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A High Thymidylate Synthase Expression is Related to Better Outcome for Advanced Gastric Cancer Patients Treated with 5-FU Chemotherapy after Curative Resection

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Background : The expressions of thymidylate synthase (TS), E2F-1, pRb, and p53 are correlated with DNA synthesis. The significance of their expressions is still controversial for predicting the outcome of 5-fluorouracil (5-FU) therapy in the patients with advanced gastric carcinoma. Furthermore, their prognostic value in the metastatic lesions of gastric carcinoma has not yet been confirmed. **Methods :** To ascertain their prognostic value, we immunohistochemically analyzed the expressions of TS, E2F-1, pRb, and p53 in the primary tumors and the related metastatic lymph nodes, and we then compared the survival between the high and low expression group of each protein. Ninety four patients with advanced gastric carcinoma who were treated by complete resection and adjuvant 5-FU chemotherapy were analyzed. **Results :** The TS expression in primary tumors was significantly correlated with that of E2F-1. The expression of these genes showed no significant difference between the primary tumors and the metastatic lymph nodes except for E2F-1, which was significantly higher in the lymph node metastasis than in the primary tumors. After complete resection and 5-FU-based adjuvant chemotherapy, patients with a high TS expression in the primary tumors showed a longer survival than those patients having primary tumors with a low TS expression (p=0.0392). **Conclusion :** A high TS expression in the primary tumors may be related to a better outcome for advanced gastric cancer patients who were treated with 5-FU chemotherapy after curative resection.

Key Words : Stomach neoplasm; Fluorouracil; Chemotherapy, adjuvant; Thymidylate synthase; E2F-1 protein

Gastric carcinoma is one of the most common cancers in Korea.[1] Some notable benefits have been obtained with using postoperative adjuvant chemoradiotherapy.[2] 5-fluorouracil (5-FU), a fluoropyrimidine analogue that acts upon thymidylate synthase (TS), is one of the most commonly used anticancer drugs for the treatment of gastric carcinoma.[3] TS is involved as the catalysis of deoxyuridine monophosphate (dUMP) methylation to deoxythymidine monophosphate (dTMP), which is a very important process for DNA synthesis in tumor tissues.[4] However, the significance of the intratumoral TS expression remains controversial.[5,6] A lot of the genetic changes observed during tumor progression are the result of multiple mutations that accumulate in different cells; they generate subclones with different characteristics.[7] This implies that the molecular characteristics of primary tumor cells are different from those of the metastatic ones. However, most of the previous studies on the TS expression were performed on primary tumors. Some investigations have indicated that the TS expression in metastatic tumors may be highly predictive of the systemic response to the 5-FU-based therapies used for treating colorectal carcinoma.[8-10] The TS expression of primary tumors has been reported to be a prognostic marker for gastric carcinoma,[11] but the significance of the TS expression in metastatic lesions has not yet been described. On the other hand, the TS expression is known to be related with the E2F-1, pRb and p53 expressions. E2F belongs to a family of transcription factors that play an important roles in cell cycle regulation. E2F-1, which normally exists as a heterodimeric complex with another protein, DP-1, stays inactive when it's bound to hypophosphorylated pRb. During the period between the G1 and S phases, hyperphosphorylated pRb is released from the E2F-1/DP-1 heterodimer, which then activates the transcription of the TS and dihydrofolate reductase (DHFR) genes, which are both involved in DNA synthesis.[12,13] Banerjee *et al.*[14] have reported that the over-expression of E2F-1 by genetic transduc-

tion leads to the up-regulation of TS and 5-FU resistance in fibrosarcoma. The close correlation between the E2F-1 and TS expressions in metastatic colon cancer has been described.[15] The previous studies described that the increased free E2F-1 levels that are induced by the loss of pRb can subsequently increase the levels of TS and DHFR, and also the resistance to antimetabolites.[12,13] p53 has an important role in DNA synthesis and it works as the 'guardian of the genome'.[16] It is normally expressed at very low levels, but the p53 expression is up-regulated in the case of DNA damage. In relation to TS, the induction of TS expression inhibits the antifolate-mediated activation of p53 and its downstream target genes. It's been suggested that the p53 expression levels are likely to play a significant role in predicting the response to chemotherapy.[17,18] However, the relationship of p53 and TS in primary and metastatic gastric carcinoma needs to be verified.

In this study, we analyzed the immunohistochemical expressions of TS, E2F-1, pRb and p53 in patients with advanced gastric carcinoma to see the followings ; 1) the relationship between the expressions of these four genes, 2) the difference of expression between primary tumors and lymph node metastases, and 3) the prognostic significance of the expressions of four genes for predicting the prognosis of patients after undergoing surgery and postoperative 5-FU chemotherapy.

MATERIALS AND METHODS

Patients

We selected 94 advanced gastric carcinoma patients who had undergone curative resection together with postoperative 5-FU-based chemotherapy from 1996 to 2000 and who were available for follow-up at Yonsei University, Wonju College of Medicine, Wonju, Korea. The postoperative adjuvant chemotherapy consisted of epirubicin at a dose of 60 mg/m^2 and intravenous mitomycin at a dose of 10 mg on day 1; this was followed by 5'DFUR (furtulone) at a dose of 1,200 mg/day for 4 weeks according to the intermittent schedule (5-days of treatment followed by a 2-day rest period). This treatment was repeated every 4 weeks and it was continued for 12 cycles.

After a review of the pathology reports and the clinical charts of the patients, we determined the stage of each patient according to the standards of the American Joint Committee on Cancer (AJCC).[19]

Tissue microarray (TMA)

The tumor areas were first identified on hematoxylin and eosin stained slides and they were subsequently marked on the corresponding paraffin blocks of the primary tumors and lymph node metastasis. Any areas with hemorrhage, necrosis, and tissue artifacts were excluded. The selected areas were sampled from the paraffin block using 5 mm-sized tip punch and these cores were re-embedded in a tissue microarray mold that could accommodate 20 cores per block (Quick-ray, Seoul, Korea).[20]

Using a microtome, the blocks were cut into 4 μm slices for immunohistochemical staining. Hematoxylin and eosin staining was also done on each tissue array block to confirm the presence of carcinoma in the tissue cores.

Immunohistochemistry

Thymidylate synthase and E2F-1

The immunohistochemical technique was used to detect TS (TS 106 antibody, NeoMarkers, Fremont, CA, USA) and E2F-1 (KH95 antibody, NeoMarkers), using the ChemMate EnVision kit (K5007, DAKO, Glostrup, Denmark). Sections 4 μm thick that were cut from the paraffin-embedded tissue array were deparaffinized with xylene and then they were gradually rehydrated in a graded series of alcohol solutions. For antigen retrieval, the tissue sections were boiled in Tris-EDTA buffer (pH 9.0) for 5 min 3 times at 100℃ with using a microwave oven, and then they were cooled for 20 min at room temperature. Endogenous peroxidase activity was blocked by soaking the sections in 3% hydrogen peroxidase for 5 min. After washing them in Tris-Buffered Saline (S3001, DAKO) for 10 min, the slides were incubated with the primary antibodies (1:50 dilution) overnight in a humid incubation box. After being washed in TBS buffer for 10 min again, the slides were incubated with dextran coupled with peroxidase and the secondary antibodies for 30 min in a humid incubation box. The slides were washed for 10 min again with TBS, and then incubated with substrate chromogen solution for 10 min. At last, the slides were washed with distilled water and then briefly counterstained with hematoxylin; they were then mounted.

p53 and pRb staining

The monoclonal antibodies for retinoblastoma gene product (pRb, 1F8, NeoMarkers) and p53 (Novo Castra, Newcastle, UK) were used at a 1:100 and 1:50 dilutions, respectively. We performed immunohistochemical staining using the Cap-Plus

Detection Kit (Zymed Laboratories, South San Francisco, USA). Tris-EDTA buffer (pH 8.0) for pRb and sodium citrate buffer (pH 6.0) for p53 were used for antigen retrieval. The staining procedure was similar to that of the ChemMate EnVision Detection kit.

Evaluation of the immunohistochemical stains

Thymidylate synthase

The grading of the immunohistochemical results were performed without any knowledge of the clinicopathological details of tumors. The TS expression was semi-quantified by using a visual grading system that was based on the intensity and the extent of the expression. The intensity was scaled from 1 to 3. The percentage of positive cells (extent) was scaled from 1 to 4 for every 25% increase; these two scales were then multiplied. The products of the two scales were sorted into four expression grades. Products 1 & 2 were categorized into expression grade 1, 3 & 4 into grade 2, 6 to 8 into grade 3 and 9 to 12 into grade 4. For the survival analysis, grades 1 & 2 were designated as low TS expressions and 3 & 4 as high TS expressions. The survival analysis was also performed according to the TS intensity and the percentage of positive cells (extent), respectively. The percentage of positive cells was also evaluated for the correlation analysis.

E2F-1, p53 and pRb

The tumor cells that stain brown in the nucleus were identified as positive, regardless of the intensity of the stain. The cells in the most well stained area were counted and the percentage of the positive cells was analyzed (Fig. 1). For the survival analysis, cases with less than the mean value of E2F1, pRb and p53 staining were designated as low expression and those with more than the mean value were designated as high expression.

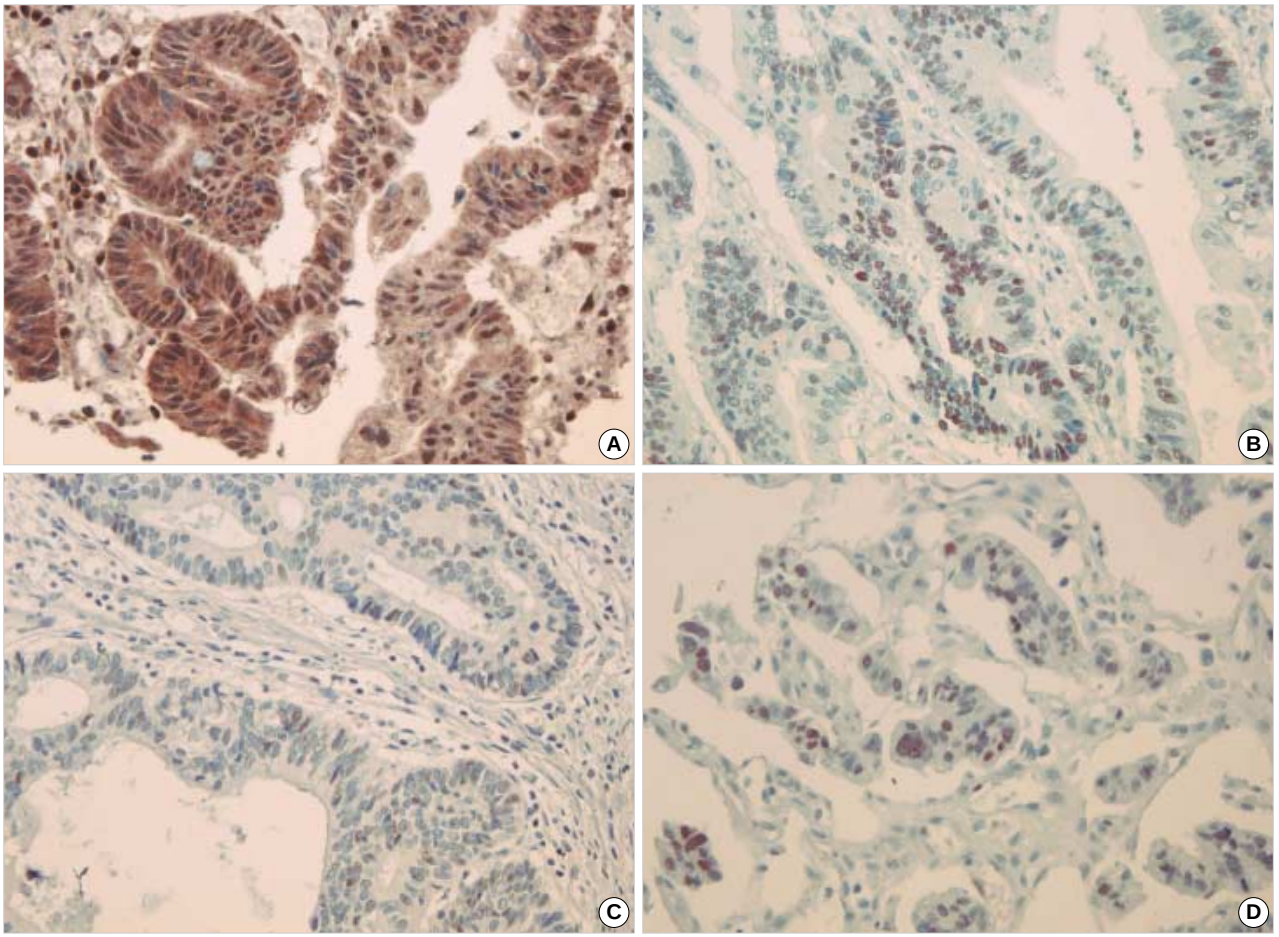

Fig. 1. Photomicrographs show the immunohistochemical stains; TS (A) expression was found in the cytoplasm and/or nucleus. p53 (B), E2F-1 (C), and pRb (D) expressions are found in the nucleus.

Statistical analysis

The continuous variables were used for Pearson correlation analysis. Wilcoxon signed rank test was used for making comparisons between the primary and metastatic lesions. Survival estimates for each group of TS, E2F-1, p53, and pRb expressions were calculated according to the method of Kaplan and Meier, and comparisons were made using the log-rank test. A p value <0.05 was considered statistically significant. The dBSTAT version 4.1 (DBSTAT Co., Chunchon, Gangwon, Korea) was used for statistical analysis.

RESULTS

Pathological findings

We analyzed 94 cases of advanced gastric carcinoma that were treated by complete resection and 5-FU based chemotherapy. The follow-up duration was more than 5 years. The primary sites of the tumor were 3 in the cardia, 29 in the body, 58 in the antrum, and 4 in the pylorus. The gross types according to the Borrmann classification were 9 polypoid, 32 ulcerofungating, 39 ulceroinfiltrative and 14 diffusely infiltrative. We histologically classified the tumor according to the WHO classification, and we found 69 cases of tubular adenocarcinoma (3 well differentiated, 23 moderately differentiated and 43 poorly differentiated), 12 signet ring cell carcinomas, 4 mucinous carcinomas, 8 mixed adenocarcinomas, and 1 adenosquamous carcinoma. The other pathological characteristics of tumors are summarized in Table 1.[21]

Table 1. Pathological characteristics of 94 cases examined

	Number of patients (%)
Histologic type (by Lauren[21])	
Intestinal	3 (3.2)
Mixed	24 (25.5)
Diffuse	62 (66.0)
Others	5 (5.3)
Depth of Invasion	
Mucosa & submucosa (T1)	4 (4.3)
Muscle & subserosa (T2)	33 (35.1)
Serosa (T3)	57 (60.6)
Lymph node metastasis	
N0 (N=0)	10 (10.6)
N1 (N=1-6)	42 (44.7)
N2 (N=7-15)	23 (24.5)
N3 (N>15)	19 (20.2)

Relationship between the expression of TS, E2F-1, p53 and pRb

The immunohistochemical expressions of TS, E2F-1, p53, and pRb are shown in Fig. 1. The expression of TS, E2F-1, pRb, and p53 were not correlated with the tumor site, histologic type, lymph node metastasis and stage. On the correlation analysis, the E2F-1 expression was significantly correlated with the TS and p53 expressions in the primary tumors. However, no significant correlation was found between the TS and pRb expressions (Table 2).

Difference of expression between the primary tumors and lymph node metastases

The immunohistochemical expression of the metastatic lesions in comparison with that of the primary tumor was significantly different only for E2F-1 (Fig. 3). The E2F-1 expression in lymph node metastasis (mean±SD, 9.68%±8.38) was significantly higher than that in the primary tumors (mean±SD, 5.45%±7.14) (Fig. 4, $p<0.0001$).

Prognostic significance

The results of the immunohistochemical stain according to stage are summarized in Table 3. The stage I cases were not included in this study. No significant difference was found in the expression of TS, E2F-1, p53, and pRb between each stage.

Table 2. Correlation analysis of immunohistochemical expression of TS, p53, E2F-1 and pRb

		TS	p53	E2F1	pRb
TS	r	1.000			
p53	r	-0.1469 (0.1577)	1.000		
E2F-1	r	0.2044 (0.0481)*	0.2482 (0.0159)*	1.000	
pRb	r	-0.0519 (0.6192)	0.1250 (0.2299)	-0.0772 (0.4595)	1.000

r, correlation coefficient, calculated by Pearson correlation analysis.
( ), p value; *, p<0.05.

Table 3. Summary of immunohistochemical results according to the stage (Mean±SD) (p>0.05)

Stage	No. of cases (%)	TS	E2F-1	pRb	p53
II	27 (28.7)	59.15± 20.07	7.48± 9.29	17.89± 19.31	27.93± 31.40
III	45 (47.9)	48.40± 23.17	5.33± 6.36	19.22± 18.29	25.47± 31.19
IV	22 (23.4)	45.75± 23.11	4.75± 5.36	21.10± 18.50	23.75± 29.29

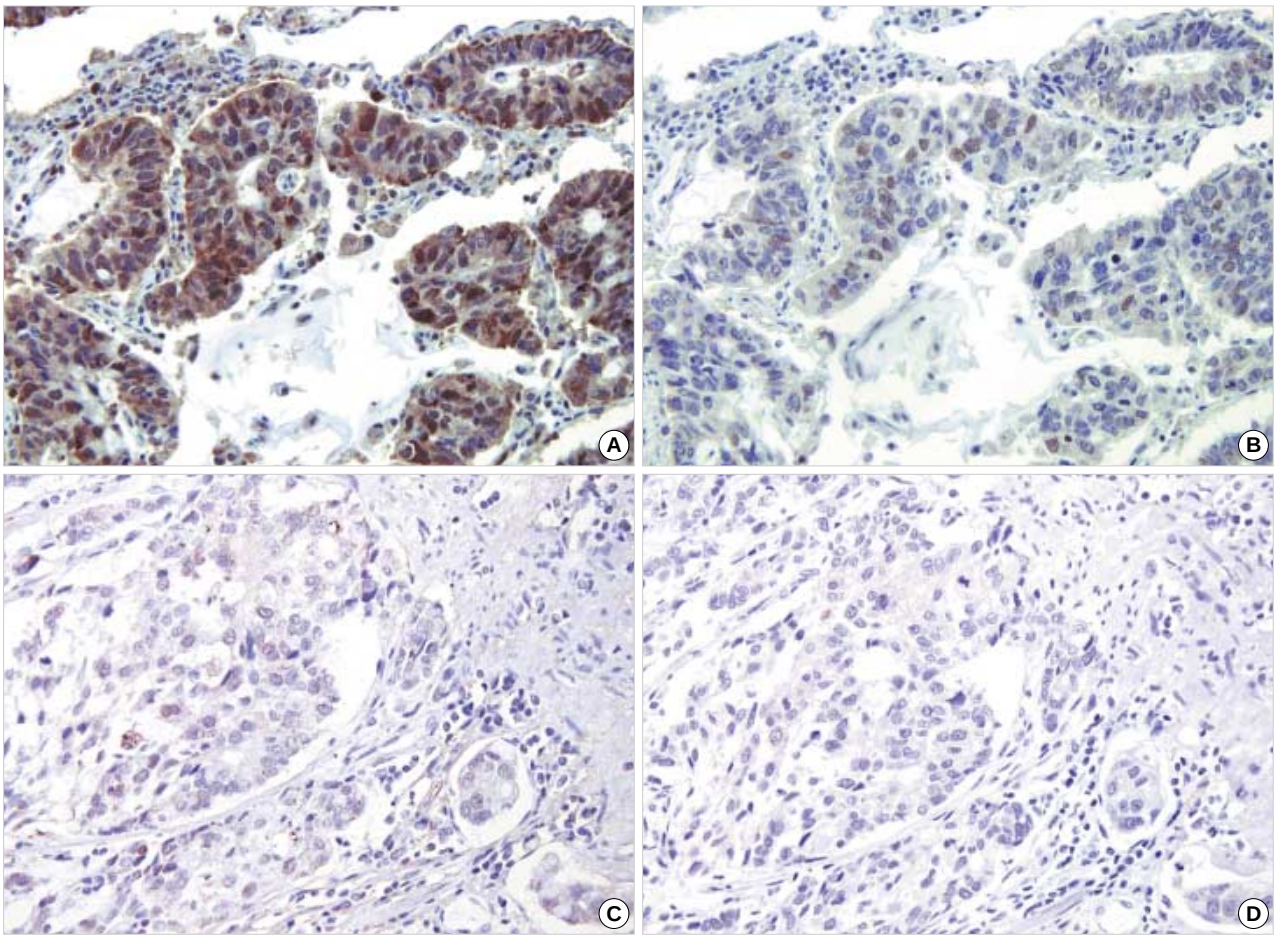

Fig. 2. Photomicrographs show that the TS expression is correlated with E2F-1 expression in primary tumor. The tumor with high TS expression (A) shows high E2F-1 expression (B). In contrast, the tumor with low TS expression (C) also shows low E2F-1 expression (D).

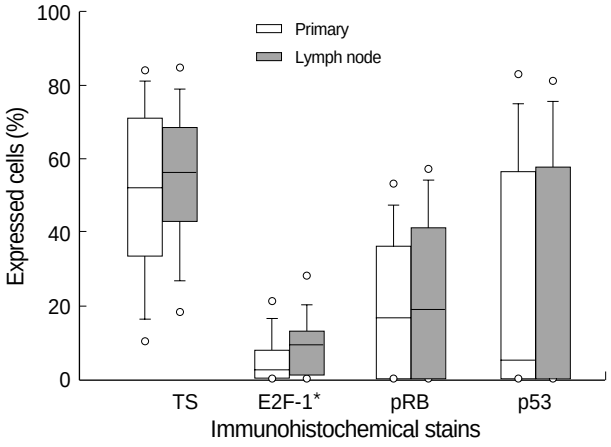

Fig. 3. Expression of TS, E2F-1, pRb and p53 in the primary tumors and lymph node metastases. The results are presented as a means ±SD (*p<0.0001). The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. Circles above and below the whiskers indicate the 95th and 5th percentiles.

On the survival analysis, there is no significant difference between the high and low TS-intensity (p=0.0713) and the high and low TS-extent in the primary tumor, respectively (p=0.1512) (Fig. 5A, B). However, there was a statistically significant difference between the TS high-expression and the low-expression group (p=0.0392). The survival rate was significantly decreased in the low TS expression group (Fig. 5C). For the survival analysis, those cases with less than 5.7% (mean value) of the tumor cells showing E2F-1 staining were designated as low expression and those cases with more than 5.7% were designated as high expression. For pRb and p53, the cases that had more than 25% (mean value) for the tumor cells were designated as high expression, and the cases that had less than 25% for tumor cells were designated as low expression. The expression of E2F-1 in the primary tumor as well as in the lymph node metastasis did not correlate with patient survival (Fig. 6, p=0.9378 and 0.9540, respectively). There was no significant difference for patient survival between the high and low expressions of p53 (p=0.8806)

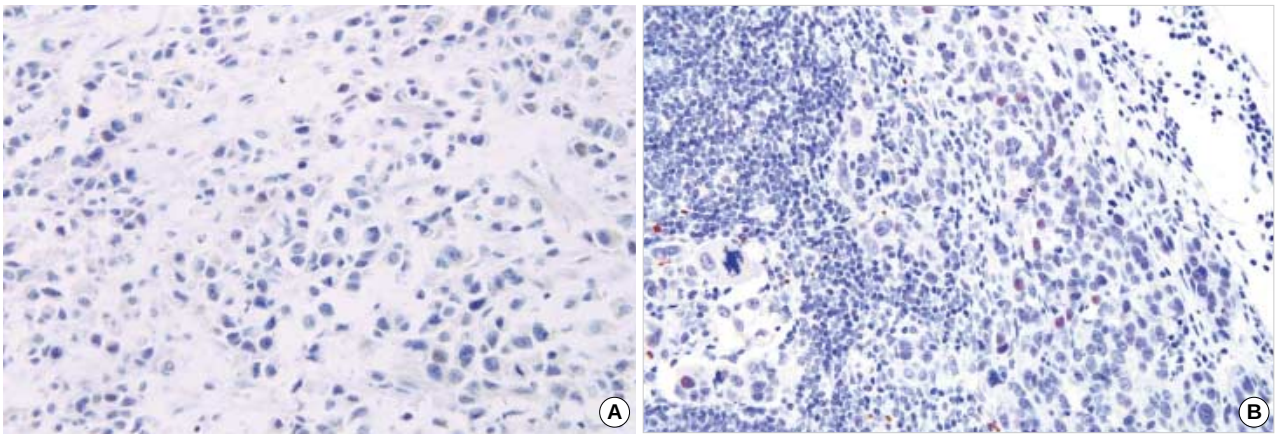

Fig. 4. The immunohistochemical expression of E2F-1 is significantly lower in primary tumor (A) than in metastasis (B).

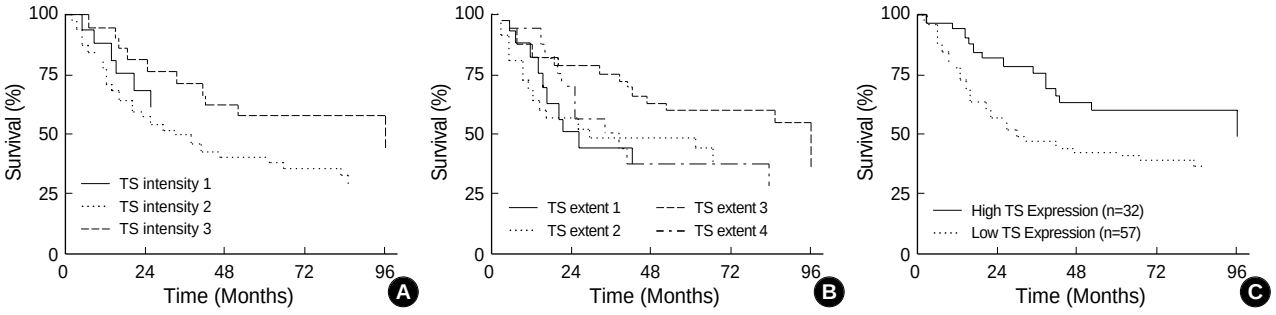

Fig. 5. Survival curves according to TS intensity (A) and extent (B). There is no significant difference between two groups (p=0.0713 for intensity & p=0.1512 for extent). However survival curves according to TS expression shows longer survival of patients with high TS expression than low TS expression (p=0.0392) (C).

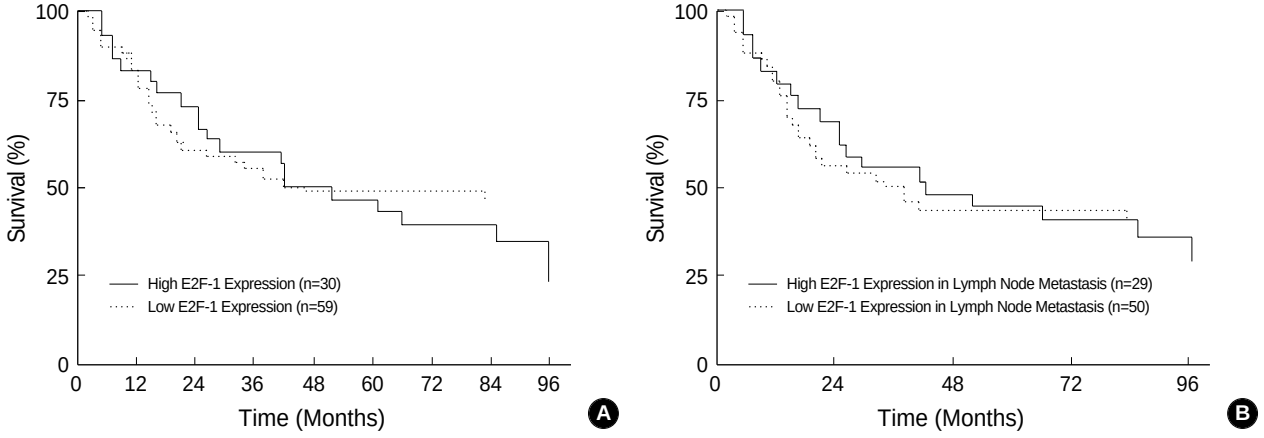

Fig. 6. Survival curves according to E2F-1 expression in primary tumor (A, p=0.9378) and lymph node metastasis (B, p=0.9540). High E2F-1 expression, more than 5.7%; low E2F-1 expression, less than 5%. There is no significant difference between two groups.

and pRb (p=0.2772) (Fig. 7).

DISCUSSION

To evaluate the relationship between the expressions of TS, E2F-1, pRb and p53 in gastric carcinoma, we immunohistochemically analyzed the primary tumors and metastatic lymph nodes from 94 patients with advanced gastric carcinoma who were treated by curative resection and adjuvant 5-FU chemotherapy. The E2F-1, pRb and p53 expressions were reported to have a relationship with the TS expression of tumors.[7,12-18,22] In the

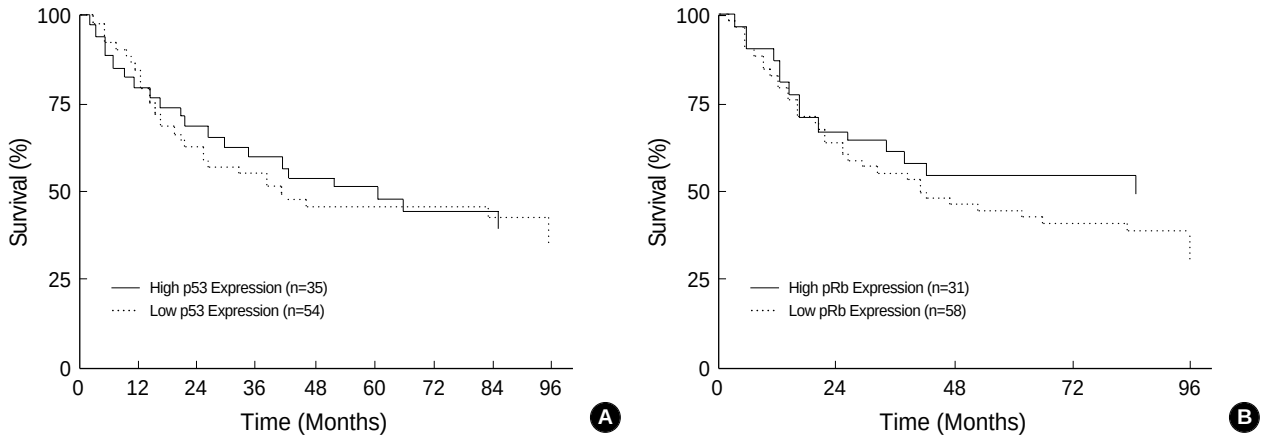

Fig. 7. Survival curves according to p53 expression. High p53 expression, more than 1+; low p53 expression, less than 1+ (p=0.8806) (A). Survival curves according to pRb expression. High pRb expression, more than 1+; low pRb expression, less than 1+ (p=0.2772) (B).

cell cycle, the functional interaction between pRb and E2F regulates the G1 to S phase transition. Cells moving into the S phase are accompanied by a concomitant increase in the levels of proteins that are required for DNA synthesis, such as DHFR, thymidine kinase (TK), TS, ribonucleotide reductase, and DNA Pol-α.[12,13] If tumors have acquired several mutations, especially in the apoptotic pathway, e.g., the p53 and p14 ARF genes, the increased E2F-1 will further stimulate cell growth.[23] The overexpression of E2F-1 via genetic transduction that leads to the up-regulation of TS and 5-FU resistance in fibrosarcoma has been reported.[14] Additional studies on the TS expression in metastatic colon cancer with using RT-PCR method indicate that there is a close correlation between E2F-1 and TS.[15] In our study, statistically significant correlation was seen only between TS and E2F-1 among all the proteins we analyzed. Broll *et al.*[24] have also reported that the p53 immunohistochemical expression had no prognostic importance for colon cancer in contrast to the TS expression. This may be related to the fact that the immunohistochemical expression of p53 is not always linked to p53 gene mutation. Evaluation of pRb hyperphosphorylation and the status of the E2F/pRb complex are needed to gain further understanding of the relationship of these genes.

TS has been described as a prognostic marker for many tumors.[8-11,25] However, the significance of the intratumoral TS expression is still controversial.[26] The role of adjuvant therapy is for eradicating circulating cancer cells.[27] After complete resection of tumor, some patients suffered local or systemic recurrence, which may be due to disseminated micrometastases that are present at the time of surgery. The remaining circulating tumor cells with their high TS expression might be more susceptible to drug-induced cell death via unknown mechanisms.[6] Many investigators have evaluated the TS expression in primary tumors as a prognostic marker for the response to therapy. However, several studies have described that the TS and E2F-1 expression in metastatic tumors might have a stronger prognostic correlation and they might be more predictive of response to 5-FU based therapies.[9,10,15] The expression of TS and the related molecules in metastatic lesions have not been studied in gastric cancer. Our results showed a significant difference for only the E2F-1 expression between the primary gastric carcinoma and its metastasis in lymph nodes. Many studies have demonstrated that clonal selection occurs during tumor growth. These genetic changes may allow tumor cells to invade or metastasize beyond the primary site.[16] The E2F-1 expression may be one of these characteristics that fosters metastases and subsequently regulates the TS expression and it then alters the tumor response to chemotherapy. The E2F-1 and TS expression in metastatic lymph nodes may predict the effect of 5-FU-based therapies. However, in our study, there was no difference of the TS expression between the primary site and the metastatic lymph nodes. Moreover, the E2F-1 expression in metastasis did not show any prognostic significance. Banerjee *et al.*[14] have reported that the lung metastasis from colon cancer had a 5-fold increase of the E2F-1 expression as compared with liver metastasis. The molecular characteristics of the tumor in distance metastasis maybe different from that of lymph node metastasis.

To evaluate the prognostic significance of the TS, E2F-1, p53 and pRb expressions in gastric cancer, we compared survival between the high and low expression groups for each protein. Formentini *et al*.[6] have described that the significance of high TS levels of primary tumor differed depending on the type of therapy. Johnstone *et al.*[28] have described that high TS levels were

associated with a poor prognosis for the patients who underwent complete primary tumor resection for rectal cancer without having received adjuvant treatment. However, adjuvant chemotherapy significantly improved the survival rate of patients with high TS levels, whereas it may not be effective for the patients with low TS levels. These results were demonstrated in patients suffering with gastric cancer by Tsujitani *et al*.[5] Our results were concordant with their results. In this study, we demonstrated that a high TS expression was related to a better outcome for the patients who received adjuvant 5-FU-based chemotherapy after complete resection for advanced gastric cancer. Patients with advanced gastric cancers who underwent palliative chemotherapy and also the patients with stage I gastric cancers were not included in our study. A recent review paper concluded that a high TS expression might benefit patients who receive adjuvant chemotherapy after complete tumor resection. On the other hand, a high TS expression may be a poor prognostic indicator for the patients with palliative chemotherapy or surgery alone.[6,25]

In regard to measurement of TS, immunohistochemical staining has several advantages. We can routinely use this method on paraffin-embedded tissue and we can perform retrospective studies. Any possible contamination by normal tissue that would result in a low TS expression could be excluded. The morphological correlation and evaluation of intratumoral heterogeneity are also possible. Several studies on the prognostic significance of the TS protein expression using immunohistochemical staining methods have been demonstrated controversial results; these were probably due to some limitations of the immunohistochemical method for the proper evaluation of the TS expression.[6,22,25,26,28,29] Even though the tissue microarray method was used in this study, intra- and interobserver variation cannot be totally avoided. We also found intratumoral heterogeneity for the TS expression. In this study, the TS expression was categorized by a visual grading system that was based on the combination of intensity and the extent of the expression. When we analyzed the TS intensity or extent, respectively, there was no significant prognostic significance. Therefore, we suggest using a visual grading system for the TS analysis to compensate for the limitations of immunohistochemical staining of TS analysis. The analysis of TS gene polymorphisms for evaluating the response rates of 5-FU chemotherapy has been recently described.[30] Additional studies to find more reliable methods to measure the TS expression needed to confirm its exact role in tumor prognosis.

In this study, the expression of TS significantly correlated with the E2F-1 expression in primary gastric carcinoma. After complete resection and 5-FU-based adjuvant chemotherapy, the patients having primary tumors with a high TS expression showed a significantly better prognosis. Therefore, the evaluating the TS expression in primary tumor is useful to predict the outcome of patients treated with 5-FU-based chemotherapy after curative resection for advanced gastric cancer.

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