Original article

Expression of thymidylate synthase and thymidine phosphorylase in recurrence and survival rates of advanced gastric cancer

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Abstract:

Background. The immunohistochemical expression of thymidylate synthase (TS) and thymidine phosphorylase (TP) was examined in a comparative study of the recurrence rates and prognoses of patients with advanced gastric cancer at the same stage.

Methods. We examined the resected specimens of 67 patients with stage IIIB gastric cancer (pT3, pN2, M0) under 70 years of age who had undergone curative gastrectomy followed by adjuvant chemotherapy with 5-fluoropyrimidines. Paraffin sections of the resected specimens were stained with human anti-TS polyclonal and anti-TP monoclonal antibodies by the avidin-biotin-peroxidase complex (ABC) method.

Results. The overall expression of TS and TP was 45.4% and 43.4%, respectively. The postoperative survival curve for the TS-positive group was significantly depressed compared with that for the TS-negative group ($P = 0.0480$). The survival curves for TP-positive and TP-negative groups did not show any difference. In regard to the combination of TS and TP expression, the best survival curve was obtained for the TS(-)/TP(+) group, followed by those for the TS(-)/TP(-), TS(+)/TP(-), and TS(+)/TP(+) groups in descending order. With regard to the recurrence site, there was no significant difference in peritoneal recurrence in relation to positivity for TS or TP. Lymph node recurrence, however, was significantly higher in the TS-positive and TP-positive groups, with P -values being 0.0466 and 0.0058, respectively, versus the corresponding negative groups. The incidence of hepatic recurrence was higher in the TP-positive group than in the TP-negative group ($P = 0.0910$). As for the total doses of 5-fluoropyrimidines given, more favorable survival curves were obtained for the high dose of negative TS and TP groups, but no significant differences were observed in their positivities.

Conclusion. The expressions of TS and TP showed different characteristics in overall survival and recurrence rate or site.

They should be used for predicting prognosis in comprehension on their properties.

Key words: thymidylate synthase, thymidine phosphorylase, gastric cancer, recurrence, survival rate

Introduction

Thymidylate synthase (TS) is involved in DNA synthesis, thymidine phosphorylase (TP) facilitates the angiogenesis of microvessels via the chemotaxis of vascular endothelial cells [1,2]. Thus, these two enzymes are being closely studied as possible factors involved in the proliferation and progression of tumors. In addition, TS is known to form a ternary complex with reduced folic acid and 5-fluorodeoxyuridine monophosphate (F-DUMP); a metabolite of 5-fluorouracil (5-FU), and to inhibit DNA synthesis [3]. TP has also been shown to activate such 5-FU prodrugs as tegafur and doxifluridine (5'-DFUR) in 5-FU-related chemotherapy for solid tumors [4].

In the present study, we investigated patients with stage IIIB advanced gastric cancer [5] who underwent curative resection and then received postoperative adjuvant chemotherapy with 5-FU or tegafur 1-(tetrahydro-2-furanyl)-5-fluorouracil [FT]) or UFT (a combination of FT and uracil at a 1:4 molar concentration). Correlations between the immunohistochemical characteristics of TS and TP, and the modes of recurrence and prognoses in patients with this limited stage of gastric cancer were then assessed.

Subjects and methods

Subjects

The records of all patients in our Division of Abdominal Surgery, treated from 1976 to 1995 with curative gastrec-

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tomy and diagnosed histologically with stage III B (pT3, pN2, M0) gastric cancer [5] were extracted from the files. Patients over the age of 70 were excluded, to reduce the effect of death of other diseases on the assessment of survival. Patients had to have been followed-up for a minimum of 3 years. As a result, 67 patients met these criteria. For the better comparative study of survival rates and recurrences, one patient who did not receive postoperative chemotherapy was excluded. Tumors were classified as either well differentiated (pap, tub) or poorly differentiated (por, sig, muc) type [6].

Postoperative adjuvant chemotherapy

Sixty-six patients received 5-fluoropyrimidines as postoperative adjuvant chemotherapy according to the protocol at the time of surgery (5-FU or FT or UFT, in general for more than 6 months, and mitomycin C [MMC], 10mg iv $\times$ 3). The daily dose of 5-FU-related drug (5-FU, 250mg i.v.; FT, 600mg p.o.; UFT (as FT), 300mg p.o.) was counted as a single unit, and the sum of units, referred to as the F-value, was used as an index of the total dose given.

Immunohistochemical staining

Tumor specimens were fixed with formalin and embedded in paraffin. Sections 3- μ m-thick were deparaffinized. According to the avidin-biotin-peroxidase complex (ABC) method [7], endogenous peroxidase was blocked with 0.3% H₂O₂, and then the sections were stained with 1000 $\times$ diluted human TS polyclonal antibody [8] or with TP monoclonal antibody [9,10]. The end products were visualized using diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

Assessment of immunohistochemical staining

Carcinoma cells stained with TS antibody were judged as positive when their cytoplasm showed positive signals compatible with those of stromal inflammatory cells used as an internal control. In the same way, when carcinoma cells were judged as TP-positive, their cytoplasmic positive signals were compatible with those of inflammatory cells, mainly lymphocytes (internal control). With TP staining, however, positive signals were sometimes observed in the nuclei of carcinoma cells as well as in the cytoplasm. Cells with the positive cytoplasmic signal were judged as TP-positive, but when only nuclear staining was observed, the cells were judged as TP-negative. The assessment of both histology and staining was performed by two of the authors (Y.K. and Y.S.) independently and the results, usually in good agreement, were discussed and sometimes corrected.

Statistical analyses

The distribution of each clinical variable was tested by a *t*-test or a χ^2 -test. Recurrence rates were analyzed by a χ^2 -test. Survival curves were drawn by the Kaplan-Meier method and tested by generalized Wilcoxon and logrank tests. Effects of variables on survival rates were analyzed using a Cox proportional hazards regression model. In all tests, a *P* value less than 0.05 was defined as significantly different.

Tables 1 and 2 show the backgrounds of patients with TS and TP positivities, respectively.

Results

Survival rates and curves

Figure 1 shows the overall survival curves in terms of TS and TP immunoreactivities and four possible combinations of the two. The survival curve for the TS(+) group was significantly lower than that of the TS(−) group (*P* = 0.0480). Although there was no significant difference between the survival curves for the TP(+) and TP(−) groups, a slightly depressed curve was observed for the TP(+) group. Of the four possible combinations of TS and TP expression [TS(+)/TP(+), TS(+)/TP(−), TS(−)/TP(+), and TS(−)/TP(−)], the survival curve for the TS(−)/TP(+) group was found to be the most favorable, while that of the TS(+)/TP(+) groups was the least favorable. The difference between these two groups was significant (*P* = 0.0352). The survival curve for the TS(−)/TP(−) group lay at the midway point of the two above-mentioned groups, and its *P* value against the TS(+)/TP(+) group was 0.0903. Thus, the findings of the present study suggest that the survival rates of patients with advanced gastric cancer treated with 5-FU-related chemotherapy were influenced by TS.

Correlation of variables with survival rates

The results of multivariate analysis using the Cox proportional hazards regression model are shown in Table 3. The clinicopathological variables in the present study (age, sex, lymphatic or venous invasion, pattern of tumor infiltration into the surrounding tissue (INF), histologic type, and F-value) did not affect the prognosis. Only TS positivity showed an independent contribution to survival (*P* = 0.0310) with a hazard ratio of 2.139, whereas no such relationship was shown for TP positivity.

Recurrence site in terms of TS and TP expression

Table 4 shows the location of recurrent tumors discovered initially during the patient's postoperative

Table 1. Clinicopathologic variables and immunohistochemical positivity for thymidylate synthase (TS)

Variables	TS-negative (n = 36)		TS-positive (n = 31)		P value
Age (years) mean $\pm$ SD	52.1 $\pm$ 11.9		57.6 $\pm$ 10.0		<0.05
Sex (M/F)	23/13		20/11		NS
Lymphatic invasion					
1	4	11.1 (%)	5	16.1 (%)	NS
2–3	32	88.9	26	83.9	
Venous invasion					
0–1	23	63.9	13	41.9	NS
2–3	13	36.1	18	58.1	
INF [6]					
β	9	25.0	9	29.0	NS
γ	27	75.0	22	71.0	
Histologic type [6]					
pap/tub	7	19.4	13	41.9	NS
por/sig/muc	29	80.6	18	58.1	
Depth of invasion					
pT3	36	100.0	31	100.0	—
Lymph node metastasis					
pN2	36	100.0	31	100.0	—
Stage					
IIIB	36	100.0	31	100.0	—
	TS-negative (n = 36)		TS-positive (n = 30)		P value
5-FU/Tegafur/UFT	36	100.0	30	100.0	—
F-value ^a					
<180	20	55.6	16	53.3	NS
≥ 180	16	44.4	14	46.7	
MMC	31	86.1	28	93.3	NS
ADM-CDDP-MTX ^b	16	44.4	20	66.7	NS

MMC, Mitomycin C; ADM, adriamycin/doxorubicin; CDDP, cis-platinum; MTX, methotrexate/amethopterin; INF, pattern of tumor infiltration into the surrounding tissue

^aDefinition of F value is described in the subjects and methods section

^bAdministered on recurrence

course and the rate of recurrence. The mortality rate, as also shown in Table 4, was significantly higher in the TS(+) group than in the TS(−) group ($P = 0.0148$), in clear contrast with the lack of difference between the TP(−) and TP(+) groups. Although there was no significant difference in peritoneal recurrence in relation to either TS or TP positivity, the rate was high in the TP(−) and low in the TP(+) group, while it was low in the TS(−) and high in the TS(+) group, showing an inverse relation between TS and TP. The rate of lymph node recurrence was significantly higher in the TS(+) group than in the TS(−) group ($P = 0.0466$) and it was much higher in the TP(+) group than in the TP(−) group ($P = 0.0058$). The rate of hepatic recurrence was higher in the TP(+) group than in the TP(−) group ($P = 0.0910$). When the results for TS were combined with those for TP, the difference between the TS(+)/TP(+) and TS(−)/TP(−) groups was sometimes significant, as seen for lymph node recurrence ($P = 0.0053$).

Recurrence rates with respect to F-value

Table 5 compares the recurrence rates in relation to TS and TP expression and their corresponding F-values. The mortality rate because of recurrence in patients with a low F-value ($F < 180$) did not show any difference between TS(−) and TS(+) groups, whereas there was a significant difference between these groups in patients with a high F-value ($F \geq 180$) ($P = 0.0095$). The mortality because of recurrence was not influenced by F-values in patients showing TP positivity. In regard to recurrence rates at different sites in the TS(−) group, the rate of peritoneal recurrence was 35.0% in patients with a low F-value and 12.5% in patients with a high F-value ($P = 0.1213$), whereas the rates were almost equal in the TS(+) group. The rates of peritoneal recurrence were low in patients with a high F-value, irrespective of TP positivity, with no significant differences from rates in those with a low F-value. Slightly significant

Table 2. Clinicopathologic variables and immunohistochemical positivity for thymidine phosphorylase (TP)

Variables	TP-negative (<i>n</i> = 37)		TP-positive (<i>n</i> = 30)		<i>P</i> value
Age (years) mean ± SD	52.6 ± 11.9		57.1 ± 10.2		NS
Sex (M/F)	21/16		22/8		NS
Lymphatic invasion					NS
1	4	10.8 (%)	5	16.7 (%)	
2–3	33	89.2	25	83.3	
Venous invasion					NS
0–1	23	62.2	13	43.3	
2–3	14	37.8	17	56.7	
INF [6]					<0.05
β	5	13.5	13	43.3	
γ	32	86.5	17	56.7	
Histologic type [6]					<0.01
pap/tub	5	13.5	15	50.0	
por/sig/muc	32	86.5	15	50.0	
Depth of invasion					—
pT3	37	100.0	30	100.0	
Lymph node metastasis					—
pN2	37	100.0	30	100.0	
Stage					—
IIIB	37	100.0	30	100.0	
	TP-negative (<i>n</i> = 37)		TP-positive (<i>n</i> = 29)		<i>P</i> value
5-FU/Tegafur/UFT	37	100.0	29	100.0	—
F-value ^a					NS
<180	20	54.1	16	55.2	
≥180	17	45.9	13	44.8	
MMC	32	86.5	27	93.1	NS
ADM-CDDP-MTX ^b	21	56.8	15	51.7	NS

Abbreviations and ^a and ^b, As in Table 1**Table 3.** Analysis of prognostic factors

Clinicopathological variables		<i>P</i> value	Hazard ratio
Age	~59/~69	0.9069	1.041
Sex	M/F	0.4120	0.741
Lymphatic invasion	1 y ₁ /1 y ₂₋₃	0.0966	2.686
Venous invasion	V ₀₋₁ /V ₂₋₃	0.8831	0.951
INF	β/γ	0.7444	1.159
Histologic type	pap, tub/por, sig, muc	0.3299	0.664
F-value	<180/≥180	0.2791	0.694
TS	−/+	0.0310	2.139
TP	−/+	0.9144	0.961

differences were shown in lymph node recurrence ($P = 0.0867$) and in hepatic recurrence ($P = 0.0941$) between TP(−) and TP(+) groups in patients with a high F-value.

Discussion

Reports on the expression of TS in patients with all stages of gastric cancer have shown that prognoses

were poorer for groups with a high TS expression, with the effect of 5-FU also being poorer for these patients [11–15]. In the present study also, the survival curve for the TS(+) group was significantly lower than that for the TS(−) group, and multivariate analysis showed that TS expression was an independent factor affecting the survival rate. Lenz et al. [12] reported that although there were no differences in TS mRNA among patients with gastric cancer at stages 0 through III, the TS expression at stage IV was lower for patients with

Fig. 1a–c. Overall survival curves for patients positive for **a** thymidylate synthase (TS), **b** thymidine phosphorylase (TP), and **c** their combinations. **a** $P = 0.0480$ (Wilcoxon); $P = 0.0477$ (Logrank). **b** $P = 0.382$ (Wilcoxon); $P = 0.482$ (Logrank). **c** A vs D, $P = 0.0903$; B vs D, $P = 0.0352$

Table 4. Recurrence sites in relation to positivity for TS and TP

	TS(-) $n = 36$		TS(+) $n = 30$		$\chi^2(P)$	TP(-) $n = 37$		TP(+) $n = 29$		$\chi^2(P)$
	Numbers	(%)	Numbers	(%)		Numbers	(%)	Numbers	(%)	
Peritoneum	9	25.0	9	30.0	0.6497	13	35.1	5	17.2	0.1052
Lymph node	3	8.3	8	26.7	0.0466	2	5.4	9	31.0	0.0058
Liver	2	5.6	3	10.0	0.4969	1	2.7	4	13.8	0.0910
Local	1	2.8	2	6.7	0.4501	2	5.4	1	3.4	0.7048
Distant	2	5.6	1	3.3	0.6661	2	5.4	1	3.4	0.7048
Death because of recurrence	17	47.2	23	76.7	0.0148	20	54.1	20	69.0	0.2185
Unrelated death	2	5.6	2	6.7	0.8506	3	8.1	1	3.4	0.2185

operable cancer than it was for those with inoperable cancer. In our previous study of stage IV gastric cancer [16], TS immunoreactivity was detectable in only 2 of 12 patients (16.7%) who had undergone non-curative gastrectomy and survived for more than 5 years. However, in the present study, we found that a TS-positive signal was detectable in 45.4% of patients with stage IIIB gastric cancer, and, in a different study, in 54.3% of

116 patients in an early stage of gastric cancer with submucosal invasion [17]. These findings suggest that TS expression could correlate, not with the stage of gastric cancer but with the characteristics of tumor cells. Although arguments for and against a relationship between TS expression and tumor histology have been reported [13,18], the present study showed a negative finding on this issue.

Table 5. Recurrence in relation to positivity for TS and TP and F-value

	(F < 180)			(F ≥ 180)			(F < 180)			(F ≥ 180)		
	TS(−) %	TS(+) %	χ^2 <i>P</i>	TS(−) %	TS(+) %	χ^2 <i>P</i>	TP(−) %	TP(+) %	χ^2 <i>P</i>	TP(−) %	TP(+) %	χ^2 <i>P</i>
Peritoneum	35.0	31.3	0.8125	12.5	28.6	0.2723	40.0	25.0	0.3428	29.4	7.7	0.1405
Lymph node	5.0	18.8	0.1924	12.5	35.7	0.1337	0.0	25.0	0.0177	11.8	38.5	0.0867
Liver	5.0	12.5	0.4185	6.3	7.1	0.9221	5.0	12.5	0.4185	0.0	15.4	0.0941
Local	5.0	6.3	0.8708	0.0	7.1	0.2769	5.0	6.3	0.8708	5.9	0.0	0.3738
Distant	10.0	6.3	0.6858	0.0	0.0	—	10.0	6.3	0.6858	0.0	0.0	—
Death because of recurrence	60.0	75.0	0.3428	31.3	78.6	0.0095	60.0	75.0	0.3428	47.1	61.5	0.4308
Unrelated death	5.0	12.5	0.4185	6.3	0.0	0.3414	15.0	0.0	0.1056	0.0	7.7	0.2448

The number of patients with recurrences in the different sites was generally high in the TS(+) group, with the rate of peritoneal recurrence being the highest. However, the rate of peritoneal recurrence in the TS(+) group, was close to that in the TS(−) group. In contrast, there was a significant difference in the rate of lymph node recurrence between the TS(+) and TS(−) groups ($P = 0.0466$). This suggests that lymph node recurrence, possibly due to an occult metastasis left at the time of surgery, could be more resistant to the 5-FU-related chemotherapy in the TS(+) group. Johnston et al. [11] reported a patient in whom a primary gastric tumor had a low level of TS expression and responded to 5-FU-based chemotherapy, whereas a lymph node metastasis with markedly high TS expression progressed despite chemotherapy. In other studies, metastatic lymph nodes had higher TS expression than that in the primary lesion in 5 of 15 patients with lung cancer [19], and metastatic lymph nodes had the highest DNA synthesis among primary and other metastatic lesions of colorectal cancer [20]. These heterogeneities in the nodal metastases could also play a role in the failure of the chemotherapy and in survival. Thus, the significant difference of overall survival curves in the present study is considered to be mainly due to the high recurrence rate in lymph nodes in the TS(+) group. As for the F-value, there was no difference in recurrence rates between patients with low and high F-values at any sites in either the TS(−) or the TS(+) group, with less possibility of chemotherapeutic effect on the peritoneal recurrence in the TS(−) group with a high F-value ($P = 0.1213$ vs low F-value). Unresolved problems, such as the dose-relationship between 5-FU and TS-intensity and the different efficacy rates at the recurrent sites could be elucidated by further clinical research.

Comparative studies on TP expression and survival curves in patients with gastric cancer have been undertaken, with some studies finding a significant correlation between TP expression and survival curves [10], while others found no significant correlation [21]. The present

study also found no significant correlation between TP expression and survival. TP expression has been shown to correlate with the proliferation of microvessels [10,21,22] and with hepatic metastasis [22]. Takebayashi et al. [10] reported that the incidence of lymph node metastasis was significantly higher in a TP(+) group than in a TP(−) group with gastric cancer, including those with stage III gastric cancer, while the prognoses were poor for all patients. In the present study, we analyzed recurrence rates together with F-values with respect to TP positivity. We found that, in contrast with results for TS, the incidence of peritoneal recurrence was lower in the TP(+) group than in the TP(−) group, while the rate of lymph node recurrence was significantly higher in the TP(+) group than in the TP(−) group ($P = 0.0058$). The incidence of hepatic recurrence was also higher in the TP(+) group, but the difference was not significant ($P = 0.0910$). These findings on recurrence were in general accord with results already reported [10,22]. In terms of F-value, similar tendencies were also observed in relation to each recurrence mode. Therefore, the higher recurrence rates in lymph node and liver, and the lower recurrence rate in peritoneum in the TP(+) group could be predicted even when patients receive the 5-FU-related chemotherapy, mainly per os, as performed in the present study. However, it would be still difficult to determine from the present results whether or not the low peritoneal recurrence rate in the TP(+) group was definitely due to a chemotherapy effect. Further studies are necessary, as mentioned above in the discussion of TS.

Many studies have reported that TP expression did not correlate with histological differentiation of gastric cancer when the cancer was classified as well- and poorly differentiated adenocarcinoma [10,21]. In the present study, a significant difference was observed in positive TP expression between well differentiated (75.0%) and poorly differentiated (31.9%) adenocarcinomas. This difference, however, did not affect the overall survival

rate when analyzed with the Cox proportional hazards model. Takahashi et al. [23] classified gastric cancer histologically into intestinal and diffuse types, and found that TP expression was higher for the infiltrating cells of the intestinal-type, and was correlated with microvessel neovascularity. The above findings suggest that classifying cells with this histological criterion may prove beneficial when cellular reactions of enzyme expression are investigated.

Furukawa et al. [24] investigated the effect of chemotherapeutic agents on gastric cancer using a histoculture drug response assay, and reported a significant difference in survival curves after postoperative chemotherapy between patients who were sensitive to 5-FU and mitomycin C and those who were not. Tumor sensitivity to 5-FU shows different overall results in terms of activities of related enzymes such as TS, TP, and dihydropyrimidine dehydrogenase (DPD). Although the present study was not identical with studies of enzyme activity, it is regarded as a retrospective evaluation of tumor sensitivity to 5-FU by means of TS and/or TP expression. When TS and TP expressions were combined, the results were sometimes much amplified, as shown by the survival curve for the TS(+)/TP(+) group being more depressed than that for either group alone. Beck et al. [25] and Etienne et al. [26] reported that in cultured cells from head-and-neck, breast, and colorectal cancers, those with low DPD and TS expression were experimentally and the patients were clinically more sensitive to 5FU. Recently, Danenberg et al. [27] reported that, of colorectal cancers treated with 5-FU, those with low TS, DPD, and TP levels showed the best survival curves among cancers expressing one or two of the three enzymes. Thus, TS, TP, and DPD have a cooperative influence on the sensitivity of cells to chemotherapeutic agents. By further study of 5-FU-related enzymes and their combined expression, the prognosis of a given patient could be more predictable and appropriate chemotherapy planned.

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