catalysis of the methylation from deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is a very important process for DNA synthesis in tumor tissues. Several investigators have elucidated the clinical importance of the inhibition of DNA synthesis that results from the inhibition of TS. On the basis of these considerations, several drugs that interact with the folate binding site of TS have been studied and have
demonstrated the possibility of the successful blockade of TS for cancer chemotherapy. Clinical trials of novel active analogs are in progress. However, TS in malignancy is clinically important not only as a chemotherapeutic target enzyme, but also as a prognostic index correlated with cancer activity and the resistance of cancer to 5-fluorouracil (5-FU). Using assays such as the polymerase chain reaction (PCR), several investigators have found that the expression of TS in cancer tissues was an important independent predictor of survival and that it predicted responses to 5-FU-based chemotherapy in gastric and colorectal carcinoma patients. In addition, with the use of an established monoclonal antibody against TS, the significant correlation between the expression of TS and the survival of rectal carcinoma patients has been demonstrated. However, little information about the significance of immunohistochemical TS analysis in gastric carcinoma was found in our extensive reviews. Thus, to determine the correlations between the expression of TS and clinicopathologic variables affecting the prognosis and survival of gastric carcinoma patients, immunohistochemical studies were performed using the newly developed anti-TS polyclonal antibody against recombinant human TS (rhTS). Immunohistochemical findings regarding the significance of TS as a prognostic indicator are also discussed in this article.

**MATERIALS AND METHODS**

**Patients**

One hundred thirty-four gastric carcinoma patients who underwent surgical treatment at the First Department of Surgery, Kobe University Hospital, Kobe, Japan, were enrolled in this study. There were 89 men and 45 women. The mean age was 63.2 years (range, 24–90 years). None of these patients received chemotherapy prior to the operation. After the operation, some patients were administered mitomycin C (MMC: 20mg/body) intravenously with oral administration of 5-FU (200mg/body/day) or tegafur-uracil (600mg/body/day), or received oral 5-FU or tegafur-uracil only. In the current study, the patients who received consecutive administration of those therapeutic agents for more than 3 months comprised the chemotherapy (+) group (n = 82), and the rest comprised the chemotherapy (-) group (n = 42).

**Clinicopathologic Evaluation**

Resected specimens of the gastric carcinomas were fixed in 4% buffered formalin and embedded in paraffin. To avoid a reduction in immunoreactivity, the fixation time did not exceed 48 hours. Four-μm tissue sections were cut and prepared for histologic examination. The histologic examinations were carried out routinely using hematoxylin and eosin stain and elastica van Gieson’s stain, and paraffin embedded blocks containing a central section of the cancer lesion were selected from each of the lesions. Eleven clinicopathologic variables (age, gender, tumor size, histologic type, depth of invasion, lymphatic and venous invasion, lymph node metastasis, peritoneal metastasis, liver metastasis, and staging) were examined according to the *Japanese Classification of Gastric Carcinoma* (first English edition). Concerning the histologic types in this study, well- and moderately differentiated adenocarcinomas were described as differentiated type, and poorly differentiated adenocarcinomas, signet ring cell carcinomas, and mucinous carcinomas were described as undifferentiated type.

**Immunohistochemical Staining for Thymidylate Synthase**

The expression of TS was studied immunohistochemically using a polyclonal antibody (immunoglobulin G) for rhTS, which was the generous gift of Dr. Masakazu Fukushima (Second Cancer Research Laboratory, Hanno Research Center, Taiho Pharmaceutical Co. Ltd., Saitama, Japan). This polyclonal antibody has been demonstrated by Western blot analysis and immunohistochemistry to react specifically with intracellular TS.

The immunohistochemical staining procedure was as follows: Four-μm sections, in parallel with a central section, were cut from the paraffin embedded cancer lesions. The sections were dewaxed with xylene and rehydrated gradually with graded alcohols. Endogenous peroxidase activity was blocked by soaking the sections in 0.3% hydrogen peroxidase for 20 minutes. After washing with tap water followed by distilled water, the sections were placed in the appropriate 10 mM citrate buffer solution. For antigen retrieval, slides were heated twice at 100°C for 5 minutes in a commercially available microwave oven (RE-121, Sharp, Japan), and then cooled for 20 minutes at room temperature. After being washed 3 times in Dulbecco’s phosphate-buffered saline (PBS), nonspecific bindings were blocked by preincubation with 10% normal bovine serum (Dakopatts, Glostrup, Denmark). The sections were then incubated with the aforementioned primary antibody of TS (dilution, 1:1000) at 36°C for 1 hour. After being washed 3 times in PBS, slides were incubated with biotinated antirabbit and antimouse immunoglobulin in PBS for 20 minutes. Then, after being washed 3 times in PBS, the sections were incubated with streptavidin conjugated to horseradish peroxidase in Tris-HCl buffer containing 15mM NaN₃ for 20 minutes. The washing proce-
dure was then repeated. The immunochemical reaction was revealed with a solution of 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris buffer (pH 7.6) containing 10 μL of 30% H2O2. The reaction was stopped after 7 minutes by the addition of tap water. The sections were then briefly counterstained with Mayer’s hematoxylin and mounted.

Evaluation of the Staining
The grade of the staining was evaluated independently by two of the authors (T.K. and T.N.) without prior knowledge of the clinicopathologic details. The expression of TS was divided in two groups based on the grade of staining: a low TS group for cases in which few or no more than 25% cancer cells were stained, and a high TS group for cases in which more than 25% cancer cells were stained. Human tumor xenograft, DLD-1/FdUrd, implanted into nude mice was used as the positive control. The negative control was made by the omission of the primary monoclonal antibody during the process of LSAB immunohistochemical staining.

Statistical Analysis
The data were analyzed with Student’s t test and the chi-square or Fisher’s exact probability calculation tests. Survival curves were estimated by the Kaplan–Meier method, and the difference between the curves was evaluated by the generalized Wilcoxon test. Multivariate analysis using the Cox proportional hazards regression model was performed with StatView J-4.5 personal computer software (Abacus Concepts, Berkeley, CA). P values less than 0.05 were considered significant.

RESULTS
Immunohistochemical Expression of Thymidylate Synthase
TS was immunohistochemically stained in the proliferative zone of gastric epithelia, the germinal center of mucosa-associated lymphoid tissues, and some of the goblet cells in normal gastric tissues (Fig. 1). As for the cancer lesions, granular cytoplasmic staining patterns of tumor cells were definitely demonstrated (Fig. 2). Although heterogeneous staining was found in the primary carcinoma lesions, there were no differences between the primary and metastatic lesions in the staining intensity of TS.

Correlation between the Expression of Thymidylate Synthase and Clinicopathologic Variables
The high and low TS groups consisted of 56 and 78 patients, respectively. Significant differences between the two groups were shown in 3 variables: depth of invasion, peritoneal metastasis, and stage classification (Table 1). There were significantly more advanced gastric carcinomas, peritoneal metastases, and advanced stages in the high TS group than in the low TS group (P < 0.05).

Univariate Analysis of Survival
The survival curve of the low TS group was significantly better than that of the high TS group (P < 0.05). The 5-year survival rate was 65.2% in the low TS group and 43.2% in the high TS group (Fig. 3). The influence of adjuvant chemotherapy on their prognoses is shown in Figure 4. As a result of the adjuvant chemotherapy, a significant difference in survival was noted between patients in the low and high TS groups who received chemotherapy (Fig. 4A). The 5-year survival rate was 60.4% in the low TS group and 33.1% in the high TS group. The prognoses of the high TS patients with advanced stage disease were significantly poor even when they received adjuvant chemotherapy. The...
5-year survival rate for the low and high TS patients who did not receive chemotherapy was 71.1% for both (Fig. 4B). Furthermore, no statistically significant difference in survival was found between the aforementioned low TS patients who received chemotherapy and the low TS patients who did not receive chemotherapy.

**Multivariate Analysis of Survival**

Eleven variables (tumor size, histologic type, depth of invasion, lymphatic invasion, venous invasion, lymph node metastasis, peritoneal metastasis, liver metastasis, staging, curability, and TS staining grade) were analyzed using the Cox proportional hazards regression model to determine the variables affecting the prognoses of the gastric carcinoma patients. The analysis revealed that 4 variables (peritoneal metastasis, lymphatic invasion, liver metastasis, and TS grade) independently contributed to survival ($P < 0.05$, Table 2). The hazard ratio of the low grade staining of TS compared with the high grade staining was 0.464.

**DISCUSSION**

TS exists as a dimer of identical subunits, each of which has a molecular weight of 36kD. The activity of this enzyme increases acutely as a cell passes from the late G1-phase to the early S-phase of the cell cycle, and plays the role of a catalyzing enzyme in the final step in dTMP synthesis. Both dUMP and 5,10-methylenetetrahydrofolate monoglutamate and polyglutamate analogs can achieve a special block of TS. This blockade of TS might contribute to an increase in intracellular dUMP pools and may inhibit the pyrimidine de novo biosynthesis of thymidylate as a precursor of DNA synthesis. In light of these possibilities,
the inhibition of TS has been thought of as a chemotherapeutic target. In addition, recent studies using TS binding assays13–15,24–26 have demonstrated that the prognoses of cancer patients and their responses to 5-FU were significantly related to the intratumoral total expression of TS. A previous investigation of PCR quantitation of TS14 also demonstrated that the mRNA level of TS influenced the response to 5-FU-based chemotherapy and the survival of patients with gastric carcinoma. These types of special assays, including FdUMP ligand assay and the reverse transcriptase (RT) PCR assay of TS mRNA, may be sensitive but may also present special technologic difficulties. The immunohistochemical evaluation of TS, by contrast, is technologically simple and easy to perform. For example, the immunohistochemical analysis of TS used in this study is visually apparent and clinically practical because paraffin embedded sections of the cancer tissue can be used. Although the appropriate fixation time for the immunohistochemical staining of TS is unclear, an excessively long fixation time should be avoided to preserve immunoreactivity, as was also reported for proliferating cell nuclear antigen (PCNA) staining.27 In our department, the fixation time for the evaluation of the PCNA labeling index in cancer lesions does not exceed 48 hours,28 and the results of the immunohistochemical staining of TS in this study were clear.

A granular cytoplasmic staining pattern was observed in the gastric carcinoma lesions in this study. The staining pattern was identical to that described in a previous report in which another polyclonal anti-TS antibody was used.17 Concerning the clinicopathologic variables, a significant correlation was found in the current study between the expression of TS and three clinicopathologic variables, namely, depth of invasion, peritoneal metastasis, and staging. The univariate analysis revealed that the expression of TS in cancer tissues was significantly correlated with the survival of gastric carcinoma patients; the patients with high grade TS staining had significantly worse prognoses than those with low grade staining. These results suggested that the immunohistochemical detection of the intratumoral expression of TS could be used as one of the indicators of tumor progression and prognosis in gastric carcinoma. Although these results were almost the same as previously reported results in which TS was investigated by other methods,7,17 the important finding of the current study is that this immunohistochemical evaluation is easy to perform and can be performed on paraffin embedded specimens.

In this study, there was a tendency for postoperative chemotherapy to be indicated for patients with advanced stages of cancer. The correlation of TS ex-
presssion with the response to chemotherapy was also investigated. As a result, oral 5-FU-based chemotherapy improved the prognoses of patients with low grade TS staining who received chemotherapy because of their advanced disease stage; the improvement brought them up to the level of prognosis for patients for whom chemotherapy was considered dispensable. Nevertheless, the patients with high grade TS staining had worse prognoses even when adjuvant chemotherapy was given.

The multivariate analysis revealed the significance of the immunohistochemical evaluation of TS as one of the independent prognostic factors; these factors also included the clinicopathologic variables of peritoneal dissemination, lymphatic invasion, and liver metastasis. The significance of lymphatic invasion might be explained by the finding that lymphatic invasion revealed the invasiveness of cancer cells regardless of whether or not lymph node metastases were present. On the other hand, lymph node metastasis and/or staging were thought not to affect survival as a result of aggressive curative lymph node dissection.

In conclusion, we propose that the immunohistochemical evaluation of TS using the newly developed anti-TS polyclonal antibody could be clinically useful in estimating the precise prognoses or survival of gastric carcinoma patients. Furthermore, the immunohistochemical evaluation of TS might be therapeutically useful as an indicator of response to chemotherapy with fluoropyrimidines. Further study of the effect of more aggressive adjuvant chemotherapy for patients with high grade TS staining is necessary.

REFERENCES