Anti-Cancer Drugs 2002, **13,** pp. 75–85

Report

Implication of thymidylate synthase in the outcome of patients with invasive ductal carcinoma of the pancreas and efficacy of adjuvant chemotherapy using 5-fluorouracil or its derivatives

Michio Takamura,[1] Yoshinori Nio,[1] Kunihiro Yamasawa,[1] Ming Dong,[1] Kazushige Yamaguchi[1] and Masayuki Itakura[1]

[1]First Department of Surgery, Shimane Medical University, Izumo 693-8501, Japan.

Thymidine synthase (TS) is a key enzyme in the synthesis of pyrimidine in the *de novo* pathway of DNA synthesis and a major target of 5-fluorouracil (5-FU), but the implications of TS regarding human pancreatic cancer have not been reported. We assessed the expression of TS in invasive ductal carcinoma (IDC) of the pancreas by immunostaining and evaluated its clinicopathological significance, especially its implications regarding the efficacy of chemotherapy with 5-FU or its derivatives. The expression of TS in the nuclei of pancreatic cancer cells in 72 primary lesions of resectable IDC and 30 distant metastases of unresectable IDC was examined by immunostaining using anti-TS polyclonal antibody and immunoreactivity was classified into three categories: negative (−), low (+) and high (2+). High TS immunoreactivity was detected in 43% (31 of 72) of the primary lesions of the resectable IDCs and in 47% (18 of 38) of the metastatic lesions of the unresectable IDCs. The high TS in primary lesions showed a significantly inverse correlation with the level of nodal involvement. High TS immunoreactivity had a significant influence on the outcome of patients with resectable IDC and the rate of survival of the high TS immunoreactivity group was significantly higher than that of the negative or low reactivity groups, although high TS immunoreactivity did not have a significant influence on survival of the patients with unresectable IDC. The implications of TS immunoreactivity regarding the efficacy of 5-FU-based adjuvant chemotherapy (ACT) was also assessed. The high TS immunoreactivity group showed significantly better survival in both the patients who received ACT and those who were treated by surgery alone, in the resectable IDC among patients with resectable IDC. In cases of unresectable IDC, there were no differences in survival between the high and low TS groups among the patients who received ACT and those who were treated by surgery. In conclusion, high TS immunoreactivity was found to be cogent in predicting the prognosis of patients with pancreatic IDC, but its implications regarding the efficacy of 5-FU-based ACT are still unclear. [© 2002 Lippincott Williams & Wilkins.]

Correspondence to Y Nio, First Department of Surgery, Shimane Medical University, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan.
Tel: (+81) 853 20 2225; Fax: (+81) 853 20 2222;
E-mail: fsurgery@shimane-med.ac.jp

Key words: 5-Fluorouracil, chemotherapy, pancreatic cancer, thymidylate synthase, UFT.

Introduction

Invasive ductal carcinoma (IDC) of the pancreas grows rapidly and is very resistant to a variety of cancer therapies, including surgery, radiation and chemotherapy. The prognosis of patients with pancreatic IDC is very poor and 85% of the patients with unresectable pancreatic IDC die within 1 year due to liver metastasis or peritoneal dissemination.[1] Even among patients who undergo curative surgery, 50% die within 1 year.[2,3] The malignant potential of pancreatic IDC is very high and the efficacy of surgical treatment is very poor. Accordingly, in order to improve the outcome of treatment for pancreatic IDC, a multimodal approach is essential and chemotherapy may play an important role in this approach. A variety of agents have been applied as chemotherapeutic agents in pancreatic IDC and 5-fluorouracil (5-FU) has been the most important agent, although the response rate of pancreatic IDC to 5-FU-based chemotherapy is still low.[4] Recently, the role of various enzymes associated with the efficacy of chemotherapy with 5-FU has been clarified and thymidylate synthase (TS) is one such enzyme.

TS is an enzyme which catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) by 5,10-methylene-5,6,7,8-tetrahydrofolate (CH2H4 folate) to produce thymidine-5-monophosphate (dTMP) and 7,8-dihydrofolate (H2 folate). TS is a dimer of identical 30–35 kDa subunits.[5,6] Two major pathways are known in the metabolism of

pyrimidine in DNA synthesis: the *de novo* pathway and the salvage pathway—TS is a key enzyme in the *de novo* pathway of pyrimidine synthesis. TS also seems to play a very important role in tumor growth; however, previous studies have reported conflicting results with respect to the clinicopathological significance of TS. Some reports have indicated that overexpression of TS in a tumor is a significant indicator for poor prognosis of patients with colorectal cancer,[7,8] ovarian cancer[9] and gastric cancer,[10–13] while others have reported that high TS activity is not always associated with a poor prognosis in colon cancer,[14] breast cancer[15] and gastric cancer.[16]

TS is a very important target for chemotherapy with fluoropyrimidines, such as 5-FU.[17] After 5-FU is metabolized to fluoro-deoxyuridine monophosphate (FdUMP), FdUMP forms a ternary complex with TS and folic acid, resulting in the inhibition of TS activity. With regard to the role of TS in the efficacy of 5-FU-based chemotherapy, although early studies indicated that TS inhibition was significantly correlated with the response to 5-FU,[18,19] these studies were controversial. Many studies indicated that low TS levels in the tumor were an indicator for a favorable response to 5-FU-based chemotherapy in colorectal or gastric cancer,[10,20–22] and head and neck cancer.[23] Other reports, however, indicated that the benefits of 5-FU-based chemotherapy were most evident in the high TS group in colorectal cancer[7,8] and breast cancer patients,[15,24] or that TS levels had no influence on the efficacy of 5-FU-based chemotherapy in gastric cancer[12,13] and in colorectal cancer.[25]

Although TS levels appear to be related to the efficacy of cancer chemotherapy, especially therapy with 5-FU or its derivatives, this suggestion remains controversial. To our knowledge, there have been no reports of TS expression in human pancreatic cancers. In the present study, we examined the expression of TS in primary and metastatic lesions of human pancreatic IDC using immunohistochemical methods, and its correlation with clinicopathological factors including clinical stage, patient survival and the efficiency of adjuvant chemotherapy including 5-FU or its derivatives after surgery.

Materials and methods

Patients

The present study included 102 patients with pancreatic cancers. Patient profiles are summarized in Table 1. All patients underwent surgery from October 1980 to December 2000 and have been followed in our department. Seventy-two patients with resectable pancreatic IDC underwent pancreatectomy, 37 pancreatoduodenectomy, 22 distal and 10 total pancreatectomy with lymphadenectomy; 35 patients had nodal involvement. Thirty patients with unresectable cancer underwent palliative surgery (30 bypass surgeries and eight exploratory laparotomies) and the specimens of distant metastases were obtained during surgery. The clinical stage of pancreatic cancer was classified according to the UICC (TNM) stage classification.[26]

Table 1. Profile of the pancreatic cancer patients

	Resectable (*n*=72)	Unresectable (*n*=38)
Gender		
male	34 (47.2%)	25 (65.8%)
female	38 (52.8%)	13 (34.2%)
Age	65.0 ± 9.9 (35–80)	67.3 ± 9.4 (47–86)
Stage		
I	10 (13.9%)	
II	4 (5.6%)	
III	36 (50.0%)	1 (2.6%)
IV	22 (30.6%)	37 (97.4%)
Histological grade		
1 (well differentiated)	35 (48.6%)	0 (0%)
2 (moderately differentiated)	32 (44.4%)	35 (92.1%)
3 (poorly differentiated)	5 (6.9%)	3 (7.9%)
Surgery		
pancreatoduodenectomy	37 (51.4%)	–
distal pancreatectomy	22 (30.5%)	–
total pancreatectomy	13 (18.1%)	–
bypass	–	30 (78.9%)
exploratory laparotomy	–	8 (21.1%)

Adjuvant chemotherapy (ACT)

In Japan, under the universal coverage of the health insurance system, the use of anticancer agents is strictly restricted by the Japanese Ministry of Health and Welfare. Accordingly, ACT mainly involves approved agents including 5-FU, mitomycin C (MMC), adriamycin (ADR), cyclophosphamide (CPA) and UFT [a mixture of uracil and ftrafur (FT) at 4:1], as summarized in Table 2. In the patients, who underwent pancreatectomy, 47 patients received ACT including fluoropyrimidines; 5-FU or its derivatives such as UFT, furutulon (5′-DFUR) and carmofur (HCFU). Twenty-five patients did not receive any ACT. Out of 30 patients with unresectable cancer, 22 patients did not receive any ACT and 16 patients received ACT using 5-FU or UFT. In Japan, gemcitabine has been approved for the treatment of pancreatic cancer since 2001; however, the present series did not include patients who received this chemotherapeutic drug.

Anti-human TS polyclonal antibody (pAb)

The anti-TS pAb was kindly supplied by Taiho Pharmaceutical (Tokyo, Japan). This anti-TS pAb was prepared from the serum of male New Zealand White rabbits immunized with recombinant human TS protein, which was prepared in a human placenta and human lung cancer cell line (Lu-99).[27] The anti-TS pAb (55 μg/ml) was diluted at 1000:1 for immunostaining.

Immunohistochemical staining

The specimens were fixed in 10% formalin and embedded in paraffin blocks. The specimens were sliced to 3 μm thickness with a microtome and prepared on the silanized slides (Dako, Tokyo, Japan). The specimens were deparaffinized in xylene for 5 min, 3 times and rehydrated in 90% ethanol for 5 min, 3 times. The specimens were then treated with 0.2% trypsin solution (0.2% trypsin and 0.2% $CaCl_2$ in 50 mM Tris–HCl buffer solution) for 15 min at room temperature. After washing in distilled water, the endogenous peroxidase was blocked with a methanol solution containing 0.3% H_2O_2 for 5 min. The specimens were washed twice in phosphate-buffered saline (PBS; Gibco, Biocult, Glasgow, UK). After washing with PBS, non-specific reaction was blocked with 10% normal rabbit serum (Nichirei, Tokyo, Japan) in a moist chamber for 10 min. The specimens were then incubated with anti-TS pAb in a moist chamber at 8°C for 12 h. After washing 3 times with PBS for 5 min each time, the specimens were incubated with anti-rabbit IgG+IgA+IgM antibody (Nichirei) diluted at 10 μg/ml for 20 min at room temperature. After washing 3 times with PBS for 5 min each time, the specimens were incubated with streptoavidin-conjugated peroxidase (Nichirei) diluted at 100 μg/ml for 10 min. The specimens were incubated with 3,3-diaminobenzidine-tetra-hydrochloride solution (Nichirei) for 20 min. The slides were then counterstained with veronal acetate-buffered 1 cm^3 methyl green solution (pH 4.0) for 1 h. After washing with 100% ethanol twice, the

Table 2. Adjuvant chemotherapy for pancreatic cancer

	Surgery	
	Pancreatectomy (*n*=72)	Palliative surgery (*n*=38)
(I) Surgery only	25	22
(II) Adjuvant chemotherapy	47	16
(a) Oral fluoropyrimidine alone	23	4
UFT	20	3
5-FU	1	1
5′-DFUR	1	0
HCFU	1	0
(b) Oral UFT combined with	24	12
oral CPA	18	7
oral CPA + 5-FU + cisplatin	2	0
oral CPA + 5-FU + MMC + ADR	1	1
oral CPA + cisplatin	0	2
oral CPA + MMC + cisplatin	0	1
5-FU + cisplatin	1	0
5-FU + MMC	1	0
5-FU + MMC + ADR	1	0
5-FU + CPA + ADR	0	1

specimens were dehydrated with 100% xylene. The slides were covered with coverglasses in mounting medium Entellan neu (Merck, Rahway, NJ). After drying, the slides were examined with an optic microscope.

Evaluation of immunoreactivity of TS

The total number of cells including both stained and unstained cells were counted in three different fields of the specimen and the mean values were used for the calculation of the positive rate of TS expression. The cells which showed strong cytoplasmic staining of TS were evaluated as positive staining and equivocally weak staining was evaluated as negative staining. In accordance with the previous studies using the same anti-TS pAb,[11,28] TS expression was classified into the categories; three degrees: (−) 0–10%, (1+) 11–25% and (2+) 25–100%. The (−) or (1+) expression was classified as low TS expression and (2+) was classified as high TS expression (Figure 1).

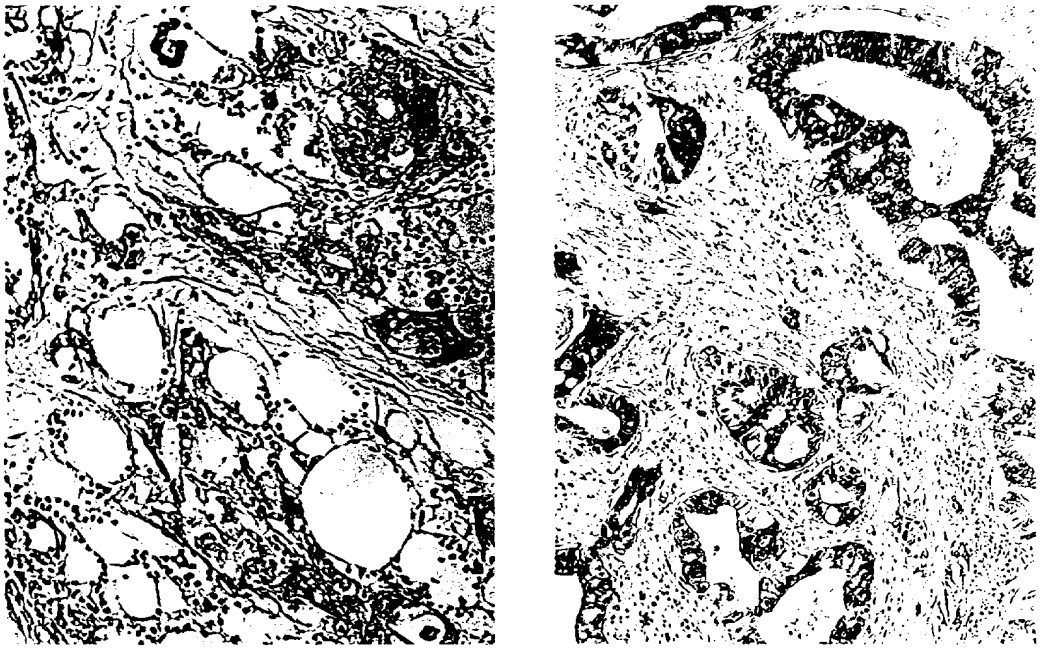
Statistics

A χ^2-test and Student's *t*-test were applied for comparison of the patients' clinicopathological backgrounds. The survival curves of the patients were calculated using the Kaplan–Meier method and were compared by the Cox–Mantel test. The correlation between TS staining and clinicopathological factors was examined using Pearson's correlation analysis. Cox's proportional hazard risk model was applied for multivariate analysis. A p value of <0.05 was considered to be significant.

Results

High TS immunoreactivity was detected in 43% (31 of 72) of the primary lesions and in 47% (18 of 38) of the metastatic lesions, and there were no differences in the level of TS immunoreactivity between the primary lesions and the metastatic lesions. The correlations between TS immunoreactivity and clinicopathological factors are summarized in Tables 3 and 4. Overall, high TS immunoreactivity was significantly correlated with age and with female gender. Immunoreactivity was not correlated with the clinical stage or histological grade of disease. In the patients with resectable IDC, high TS immunoreactivity showed a significantly inverse correlation with the level of nodal involvement.

TS immunoreactivity had a significant influence on the outcome of patients with resectable IDC and



(a) (b)

Figure 1. Representative TS expression. (a) Low TS expression. (b) High TS expression.

Table 3. TS expression in IDCs of the pancreas

	Numbers of high TS expression (%)		
	Resectable (n=72)	Unresectable (n=38)	Overall (n=110)
Overall	31/72 (43.1)	18/38 (47.4)	49/110 (44.5)
Gender			
male	11/34 (32.3)	9/25 (36.0)	20/59 (33.9)
female	20/38 (52.6)	9/13 (69.2)	29/51 (56.9)
Age			
$\leqslant$70	18/47 (38.3)	8/25 (32.0)	26/72 (36.1)
$>$71	13/25 (52.0)	10/13 (76.9)	23/38 (60.5)
Stage			
I	6/10 (60.0)	–	6/10 (60.0)
II	3/4 (75.0)	–	3/4 (75.0)
III	13/36 (36.1)	1/1 (100)	14/37 (37.8)
IV	9/22 (40.9)	17/37 (45.9)	26/59 (44.1)
Histological grade			
1 (well differentiated)	15/35 (42.9)	0 (0)	15/35 (42.9)
2 (moderately differentiated)	12/22 (54.5)	15/35 (92.1)	27/57 (47.4)
3 (poorly differentiated)	1/5 (20.0)	3/3 (100)	4/8 (50.0)
Surgery alone	11/25 (44.0)	11/22 (50.0)	11/47 (46.8)
Adjuvant chemotherapy	20/47 (42.6)	7/16 (43.8)	27/63 (42.9)

Table 4. Correlation between TS expression and clinicopathological factors

	Correlation coefficient (p value)		
	Overall (n=110)	Resectable (n=72)	Unresectable (n=38)
Age	0.216 (0.0235)	0.154 (0.1978)	0.314 (0.0662)
Gender	–0.230 (0.0152)	–0.204 (0.0850)	–0.318 (0.0626)
Histological grade	0.128 (0.1827)	0.087 (0.4688)	0.315 (0.0650)
TNM stage	–0.079 (0.4132)	–0.129 (0.2809)	–0.176 (0.3131)
pT	–	–0.076 (0.5287)	–
pN	–	–0.451 ($<$0.0001)	–
M	–	–0.041 (0.7337)	–

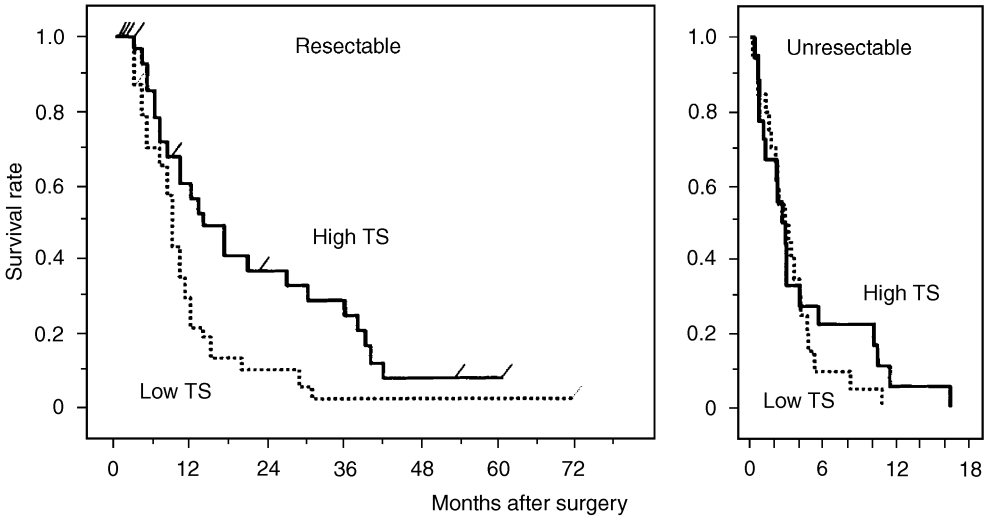


Figure 2. TS immunoreactivity and survival after surgery. High TS indicates (2+) immunoreactivity and low TS indicates (+) or (–) immunoreactivity. Resectable IDC (n=72): high TS (n=31) versus low TS (n=41), p=0.0078. Unresectable IDC (n=38): high TS (n=18) versus low TS (n=20), p=0.4970.

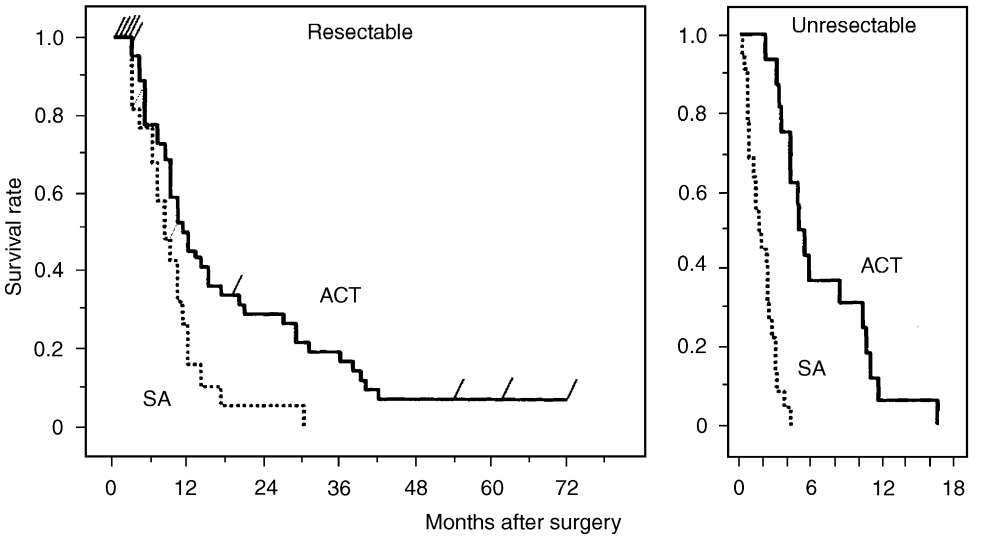


Figure 3. Adjuvant chemotherapy and survival after surgery. ACT, adjuvant chemotherapy group; SA, surgery alone group. Resectable IDC (n=72): ACT (n=47) versus SA (n=25), p=0.0081. Unresectable IDC (n=38): ACT (n=16) versus SA (n=22), $p < 0.0001$.

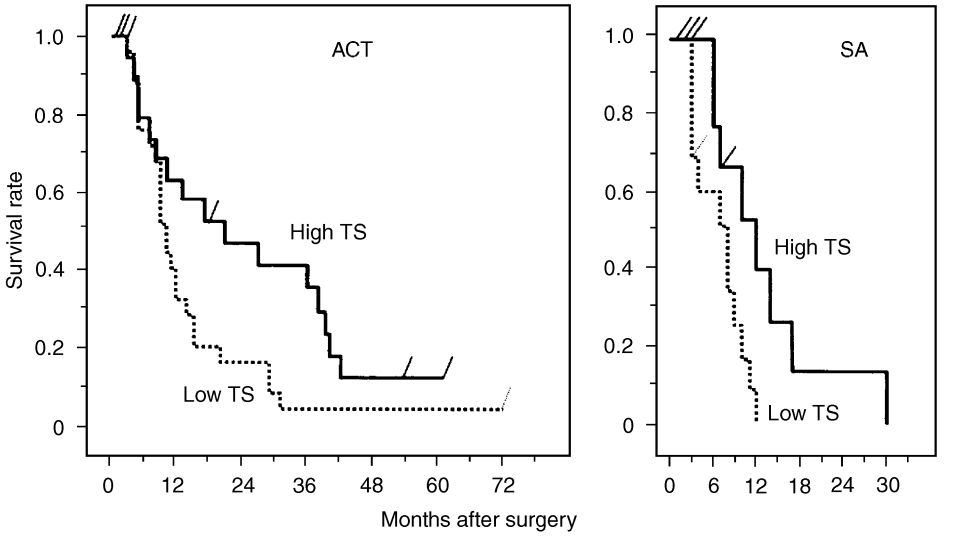


Figure 4. TS expression and the effect of adjuvant chemotherapy in resectable IDC. ACT group (n=47): high TS (n=20) versus low TS (n=27), p=0.0353. Surgery alone group (n=25): high TS (n=11) versus low TS (n=14), p=0.0208.

survival of the high TS immunoreactivity group was significantly better than that of the low TS reactivity group (p=0.0078) (Figure 2). However, in the patients with unresectable IDC, there were no significant differences in survival between the high TS group and low TS group (p=0.4970).

In the present study, ACT improved the survival after surgery in comparison with surgery alone in both the patients with resectable IDC (p=0.0081) and those with unresectable IDC ($p < 0.0001$) (Figure 3). Furthermore, the influence of TS expression on the efficacy of ACT was analyzed. In the patients with resectable IDC, the survival of the high TS expression group was significantly higher than that of the low TS group both in the patients who received ACT (p=0.0353) and those who were treated with surgery alone (p=0.0208) (Figure 4). By contrast, in the patients with unresectable IDC, the survival of the high TS immunoreactivity group tended to be better than that of the low TS group in

the patients who received ACT ($p=0.0844$), but there were no differences in survival between the high TS group and the low TS group among patients who were treated with surgery alone ($p=0.4082$) (Figure 5).

The significance of TS immunoreactivity in relation to patient outcome was analyzed by multivariate analysis and the results are summarized in Table 5. Overall, resection, ACT, histological grade, TS immunoreactivity and clinical stage were significant variables affecting patient outcome. In the patients with resectable IDC, clinical stage, histological grade and TS immunoreactivity were the significant variables, and in the patients with unresectable IDC, the ACT was the only significant variable.

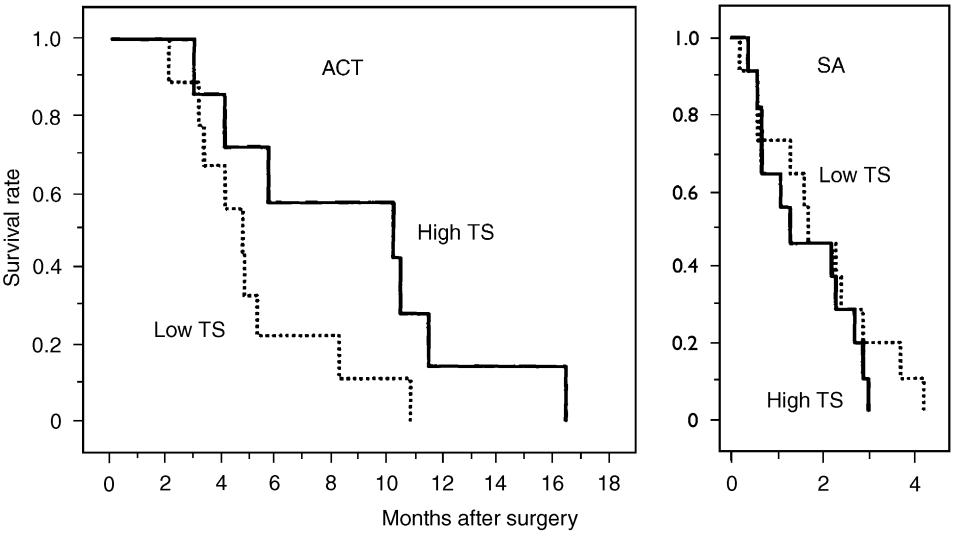


Figure 5. TS expression and the effect of adjuvant chemotherapy in unresectable IDC. ACT group ($n=16$): high TS ($n=7$) versus low TS ($n=9$), $p=0.0844$. Surgery alone group ($n=22$): high TS ($n=11$) versus low TS ($n=11$), $p=0.4082$.

Table 5. Relationship between various clinicopathological factors, including TS expression and prognosis of patients with pancreatic IDC (multivariate analysis by Cox's proportional hazard risk model[a])

Variables	Conditional risk ratio (95% confidence limit)	p value (χ^2)
Overall cases		
resection	0.275 (0.152(0.499)	<0.0001
adjuvant chemotherapy	0.282 (0.177(0.450)	<0.0001
histological grade	2.135 (1.350–3.377)	0.0012
TS expression	0.489 (0.311(0.771)	0.0020
stage	1.551 (1.132–2.124)	0.0062
age	0.998 (0.976–1.020)	0.8692
gender	0.837 (0.524–1.337)	0.4567
Resectable cases		
stage	1.698 (1.225–2.355)	0.0015
histological grade	2.046 (1.261–3.319)	0.0034
TS expression	0.484 (0.266(0.882)	0.0177
adjuvant chemotherapy	0.589 (0.313–1.108)	0.1005
age	1.014 (0.982–1.048)	0.3934
gender	0.998 (0.554–1.798)	0.9938
Unresectable cases		
adjuvant chemotherapy	0.102 (0.037(0.279)	<0.0001
stage	0.147 (0.014–1.571)	0.1127
gender	0.553 (0.231–1.323)	0.1831
TS expression	0.588 (0.254–1.359)	0.2136
histological grade	2.291 (0.562–9.349)	0.2479
age	0.995 (0.960–1.031)	0.7683

[a]Dependent variable=month, censoring variable=death due to pancreatic cancer.

Discussion

Various methods have been developed to evaluate the activity of TS in tumors. TS activity was first determined by PCR or Western blotting. These methods, however, are costly and time consuming, and cannot be widely applied. Recently, anti-TS antibodies have become available and it was reported that the level of TS gene expression, which was evaluated by PCR, was correlated with the expression of TS protein, which was evaluated by immunohistochemical methods.[21,28] Therefore, immunohistochemical methods have become the most popular techniques for evaluating the expression and distribution of TS in tumors.

Regarding the significance of high TS high immunoreactivity in the prognosis of the patients with IDC and the response to 5-FU-based chemotherapy, the present results were contradictory. The present study demonstrated that high TS immunoreactivity was associated with a better prognosis in the patients with resectable pancreatic IDC, but not in the patients with unresectable IDC. Furthermore, high TS tended to improve the response to 5-FU-based ACT in unresectable IDC, but not in resectable IDC. The previous reports have also been contradictory as summarized in Table 6.

Regarding the clinicopathological significance of TS, the present study demonstrated that high TS immunoreactivity was correlated with age and female gender, and also correlated with less frequent nodal involvement. These correlations remain controversial. In gastric cancer, high TS immunoreactivity was reported to be correlated with male gender and low-grade histology,[16] while other reports have found no correlation between TS immunoreactivity and patient gender, age or histology.[11,13] On the other hand, high TS immunoreactivity has been correlated with nodal involvement,[13] while others have reported no correlation between TS immunoreactivity and nodal involvement.[11,16] In colorectal cancer, no correlation has been found between the level of TS expression and the clinicopathological factors. In breast cancer, it was reported that high TS levels were correlated with advanced disease stages and nodal involvement,[29] but other reports have indicated no correlation.[24,30] Therefore, correlations between the level of TS expression and clinicopathological factors remain unclear.

The influence of TS expression on prognosis is also controversial and unclear in colorectal cancer, although several studies have agreed with the early publications by Johnston's group that high TS is a significant indicator for poor prognosis.[8,31] However, some recent publications have disagreed, reporting that TS status did not have any significant influence on survival,[22] but that TS immunointensity was correlated with the Dukes disease stage and the prognosis of the low TS group was significantly poorer than that of the high TS group.[14] In studies of gastric cancer, many reports have indicated that high TS was associated with rapid disease progression and poor prognosis,[10–13] while one report indicated that the level of TS expression had no influence on the prognosis of patients with gastric cancer.[16] In lung cancer and ovarian cancer, high TS was an indicator

Table 6. Implications of intratumoral TS expression in various solid malignancies

Malignancy	Prognosis of high TS group	Response of high TS to chemotherapy	Reference
Colorectal	no differences	–	22
	–	poor	31
	good	–	14
	poor	good	8
	–	poor	25
	–	poor	35
Rectal	poor	good	7
Gastric	poor	no differences	13
	–	no differences	16
	poor	no differences	12
	poor	–	11
	–	poor	10
Head and neck	poor	–	23
Breast	good	good	15
	–	poor	33
	poor	good	24
Lung	poor	–	32
Ovarian	poor	–	9
Pancreatic	good	good	This study

for poor prognosis.[9,32] However, in breast cancer it was reported that high TS activity was associated with slow disease progression.[15] In contrast, another study reported that extremely high TS levels were accompanied by an unfavorable prognosis.[33] Therefore, the implications of intratumoral TS levels in prognosis remain very controversial.

Regarding the role of TS in the efficacy of 5-FU-based chemotherapy, the results of many studies have similarly been contradictory: although several studies have indicated that high TS levels are responsible for resistance to TS inhibitors such as fluoropyrimidines or antifolates, but other studies have demonstrated that high TS levels were associated with a favorable response to 5-FU-based chemotherapy. The early publications following the original reports by Johnston's group indicated that low TS levels in a tumor were an indicator of favorable response to 5-FU-based chemotherapy in colorectal or gastric cancer,[10,20,21] and head and neck cancer.[23] On the other hand, Johnston also reported contradictory results that the benefits of 5-FU-based chemotherapy are most evident in the high TS group in Dukes' B and C rectal cancer.[7] Recent studies of TS report similarly controversial results. Some studies indicated that low TS was an indicator for favorable response to 5-FU or its derivatives in colorectal cancer,[22,34,35] but others indicated that high TS activity was a predictor of responsiveness to 5-FU-based chemotherapy in colon cancer[8] and in breast cancer.[15] Furthermore, TS has been reported to have no significant influence on the efficacy of chemotherapy in gastric cancer[13] and that TS had no predictive value for the response of colorectal cancer to chemotherapy.[25] In the present study different results were demonstrated between resectable IDC and unresectable IDC. Moreover, the implications of TS level in the efficacy of 5-FU-based chemotherapy were also controversial.

Several factors may account for these conflicting results regarding the implication of TS status in the progression of malignant disease and the response to chemotherapy. Differences in the antibodies used for immunostaining, the criteria delineating the cut-off point for TS overexpression and the chemotherapy regimens are all possible factors. One of the most important factors, however, is likely the heterogeneic distribution of TS in primary and metastatic lesions. TS expression has been reported to differ between the primary lesions, the corresponding metastases and the recurrent lesions in the same patients.[25,30,33] The designs of previous studies have varied and have involved different subjects: some studies have evaluated curatively resected cases, while others have studied metastatic diseases. If the primary tumors are completely removed by surgery, the prognosis of the patients will be affected by the progression of metastatic tumors. In contrast, prognosis in patients with unresectable primary tumors will be affected by the progression of primary tumors. Accordingly, if 5-FU-based chemotherapy is applied on the basis of TS activity, the TS activity should be assessed in the target lesions. This may be one of the factors responsible for the dissociated results between the resectable and unresectable IDC cases in the present study. In unresectable IDC, TS expression in the metastatic lesions may reflect prognosis, but in resectable IDC, the possible presence of residual lesions after surgery may mask any influence of TS overexpression.

Furthermore, it is not clear whether TS activity really reflects the growth ability of tumors. While TS is one of the key enzymes in the *de novo* pathway of DNA synthesis, its activity does not reflect DNA synthesis in the salvage pathway. Accordingly, even if TS activity is low or disturbed, DNA synthesis via the salvage pathway may be activated and may work as the main pathway of DNA synthesis. Accordingly, it may be useful to co-evaluate the activity of TS with the activity of thymidine phosphorylase, which is a key enzyme for the phosphorylation of pyrimidine in the salvage pathway of DNA synthesis.

Another important factor is that the sensitivity of tumor cells to fluoropyrimidines is not affected by TS alone and other factors such as a dihydropyrimidine dehydrogenase (DPD), an enzyme that catabolizes 5-FU to 5-fluoro-dihydrouracil, are also involved in 5-FU metabolism. DPD activity has been reported to more accurately reflect 5-FU sensitivity than TS activity.[36] Accordingly, co-evaluation of intratumoral TS and DPD has been reported to be useful in the accurate prediction of responsiveness to 5-FU-based chemotherapy.[32] Furthermore, the metabolism of 5-FU is affected by the amount of uracil in the tissue. In previous experiments using murine tumors, we demonstrated that the percent inhibition of TS activity by fluoropyrimidines was not correlated with the tumor-inhibitory effects, and that uracil in the tumor played an important role in both antitumor mechanisms and the metabolism of fluoropyrimidines.[37]

Differences in the chemotherapy regimens are also important factors. In Japan, 5-FU-based chemotherapy differs from that in Western countries, because oral fluoropyrimidines such as UFT are widely used for adjuvant chemotherapy. As such, most of the patients in the adjuvant chemotherapy group in the present study were administered oral UFT, which is the only oral fluoropyrimidine approved for

pancreatic cancer under coverage of the health insurance system in Japan. Intravenous 5-FU results in relatively high concentrations in the tumor, but the retention time of the drug is short. Accordingly, i.v. 5-FU may inhibit intratumoral TS rapidly, but temporarily. Furthermore, i.v. 5-FU has also been administered in various regimens using different doses, intervals and methods (bolus or continuous), and for different purposes (adjuvant or salvage) or in different combinations with other agents. Accordingly, these variations may also affect the efficacy of chemotherapy. By contrast, oral UFT results in continuous accumulation of FT and FT is metabolized into 5-FU within the tumor. Accordingly, oral UFT inhibits TS slowly but continuously. Our previous study demonstrated that preoperative administration of oral UFT in patients with gastric cancer resulted in the accumulation of higher concentrations of 5-FU in cancerous tissue than in normal tissue, and over a 3-week administration period the average 5-FU concentration was 0.04–0.07 μg/g in the gastric cancer tissue and 0.01−0.04 μg/g in non-malignant stomach tissue.[38] The clinical effects of oral UFT have been recently studied in Western countries and oral UFT in combination with leucovorin (LV) resulted in a relatively high response compared to i.v. 5-FU in colorectal cancer.[39,40] In pancreatic cancer, the standard regimen for the last decade has been i.v. administration of 5-FU and LV in combination with radiotherapy.[4] Some recent new regimens for pancreatic cancer have included gemcitabine as well as oral UFT.[41,42] The influence of intratumoral TS activity on the antitumor efficacy of UFT remains unclear; however, the results of the present study may contribute to clarification of this issue.

In conclusion, the present study demonstrated conflicting results regarding the implication of high TS immunoreactivity in patient prognosis and response to 5-FU-based ACT in human pancreatic IDC. It is not clear whether the conflicting results derive from the different biologic characteristics of pancreatic IDC compared to other digestive organ cancers or not. This is the first report of TS expression in human pancreatic IDC and subsequent studies of human pancreatic IDC may clarify the role of TS in treatment and progression of this disease.

Acknowledgments

We gratefully acknowledge Ms Tomoko Toga, Ms Miyuki Ishihara, Ms Yasuko Sonoyama and Ms Yuka Maniwa for their excellent assistance.

References

1. Johnson C. Prognosis in pancreatic cancer. *Lancet* 1997; **349**: 1027–8.
2. Nagakawa T, Konishi I, Ueno K. Surgical treatment of pancreatic cancer. The Japanese experience. *Int J Pancreatol* 1991; **9**: 135–43.
3. Hirata K, Sato T, Mukaiya M, *et al*. Results of 1001 pancreatic resections for invasive ductal adenocarcinoma of the pancreas. *Arch Surg* 1997; **132**: 771–6.
4. Ghaneh P, Kawesha A, Howes N, Jones L, Neoptolemos JP. Adjuvant therapy for pancreatic cancer. *World J Surg* 1999; **23**: 937–45.
5. Ivanetich KM, Santi DV. Bifunctional thymidylate synthase-dihydrofolate reductase in protozoa. *FASEB J* 1990; **4**: 1591–7.
6. Schiffer CA, Davisson VJ, Santi DV, Stroud RM. Crystallization of human thymidylate synthase. *J Mol Biol* 1991; **219**: 161–3.
7. Johnston PG, Fisher ER, Rockette HE, *et al*. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J Clin Oncol* 1994; **12**: 2640–7.
8. Takenoue T, Nagawa H, Matsuda K, *et al*. Relation between thymidylate synthase expression and survival in colon carcinoma, and determination of appropriate application of 5-fluorouracil by immunohistochemical method. *Ann Surg Oncol* 2000; **7**: 193–8.
9. Fujiwaki R, Hata K, Nakayama K, Fukumoto M, Miyazaki K. Thymidylate synthase expression in epithelial ovarian cancer: relationship with thymidine phosphorylase expression and prognosis. *Oncology* 2000; **59**: 152–7.
10. Lenz HJ, Leichman CG, Danenberg KD, *et al*. Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. *J Clin Oncol* 1996; **14**: 176–82.
11. Kuniyasu T, Nakamura T, Tabuchi Y, Kuroda Y. Immunohistochemical evaluation of thymidylate synthase in gastric carcinoma using a new polyclonal antibody: the clinical role of thymidylate synthase as a prognostic indicator and its therapeutic usefulness. *Cancer* 1998; **83**: 1300–6.
12. Suda Y, Kuwashima Y, Tanaka Y, Uchida K, Akazawa S. Immunohistochemical detection of thymidylate synthase in advanced gastric cancer: a prognostic indicator in patients undergoing gastrectomy followed by adjuvant chemotherapy with 5-fluoropyrimidines. *Anticancer Res* 1999; **19**: 805–10.
13. Tsujitani S, Konishi I, Suzuki K, *et al*. Expression of thymidylate synthase in relation to survival and chemosensitivity in gastric cancer patients. *J Exp Clin Cancer Res* 2000; **19**: 189–95.
14. Umehara A, Yoshimatsu K, Endo S, Kato H, Haga S. The correlation between the intratumoral thymidylate synthase levels and clinicopathological factors in colorectal cancer patients. *J Tokyo Wom Med Univ* 2000; **70**: 621–9.
15. Foekens JA, Romain S, Look MP, Martin PM, Klijn JG. Thymidine kinase and thymidylate synthase in advanced breast cancer: response to tamoxifen and chemotherapy. *Cancer Res* 2001; **61**: 1421–5.

16. Choi J, Lim H, Nam DK, *et al*. Expression of thymidylate synthase in gastric cancer patients treated with 5-fluorouracil and doxorubicin-based adjuvant chemotherapy after curative resection. *Br J Cancer* 2001; **84**: 186–92.
17. VanTriest B, Peters GJ. Thymidylate synthase: a target for combination therapy and determinant of chemotherapeutic response in colorectal cancer. *Oncology* 1999; **57**: 179–94.
18. Spears SP, Gustavsson BG, Mitchell MS, *et al*. Thymidylate synthase inhibition in malignant tumors and normal liver of patients given intravenous 5-fluorouracil. *Cancer Res* 1984; **44**: 4144–50.
19. Swain SM, Lippman ME, Egan EF, Drake JC, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J Clin Oncol* 1989; **7**: 890–9.
20. Peters GJ, van der Wilt CL, van Groeningen CJ, Smid K, Meijer S, Pinedo HM. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: implications for treatment with fluorouracil. *J Clin Oncol* 1994; **12**: 2035–42.
21. Johnston PG, Lenz HJ, Leichman CG, *et al*. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res* 1995; **55**: 1407–12.
22. Paradiso A, Simone G, Petroni S, *et al*. Thymidylate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. *Br J Cancer* 2000; **82**: 560–7.
23. Johnston PG, Mick R, Recant W, *et al*. Thymidylate synthase expression and response to neoadjuvant chemotherapy in patients with advanced head and neck cancer. *J Natl Cancer Inst* 1997; **89**: 308–13.
24. Pestalozzi BC, Peterson HF, Gelber RD, *et al*. Prognostic importance of thymidylate synthase expression in early breast cancer. *J Clin Oncol* 1997; **15**: 1923–31.
25. Aschele C, Debernardis D, Tunesi G, Maley F, Sobrero A. Thymidylate synthase protein expression in primary colorectal cancer compared with the corresponding distant metastases and relationship with the clinical response to 5-fluorouracil. *Clin Cancer Res* 2000; **6**: 4797–802.
26. American Joint Committee on Cancer. TNM committee of the International Union Against Cancer. *Staging of cancer from the manual for staging of cancer*, 5th edn. AJCC 1997.
27. Okabe H, Tsujimoto H, Fukushima M. Preparation of the antibodies against recombinant human thymidylate synthase for the detection of its intratumoral level and the application to sensitivity-study of 5-fluorouracil. *Oncol Rep* 1997; **4**: 685–90.
28. Otake Y, Tanaka F, Yanagihara K, *et al*. Expression of thymidylate synthase in human non-small cell lung cancer. *Jpn J Cancer Res* 1999; **90**: 128–53.
29. Ebuchi M, Sakamoto S, Kudo H, Nagase J, Endo M. Clinicopathological stages and piromidine nucleotide synthesis in human mammary carcinomas. *Anticancer Res* 1995; **15**: 1481–4.
30. Komaki K, Kamamura Y, Ohmine Y, *et al*. Differences in thymidylate synthetase activity in involved nodes compared with primary tumor in breast cancer patients. *Breast Cancer Res Treat* 1995; **35**: 157–62.
31. Tachikawa D, Arima S, Futami K. Immunohistochemical expression of thymidylate synthase as a prognostic factor and as a chemotherapeutic efficacy index in patients with colorectal carcinoma. *Anticancer Res* 2000; **20**: 4103–7.
32. Huang CL, Yokomise H, Kobayashi S, Fukushima M, Hitomi S, Wada H. Intratumoral expression of thymidylate synthase and dihydropyrimidine dehydrogenase in non-small cell lung cancer patients treated with 5-FU-based chemotherapy. *Int J Oncol* 2000; **17**: 47–54.
33. Nishimura R, Nagao K, Miyayama H, *et al*. Thymidylate synthase levels as a therapeutic and prognostic predictor in breast cancer. *Anticancer Res* 1999; **19**: 5621–6.
34. Davies MM, Johnston PG, Kaur S, Allen-Mersh TG. Colorectal liver metastasis thymidylate synthase staining correlates with response to hepatic arterial floxuridine. *Clin Cancer Res* 1999; **5**: 325–8.
35. Cascinu S, Aschele C, Barni S, *et al*. Thymidylate synthase protein expression in advanced colon cancer: correlation with the site of metastasis and the clinical response to leucovorin-modulated bolus 5-fluorouracil. *Clin Cancer Res* 1999; **5**: 1996–9.
36. Nita ME, Tominaga O, Nagawa H, Tsuruo T, Muto T. Dihydropyrimidine dehydrogenase but not thymidylate synthase expression is associated with resistance to 5-fluorouracil in colorectal cancer. *Hepato-Gastroenterology* 1998; **45**: 2117–22.
37. Nio Y, Shiraishi T, Tsubono M, *et al*. Relationship of *in vivo* antitumor activities of fluoropyrimidines to thymidylate synthase activity and intratumoral concentrations of 5-fluorouracil and uracil. *Anticancer Res* 1991; **11**: 607–12.
38. Nio Y, Sato Y, Nagami H, *et al*. Neoadjuvant chemotherapy of gastric cancer with oral UFT (a mixture of uracil and futrafur) during the waiting period for surgery. *Anticancer Res* 1998; **18**: 523–30.
39. Pazdur R, Lassere Y, Rhodes V, *et al*. Phase II trial of uracil and tegafur plus oral leucovorin: an effective oral regimen in the treatment of metastatic colorectal carcinoma. *J Clin Oncol* 1994; **12**: 2296–300.
40. Saltz LB, Leichman C, Young CW, *et al*. A fixed-ration combination of uracil and ftrafur (UFT) with low dose leucovorin. *Cancer* 1995; **75**: 782–5.
41. Feliu J, Lopez Alvarez MP, Jaraiz MA, *et al*. Phase II trial of gemcitabine and UFT modulated by leucovorin in patients with advanced pancreatic carcinoma. The ONCOPAZ Cooperative Group. *Cancer* 200; **89**: 1706–13.
42. Childs III, HA, Spencer SA, Raben D, Bonner JA, Newsome J, Robert F. A phase I study of combined UFT plus leucovorin and radiotherapy for pancreatic cancer. *Int J Radiat Oncol Biol Phys* 2000; **47**: 939–44.

(Received 2 October 2001; accepted 16 October 2001)