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Thymidylate synthase predicts for clinical outcome in invasive breast cancer

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Summary. Thymidylate synthase (TS) is a major target of 5-fluorouracil (5-FU) and dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme in the degradation of 5-FU. Whether TS or DPD could be used as valuable parameters for 5-FU sensitivity in clinical patients are largely unknown. We analyzed TS and DPD expression in breast carcinomas to evaluate the clinicopathological significance of these enzymes in patients with invasive breast cancer receiving 5-FU-based chemotherapy. A total of 197 patients with invasive ductal carcinoma were included in our study. Both the TS and DPD expression were analyzed using immunohistochemical method for all the surgical samples. Sixty-three out of 197 (31.97%) patients are positive for TS expression, and 77 out of 197 (39.09%) patients are positive for DPD expression. TS expression was not correlated with DPD expression. Patients with TS-positivity had aggressive phenotype including large tumor size, low differentiation and nodal metastasis. DPD expression is not related with phenotype or prognosis. Multivariate analysis demonstrated that TS expression was an independent prognostic factor for both disease-free and overall survival. The current study demonstrated that TS but not DPD expression was associated with both progression and prognosis in breast cancer receiving 5-FU-based chemotherapy. TS expression in the primary tumor might be useful as a predictive parameter for the efficacy of 5-FU-based chemotherapy for breast cancer.

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Introduction

Although adjuvant chemotherapy improves survival of radically resected breast cancer, approximately 50% of all patients will eventually relapse (Harris et al., 1992). Resistance to anticancer agents is thought to be responsible for chemotherapy failure in breast cancer. The antimetabolite, 5-flurouracil (5-FU), has been widely used in the treatment of breast cancer, either singly or in combination with other cytostatics. It would be highly desirable to identify most patients who are likely to benefit from adjuvant 5-FU treatment before the initiation of such treatment. Recently, increased interest has been focused on identifying biochemical response determinants of this drug (Yang et al., 1999, 2000, 2002; Sohn et al., 2004). If such determinants could be measured in tumors before treatment, patients who are judged unlikely to respond to 5-FU would then have the option to be treated, instead, with another agent to which they might respond, whereas those patients with favorable 5-FU response indices could anticipate a higher than average probability of response.

Thymidylate Synthase (TS) and dihydropyrimidine dehydrogenase (DPD) may be candidate response determinants of 5-FU. 5-FU irreversibly blocks TS after conversion to its active metabolite 5-fluorodl-UMP (Danenberg 1977; Radparvar et al., 1988) TS is a dimeric cytosolic enzyme that catalyzes the reductive methylation of deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP) (Radparvar et al., 1988). This reaction, where a methyl group is transferred from the donor cofactor 5,10-methylene-tetrahydrofolate to the position 5 of the

pyrimidine ring, provides the only de novo source of dTMP and hence deoxythymidine-5'-triphosphate (dTTP), a nucleotide necessary for DNA synthesis. TS is therefore a rate-limiting enzyme in the DNA synthetic pathway and has represented an attractive target for drug inhibition. DPD is the initial and rate-limiting enzyme of 5-FU catabolism, 85% of an administered dose of 5-FU is degraded to inactive metabolites by DPD, with only 1-3% of the drug anabolized. While anabolism is essential for the antitumor activity of 5-FU, anabolism by indirectly controlling the availability of 5-FU for anabolism is a critical determinant of 5-FU cytotoxicity (Fischel et al., 1995; Mattison et al., 2002).

Although the significance of TS and DPD expression has been extensively analyzed in gastrointestinal (Mizutani et al., 2001; Etienne et al., 2002; Kornmann et al., 2003; Yoshinare et al., 2003), bladder (Mizutani et al., 2001), lung (Shintani et al., 2004) cancer, very few reports about the expression of TS and DPD genes in breast carcinomas are available in the literature. In this study, we analyzed intratumoral TS and DPD expression in a series of breast cancer specimens to correlate their expression with clinicopathological parameters and investigate if the expression levels of these genes could serve as prognostic factor in patients with breast cancer receiving 5-FU-based therapy.

Materials and methods

Patients and samples

Surgical specimens from 197 consecutive patients with primary breast cancer were included in this study. The patients underwent surgery in Affiliated Hospital of Shandong University between 1990 and 1993. All had histological evidence of invasive ductal carcinoma and none had a family history in first-degree relatives as judged by questioning at the time of admission for surgery. The patients had received mastectomy with axillary lymph node dissection. All of our patients received post-operative adjuvant therapy consisting of combination chemotherapy with six cycles of conventional adjuvant CMF (cyclophosphamide, methotrexate and fluorouracil) therapy.

Immunohistochemical studies

The history of all cases was reviewed and representative sections from the deepest areas of each tumor were selected for immunohistochemistry. For the immunohistochemical study, 4 µm thick sections on silane-coated slides were dewaxed with xylene and rehydrated through a graded alcohol series. Then, endogenous peroxidase activity was blocked in absolute methanol solution containing 0.3% hydrogen peroxide for 35 min and the slides were washed in 10 mM phosphate-buffered saline (PBS), PH 7.4. For antigen retrieval, they were immersed in 1mM citrate-phosphate buffer, and microwaved at 100°C for 15 min. After the buffer had cooled, 10% fetal serum was reacted with the slides for 15 min to eliminate non-specific immunostaining. The sections were then incubated with anti-TS (Taiho Pharmaceutical Co. Ltd., Saitama, Japan) and anti-DPD polyclonal antibodies (Taiho Pharmaceutical Co. Ltd., Saitama, Japan) overnight at 4°C in a humidified chamber. Biotinylated goat anti-rabbit IgG was applied as a secondary antibody for 20 min at room temperature, followed by streptavidin-biotinylated peroxidase complex for 20 min at room temperature. Peroxidase activity was visualized with a diaminobenzidine as the chromogen. Replacement of the primary antibody with PBS was used as a negative control. Immunoreactivity in the cytoplasm of cancer cells was observed to evaluate both TS and DPD. When more than 25% of the cancer cells were stained, the specimen was defined as positive.

Covariates

Adequate clinicopathological data and follow-up information were obtained for all the cases. Age at diagnosis was considered as the patient's age. The size of the primary tumor was considered to be the largest tumor diameter observed after surgical excision. Lymph node status was determined by counting the number of axillary lymph nodes with histological evidence of metastatic breast carcinoma. Histological typing and histological grading were done according to the WHO classification (WHO) and the Nottingham scheme (Elston and Ellis, 1991), respectively.

Statistical analysis

Descriptive statistics comparing TS, DPD expression with other biological or conventional markers were analyzed by standard chi-square tests, or, when appropriate, Fisher's exact test. Estimates of disease free survival (DFS) and overall survival (OS) were calculated by the Kaplan-Meier product-limit method and the differences assessed by the log rank test. Probabilities of DFS (OS) were calculated from the date of breast carcinoma diagnosis to either the date at which relapse (death) from breast carcinoma was clinically identified or the date of last contact. Multivariate survival analysis using Cox's proportional hazard regression model was carried out to assess the independent contribution of each variable to DFS and OS. All p values were two-tailed and the 0.05 level was considered statistically significant. A computer program package (StatView 5.0, Abacus Concepts, Berkeley, CA, USA) was used for all statistical testing and management of the database.

Results

Demographics and Clinical Data

The median age at diagnosis for the 197 subjects was 51 years (range, 29-76 Years). Seventy-eight percent of the patients were younger than 50 years (n=154), and

90.9% (n=179) of the patients had lymph node metastases at the time of surgery. Of the 197 invasive ductal carcinomas, 50 were evaluated as histological grade 1, 107 as grade 2, and 40 as grade 3. Median follow-up time for the 197 subjects was 142 months (range, 28-176 months). One hundred fifty-five subjects had relapsed by the time of last follow-up. Eighty-seven patients died of breast carcinoma.

Expression of TS and DPD

The representative immunostaining results for TS and DPD were shown in Figure 1. According to the criteria for TS and DPD immunohistochemical

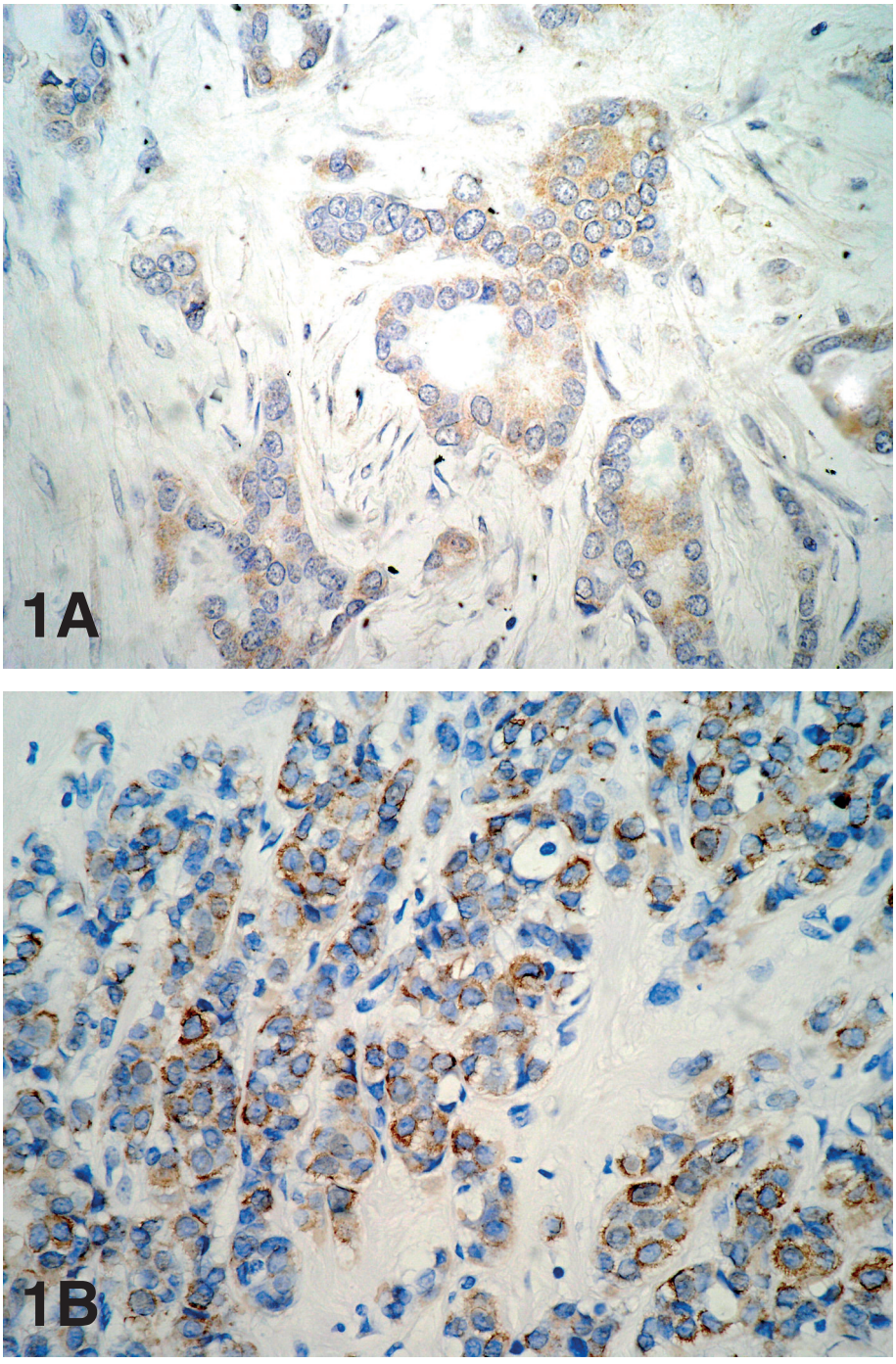

Fig. 1. Immunohistochemical staining of breast cancers using anti-TS **(A)** and anti-DPD **(B)** polyclonal antibodies. The tumor cells showed diffuse cytoplasm staining both anti-TS and anti-DPD. x 400

evaluation, 63 (31.93%) and 77 (39.09%) of 197 breast cancers were evaluated as positive for TS and DPD, respectively. The relationship between TS and DPD positivity is shown in Table 1. TS protein expression level was not correlated with DPD protein expression level in breast cancer (p=0.6416).

Associations of TS, DPD expression with conventional prognostic factors

We compared the expression levels of TS, DPD protein expression with the clinicopathological profiles of the 197 patients with sporadic breast cancer. The profile included age, primary tumor size, nodal involvement, histological grading, and biological markers including ER and PR. As shown in table 2, positive TS immunoreactivity was associated with large tumor size (p<0.0001), high histological grade (p<0.0001), nodal metastasis (p=0.009). However, no significant association between the DPD expression level and the conventional prognostic factors was found.

Table 1. Correlation between TS and DPD Expression in Breast Cancer.

	DPD		
	Negative	Positive	p
TS			0.6416
Negative	80	54	
Positive	40	23	

Table 2. Association of TS,DPD Expression with Features of Breast Cancer.

FEATURES	TS			DPD		
	Negative	Positive	p	Negative	Positive	p
Age (years)			0.8546			0.9457
<50	104	50		94	60	
≥ 50	30	13		26	17	
Tumor size (cm)			<0.0001			0.8737
≤ 2	56	2		36	22	
>2	78	61		84	55	
Nodal status			0.009			0.2038
Negative	18	0		8	10	
Positive	116	63		112	67	
Histological grade			< 0.0001			0.2081
I&II	121	36		92	65	
III	13	27		28	12	
ER			0.9529			0.2699
Negative	108	51		100	59	
Positive	26	12		20	18	
PR			0.0702			0.9415
Negative	86	49		82	53	
Positive	48	14		38	24	

TS and DPD in univariate and multivariate analysis of survival

The survival analysis was performed on 197 patients and took into account the following variables: TS, DPD, patient's age, tumor size, histological grade, lymph node status, ER and PR. As shown in Table 3, univariate analysis focusing on DFS revealed tumor size (p<0.0001, logrank test), axillary lymph node status (p<0.0001, logrank test), histological grade (p<0.0001, logrank test), and TS (p<0.0001, logrank test) to be significant prognostic factors. Univariate analysis focusing on OS revealed TS (p<0.0001), tumor size (p<0.0001), nodal status (p=0.0024), histological grade (p<0.0001) to be significant prognostic factors. There was a statistically significant difference in both DFS and OS between patients with tumors showing positive TS immunoreactivity and those whose tumors did not (Fig. 2). We failed to identify DPD immunostaining as valuable prognostic factor for DFS and OS. Multivariate Cox regression analysis of the TS, tumor size, lymph node status and histological grade identified TS as an independent statistically prognostic factor for both DFS and OS (Table 4). The odds ratio of DFS for TS is 8.4. The risk of patients with TS-positivity relapse within a specific time was 8.4 times as high than the risk of patients (to relapse within the same time course) with TS-negativity. Moreover, the risk of patients with TS

Table 3. Univariate analysis of DFS and OS by various clinicopathological factors.

FACTOR		DFS		OS	
	No.	No. (%)	p	No. (%)	p
Age (years)			0.8012		0.6094
<50	154	38 (25.68)		84 (54.55)	
≥ 50	43	7 (16.28)		26 (60.47)	
Tumor size (cm)			<0.0001		<0.0001
≤ 2	58	28 (48.28)		50 (86.21)	
>2	139	17 (12.23)		60 (43.17)	
Nodal status			<0.0001		0.0024
Negative	18	15 (83.33)		17 (94.44)	
Positive	179	30 (16.76)		93 (51.96)	
Histological grade			<0.0001		<0.0001
I&II	157	40 (25.48)		102 (64.97)	
III	40	5 (12.50)		8 (20.00)	
ER			0.2888		0.7951
Negative	159	38 (23.90)		89 (55.98)	
Positive	38	7 (18.42)		21 (55.26)	
PR			0.2380		0.6747
Negative	135	27 (20.00)		74 (54.82)	
Positive	62	18 (29.03)		36 (58.07)	
TS			<0.0001		<0.0001
Negative	134	44 (32.84)		106 (79.10)	
Positive	63	1 (1.59)		4 (6.35)	
DPD			0.2265		0.6755
Negative	120	30 (25.00)		69 (57.50)	
Positive	77	15 (19.48)		41 (53.25)	

Table 4. Results of Multivariate Cox Regression Analysis for DFS and OS.

VARIABLE	DFS		OS	
	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
Tumor size		0.0008		0.0731
≤ 2	1.0 (referent)		1.0 (referent)	
>2	2.0877 (1.3569-3.2154)		2.0534 (0.9337-4.5249)	
Histological grade		<0.0001		<0.0001
I&II	1.0 (referent)		1.0 (referent)	
III	2.5974 (1.7331-3.8911)		3.0960 (1.9570-4.9020)	
Nodal status		0.0003		0.1070
Negative	1.0 (referent)		1.0 (referent)	
Positive	8.6207 (2.7174-27.0270)		5.1813 (0.7001-38.4615)	
TS		<0.0001		<0.0001
Negative	1.0 (referent)		1.0 (referent)	
Positive	8.4034 (5.6180-12.5000)		9.1743 (5.4645-15.3846)	

95% CI: 95% confidence interval.

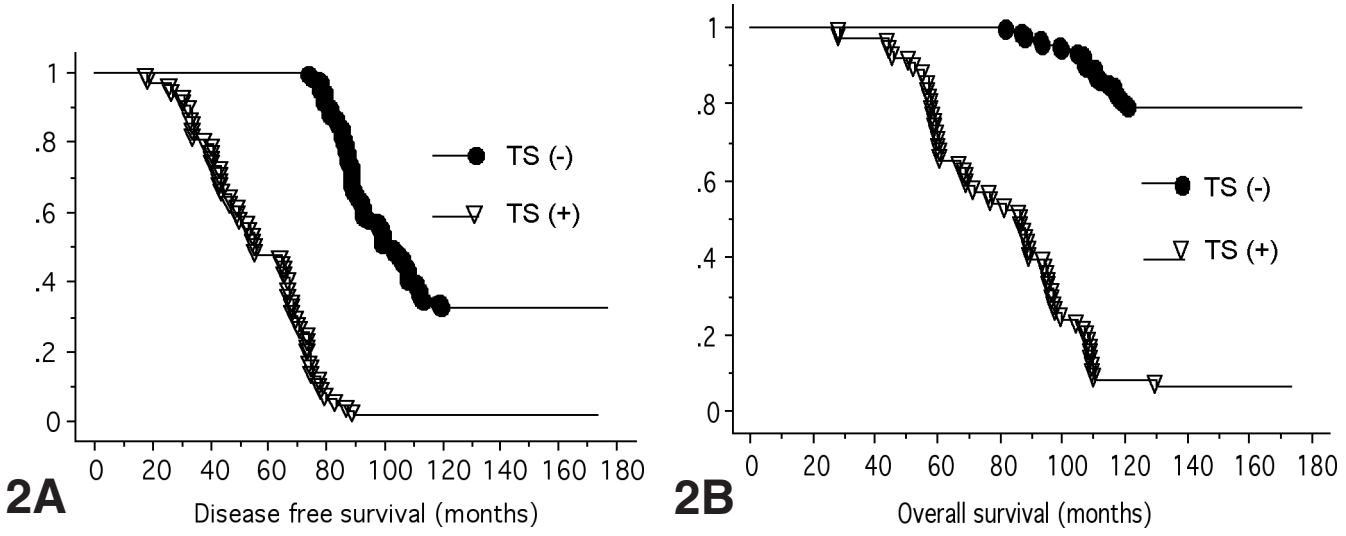

Fig. 2. Survival curves in the whole cohort of patients. TS is significantly related to recurrence (**A**, p<0.0001) and death (**B**, p<0.0001).

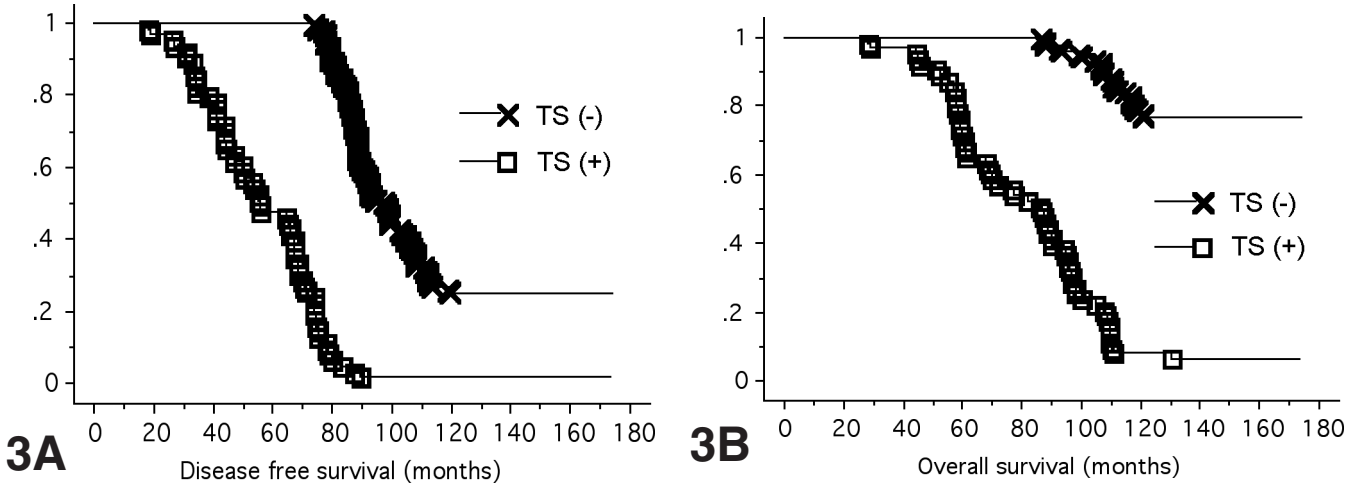

Fig. 3. Survival curves in the node-positive patients. TS is significantly related to recurrence (**A**, p<0.0001) and death (**B**, p<0.0001).

positivity die within a specific time was 9.2 times as high than the risk of patients (to die within the same time course) with TS-negativity.

To further test if TS was independent of nodal status, we performed additional analyses on subgroup of patients adjusted by nodal status. In our series, no node-negative patients had TS-positive tumors, therefore, we did not perform further analysis on subgroup of patients with nodal-negative status. As shown in Figure 2, in the nodal-positive patients, low TS expression was associated with prolonged DFS ($p<0.0001$) and OS ($p<0.0001$).

Discussion

5-FU is one of the most commonly prescribed anticancer agents having notable activity in the treatment of cancers arising from the breast, gastrointestinal tract, and head and neck. The metabolism, mechanisms of action and resistance, and pharmacokinetics of 5-FU have been extensively investigated since its synthesis over 4 decades ago. TS is a key enzyme in de novo DNA synthesis in addition to being a major target of 5-FU, and DPD is the first and rate-limiting enzyme in the catabolism of 5-FU (Diasio and Harris, 1989). Recently, increased interest has been focused on the roles of TS and DPD as responsive determinants for cancer patients with 5-FU-based therapy (Aschele et al., 2002). The methodologies used for measurement of TS and DPD expression have varied greatly, including biochemical assays, immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), reverse transcriptional polymerase chain reaction (RT-PCR), and real-time RT-PCR method (Aschele et al., 2002). Immunochemistry has the advantage of permitting the evaluation of protein expression in situ using paraffin-embedded blocks of specimens, which has been frequently used in clinical studies (Pestalozzi et al., 1997; Kuniyasu et al., 1998; Otake et al., 1999; Edler et al., 2002). We used this method to analyze TS and DPD gene expression in archival breast cancer samples. Our study is the first time that TS and DPD are analyzed together in a series of breast cancer patients receiving 5-FU-based chemotherapy with long-term follow-up information.

We demonstrated that intratumoral TS-positivity correlates with aggressive phenotypes including large tumor size, nodal metastasis and high histological grade. The findings support that TS exert an oncogene role in breast cancer, which has been indicated before (Rahman et al., 2004). Our results are consistent with previous reports (Ebuchi et al., 1995; Romain et al., 1997; Nishimura et al., 1999). These findings suggest that positive TS gene expression is associated with high cell proliferation, poor tumor differentiation and high metastatic potential in breast cancer. The precise reasons responsible for the relationship between TS gene expression and tumor aggression is still unknown. The TS-dependent conversion of dump to TMP is an essential step for providing the necessary dTTP components required for DNA replication and for maintaining a proper nucleotide balance within the cells. Overexpression of TS might generate an imbalance of dNTP pools that may have multifactorial effects on cell homeostasis, including an increase in the mutational rates or an impairment of DNA repair mechanisms (Bradley and Sharkey, 1978; Davidson and Kaufman, 1978; Meuth, 1989), which may affect cell proliferation and metastatic potential. It has been reported that the level of TS activity increases 20-fold when the cells enter the S phase from the G0 phase in synchronized cells (Navalgund et al., 1980). Furthermore, TS has been shown to be involved in the coordinate regulation of many genes, since TS binds to the c-myc mRNA as a part of a ribonucleoprotein complex (Chu et al., 1994). Further studies are needed to determine the biologic interaction between these genes and tumor development and aggression of breast cancer.

It is very important for practical medical purposes to clarify whether TS expression will really prove to be a prognostic indicator for breast cancer. Notably, TS-positivity was significantly associated with poor survival of patients with breast cancers. The survival curves determined by the Kaplan-Meier method showed that outcomes in patients with TS-positivity had both poor disease-free survival and over-all survival. Furthermore, multivariate analysis using the Cox proportional hazard model demonstrated that TS-positivity was still related to poor survival after consideration of other prognostic factors. The TS expression thus appears to be a reliable prognostic biomarker. These results presented here are similar to those published before (Pestalozzi et al., 1997; Nishimura et al., 1999; Romain et al., 2000; Li et al., 2004). The fact that patients with TS-positivity have worse prognosis may be partially explained by the fact that TS-positivity was related with aggressive breast cancer phenotye including large tumor size, poor differentiation, nodal metastasis. In addition, a high expression of TS is related to less sensitivity of 5-FU or results in the development of resistance to 5-FU (Johnston et al., 1992). Because all of our patients received 5-FU-based treatment, it is reasonable that patients with TS-negativity have longer DFS and OS than those with TS-positivity in our study. Novel drugs such as 1-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl) uracil may be a therapeutic agent in tumors with high TS that are less likely to respond to 5-fluorouracil treatment (Eiseman et al., 2004).

Although Horiguchi et al. (2002) reported that a high expression level of DPD correlated with aggressive phenotype and short disease-free survival, Li et al. (2004) showed that DPD was not a prognostic factor. In agreement with Li's findings, our results showed that DPD was not related with breast cancer malignancy or patients' survival receiving 5-FU-based chemotherapy. One of the reasons for this controversy about the clinicopathological significance of intratumoral levels of DPD is that methodologies and evaluations are different for different studies. Another reason is that various

background biases such as treatment methods are also different for different studies. To clarify the clinical significance of intratumoral DPD expression in breast cancer, a prospective study are needed.

In conclusion, the current study is the first study to analyze TS and DPD expression simultaneously in invasive breast cancer receiving 5-FU-based chemotherapy with long-term follow-up information. Our study showed that TS but not DPD in breast cancer was correlated with aggressive phenotype, and TS-positivity was associated with short disease-free survival and overall survival. These findings suggest that the evaluation of TS expression may provide a tool to separate patients who are likely to benefit from 5-FU-based chemotherapy (TS-negativity) from those who are unlikely to benefit (TS-positivity). However, the conclusions are drawn from a limited retrospective study. Prospectively randomized translational treatment trials are needed to confirm our results.

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