

Prognostic significance of thymidylate synthase, thymidine phosphorylase and dihydropyrimidine dehydrogenase expression in biliary tract cancer patients receiving adjuvant 5-fluorouracil-based chemotherapy

KWAN WOO KIM¹, HYUK-CHAN KWON², SUNG-HYUN KIM², SUNG YONG OH², SUEE LEE²,
JI HYUN LEE², MYUNG HWAN ROH², MIN CHAN KIM³, KI HAN KIM³, YOUNG HOON KIM³,
YOUNG HOON ROH³, JIN SOOK JEONG⁴ and HYO-JIN KIM¹

¹Department of Surgery, Haeundae Paik Hospital, College of Medicine, Inje University;
Departments of ²Internal Medicine, ³Surgery and ⁴Pathology, College of Medicine,
Dong-A University, Busan, Republic of Korea

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Abstract. Biliary tract cancer (BTC) is a relatively uncommon type of cancer, accounting for ~4% of the malignant neoplasms of the gastrointestinal tract. The aim of this study was to determine whether the expression of thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) predict clinical outcome in BTC patients treated with adjuvant 5-fluorouracil (5-FU)-based chemotherapy. TS and TP expression were found to be significantly correlated with cancer location ($P=0.044$ and 0.031 , respectively). The multivariate analysis revealed that age [hazard ratio (HR)=2.157, $P=0.008$], stage (HR=2.234, $P<0.001$), resection margin status (HR=2.748, $P=0.004$) and TP expression (HR=2.014, $P=0.039$) were independently associated with overall survival (OS).

Introduction

Biliary tract cancer (BTC), including cancer of the gallbladder, bile ducts and ampulla, is a relatively uncommon type of cancer, accounting for ~4% of the malignant neoplasms of the gastrointestinal tract. This type of cancer is more common in Asia (1). Based on the data of the National Cancer Registry in 2009, BTC is the eighth most common cancer in Korea, with an annual incidence of 4,782 per 100,000 cancer cases (2). Surgical resection offers patients with resectable BTC the only option for cure and long-term survival. However, the reported

overall 5-year survival rate following surgical resection was 33.1% for bile duct, 52.8% for ampullary and 41.6% for gallbladder cancer (3). The prognosis for BTC remains poor, even after extensive surgical resection, due to the high recurrence rate. Therefore, effective adjuvant therapy is required to prolong the survival of BTC patients.

However, the role of adjuvant treatment remains controversial, since the results mentioned above are based on small-scale studies, rather than large-scale, controlled clinical trials. The majority of the retrospective trials, which included limited sample sizes, heterogeneous patient populations and non-standardized therapies, suggested a marginal benefit of chemotherapy in reducing recurrence and an uncertain effect on survival (4,5). The only two prospective randomized controlled trials (RCT) currently available concluded that adjuvant chemotherapy did not improve survival (6,7).

Since it was originally synthesized in 1957, 5-fluorouracil (5-FU) has been widely applied in clinical practice. 5-FU is the most extensively investigated drug for BTC, as a single agent or in combination with other treatment modalities (4-7). Several mechanisms of resistance to 5-FU were reported in cholangiocarcinoma cell lines. It was previously demonstrated that increases in UNG1 and BIRC5 expression and decreases in TP73 expression may be associated with 5-FU resistance (8). Previous studies also demonstrated that the development of resistance of cancers to 5-FU may involve mechanisms including alterations in the expression of several 5-FU metabolic enzyme genes, including thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) in colorectal cancer patients (9,10).

TS is the target enzyme for 5-FU and catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is an important process of DNA biosynthesis (11). Elevated TS protein levels may interfere with the mechanisms of action of fluoropyrimidines (12). A recent meta-analysis confirmed a poorer overall survival of patients with enhanced TS activity,

Correspondence to: Dr Sung Yong Oh, Department of Internal Medicine, College of Medicine, Dong-A University, 3-1 Dongdaeshin-dong, Seo-gu, Busan 602-715, Republic of Korea
E-mail: drosy@dau.ac.kr

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compared to those with low TS activity (13). However, conflicting results have been reported regarding TS expression in gastric cancer (14,15).

DPD is the initial and rate-limiting enzyme responsible for the inactivation of 5-FU (11). DPD activity is highly variable in cancer tissues, which may affect the antitumor efficacy of 5-FU. A previous *in vitro* study demonstrated that DPD expression in cancer cell lines confers resistance to 5-FU (16). It was also reported that the intratumoral gene expression levels of DPD are associated with tumor response to 5-FU (17).

TP is a key enzyme in the metabolic activation of fluoropyrimidines by conversion of doxifluridine (5'-DFUR), which is an intermediate metabolite of capecitabine, to 5-FU (11). Thus, administration of 5'-DFUR in cases of tumors with a high TP expression is expected to yield high concentrations of 5-FU in tumor tissues and thereby a good chemotherapeutic response. The clinical efficacy of 5'-DFUR was demonstrated in colorectal cancer patients with high TP expression tumors, who exhibited a better survival compared to patients with low TP tumors (18). However, TP was also identified as an angiogenic factor, identical to the platelet-derived endothelial cell growth factor (19). Another previous study reported that high TP immunostaining correlated with more extensive angiogenesis and poor clinical outcome in colorectal cancer patients (20).

Due to their involvement in 5-FU metabolism, the expression and activity levels of TS, DPD and TP are potentially important as predictive markers for the response to 5-FU and as prognostic factors in colorectal cancer patients (9,10). However, there is currently no study available on the significance of these proteins in BTC. The aim of this study was to determine whether the expression of TS, TP and DPD predicts clinical outcome in BTC patients treated with adjuvant 5-FU-based chemotherapy.

Patients and methods

Patients. A total of 99 patients who underwent curative surgery for extrahepatic bile duct, ampullary or gallbladder cancer at Dong-A University Medical Center between November, 1999 and February, 2009 were evaluated. Patients with intrahepatic cholangiocarcinoma were excluded, since this type of cancer has been known to exhibit different clinicopathological characteristics from other types of BTC.

Of the 99 patients, 39 (39.4%) had been diagnosed with gallbladder cancer, 43 (43.4%) with extrahepatic bile duct cancer and 17 (17.2%) with ampullary cancer. Patients with extrahepatic bile duct and ampullary cancer typically underwent pancreatoduodenectomy, with or without pyloric preservation, whereas the surgical procedure for gallbladder cancer patients almost always included cholecystectomy, with or without major hepatectomy. The patients also underwent regional lymph node dissection. However, dissection of para-aortic lymph nodes was not routinely performed.

Following tumor resection, the specimens were pathologically examined and each tumor was classified as well-, moderately- or poorly-differentiated adenocarcinoma, according to the predominant pathologic grading of differentiation. Pancreatic, duodenal and hepatic invasion and lymph node metastasis were pathologically determined. The surgical

margins were considered positive when infiltrating adenocarcinoma was present at the proximal hepatic transection line, distal bile duct transection line, or dissected periductal soft tissue margins. The final stage of biliary carcinoma was pathologically determined, according to the tumor-node-metastases staging system of malignant tumors, published by the American Joint Committee on Cancer (AJCC), 6th edition.

Clinical records and pathological reports were retrospectively reviewed. Clinical outcomes were followed from the date of surgery to either the date of death or August, 2012. The study was approved by the Institutional Review Board of Dong-A University Medical Center. Patient consent was obtained from either the patient or the patient's family.

Adjuvant chemotherapy. The eligibility criteria for adjuvant therapy included: i) histologically confirmed preoperative diagnosis of carcinoma of the gallbladder, extrahepatic bile duct, or ampulla; ii) stage I-III disease; iii) confirmed resection of the primary lesion; iv) age <75 years; v) Eastern Cooperative Oncology Group performance status of 0-2; vi) no previous surgery or chemotherapy; vii) no serious concomitant disease; viii) no concurrent or non-concurrent multicentric tumor or double tumor; and ix) at treatment initiation, a leukocyte count of $\geq 4,000/\text{mm}^3$, a platelet count of $\geq 100,000/\text{mm}^3$, liver enzymes [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] ≤ 100 units and negative urinary protein. External beam radiation or intraoperative irradiation was not administered to any of the patients during the study period.

The chemotherapy regimens were FP (5-FU plus cisplatin) or oral 5-FU (doxifluridine). The FP regimen was as follows: cisplatin 60 mg/m² was administered intravenously on day 1 and 5-FU 1,000 mg/m² was administered intravenously on days 1-5. This regimen was repeated every three weeks for 6 cycles. Oral chemotherapy was initiated at 4 weeks after surgery; 460 mg/m²/day of doxifluridine was administered daily for 1 year. Chemotherapy was discontinued for recurrent disease, unacceptable treatment toxicity or at patient request.

Patient follow up. The postoperative baseline and follow-up investigations were standardized. Prior to adjuvant chemotherapy, the baseline assessments included medical history and physical examination, complete blood count (CBC), renal and liver function tests, urinalysis, ECG, chest X-ray, radio-nuclide bone scan and abdominal computed tomography (CT). Follow-ups for the patients occurred at 3-month intervals for 2 years, at 6-month intervals for the following 3 years and annually thereafter. The follow ups comprised physical examination, CBC, renal and liver function tests and abdominal ultrasonography or CT scan.

Immunohistochemistry and assessment of immunostaining. Immunohistochemical study for the detection of TS, TP and DPD expression was performed on three core cancer tissues (2 mm in diameter) from each individual, which were arranged in tissue array blocks. The 4-5- μm sections were mounted on Superfrost Plus glass slides (Thermo Scientific, Braunschweig, Germany). Using the Discovery XT automated immunohistochemistry stainer (Ventana Medical Systems, Tucson, AZ, USA), the slides were stained according to the following procedure: tissue sections were deparaffinized using the EZ Prep

Table I. Correlation between patient characteristics and chemotherapy regimen.

Characteristics	Patient no. (%)		P-value	Patient no. (%)		P-value	Patient no. (%)		P-value
	TS (low)	TS (high)		TP (low)	TP (high)		DPD (low)	DPD (high)	
Age, years									
<65 (n=59)	45 (76.3)	14 (23.7)	0.841	19 (32.2)	40 (67.8)	0.504	18 (30.5)	41 (69.5)	0.164
≥65 (n=40)	29 (72.5)	11 (27.5)		10 (25.0)	30 (75.0)		7 (17.5)	33 (82.5)	
Gender									
Male (n=48)	40 (83.3)	8 (16.7)	0.067	14 (29.2)	34 (70.8)	1.000	13 (27.1)	35 (72.9)	0.818
Female (n=51)	34 (66.7)	17 (33.3)		15 (29.4)	36 (70.6)		12 (23.5)	39 (76.5)	
Location									
Gallbladder (n=39)	24 (61.5)	15 (38.5)	0.044	14 (35.9)	25 (64.1)	0.031	11 (28.2)	28 (71.8)	0.351
EHBD (n=43)	35 (81.4)	8 (18.6)		7 (16.3)	36 (83.7)		8 (18.6)	35 (81.4)	
AOV (n=17)	15 (88.2)	2 (11.8)		8 (47.1)	9 (52.9)		6 (35.3)	11 (64.7)	
T stage									
1 (n=18)	13 (72.2)	5 (27.8)	0.902	9 (50.0)	9 (50.0)	0.067	6 (33.3)	12 (66.7)	0.060
2 (n=45)	33 (73.3)	12 (26.7)		14 (31.1)	31 (68.9)		15 (33.3)	30 (66.7)	
3 (n=29)	22 (75.9)	7 (24.1)		4 (13.8)	25 (86.2)		2 (6.9)	27 (93.1)	
4 (n=7)	6 (85.7)	1 (14.3)		2 (28.6)	5 (71.4)		2 (28.6)	5 (71.4)	
N stage									
N0 (n=75)	56 (74.7)	19 (25.3)	1.000	25 (33.3)	50 (66.7)	0.133	20 (26.7)	55 (73.3)	0.788
N1 (n=24)	18 (75.0)	6 (25.0)		4 (16.7)	20 (83.3)		5 (20.8)	19 (79.2)	
Stage ^a									
I (n=50)	36 (72.0)	14 (28.0)	0.804	20 (40.0)	30 (60.0)	0.026	17 (34.0)	3 (66.0)	0.041
II (n=41)	32 (78.0)	9 (22.0)		6 (14.6)	35 (85.6)		5 (12.2)	36 (87.8)	
III (n=8)	6 (75.0)	2 (25.0)		3 (37.5)	5 (62.5)		3 (37.5)	5 (62.5)	
Differentiation									
High (n=56)	42 (75.0)	14 (25.0)	0.281	15 (26.8)	41 (73.2)	0.678	19 (33.9)	37 (66.1)	0.061
Moderate (n=28)	23 (82.1)	5 (17.9)		10 (35.7)	18 (64.3)		3 (10.7)	25 (89.3)	
Poor (n=15)	9 (60.0)	6 (40.0)		4 (26.7)	11 (73.3)		3 (20.0)	12 (80.0)	
Lymphovascular invasion									
- (n=83)	64 (77.1)	19 (22.9)	0.224	24 (28.9)	59 (71.1)	1.000	18 (21.7)	65 (78.3)	0.112
+ (n=16)	10 (62.5)	6 (37.5)		5 (31.2)	11 (68.8)		7 (43.8)	9 (56.2)	
Resection margin									
- (n=86)	65 (75.6)	21 (24.4)	0.733	27 (31.4)	59 (68.6)	0.335	23 (26.7)	63 (73.3)	0.507
+ (n=13)	9 (69.2)	4 (30.8)		2 (15.4)	11 (84.6)		2 (15.4)	11 (84.6)	

Table I. Continued.

Characteristics	Patient no. (%)		P-value	Patient no. (%)		P-value	Patient no. (%)		P-value
	TS (low)	TS (high)		TP (low)	TP (high)		DPD (low)	DPD (high)	
Chemotherapy regimen			0.615			0.235			0.615
Oral 5-FU (n=70)	51 (72.9)	19 (27.1)		18 (25.7)	52 (74.3)		19 (27.1)	51 (72.9)	
FP (n=29)	23 (79.3)	6 (20.7)		11 (37.9)	18 (62.1)		6 (20.7)	23 (79.3)	

^aAmerican Joint Committee on Cancer staging manual, 6th edition. TS, thymidylate synthase; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase; EHBD, extrahepatic bile duct; AOV, ampullar of Vater; FU, fluorouracil; FP, fluorouracil + cisplatin.

solution (Ventana Medical Systems). For antigen retrieval, CCl standard buffer (pH 8.4), containing Tris/Borate/EDTA, (Ventana Medical Systems) was used for 30 min. Inhibitor D of endogenous peroxidase (3% H₂O₂, Ventana Medical Systems) was applied for 4 min at a temperature of 37°C. The slides were incubated with anti-thymidine synthase antibody (clone TS106; Millipore Co., CA, USA; dilution, 1:25), anti-thymidine phosphorylase antibody (sc-47702; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; dilution, 1:100) and anti-dihydropyrimidine dehydrogenase antibody (sc-50521, Santa Cruz Biotechnology, Inc.; dilution, 1:50) for 1 h at 37°C, followed by incubation with an HRP-conjugated anti-rabbit/mouse secondary antibody for 8 min at 37°C. The reaction was detected with the Dako REAL™ EnVision™ system (Dako, Glostrup, Denmark). The slides were incubated in DAB+H₂O₂ substrate using the Ventana Chromo Map kit (Ventana Medical Systems) for 8 min at 37°C, followed by hematoxylin and bluing agent counterstaining.

For the assessment of immunostaining, the intensity and distribution percentage of stained cancer cells were initially evaluated. The intensity scores of immunostaining were divided into negative and positive staining, which were classified as: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining intensity. The distribution scores of immunostaining were divided into 3 groups as follows: 1, ≤33% of the tumor cells; 2, 33-66% of the tumor cells; and 3, 66-100% of the tumor cells. Thereafter, sum scores were calculated by adding two parameters and were then segregated into low and high expression groups.

Statistical analysis. The association of the expression of TS, DPD and TP with the clinicopathological parameters was assessed using the χ^2 or Fisher's exact tests. Overall survival (OS) was defined as the length of time from surgery to death. The Kaplan-Meier method was used to construct curves for OS. The log-rank test was used to compare distributions. To identify independent factors significantly associated with patient prognosis, the Cox's proportional hazard analysis was used with a stepwise procedure. The tests were two-sided and P<0.05 was considered to indicate a statistically significant difference. Analyses were performed using SPSS software version 19.0 (SPSS Inc, Chicago, IL, USA).

Results

Patient characteristics. The demographic details of the patients included in this study are presented in Table I. The patients included 51 (51.5%) females and 48 (48.5%) males, with a mean age of 61 years (range, 37-75 years). As regards differentiation, 56 patients (56.6%) presented with highly, 28 (28.3%) with moderately and 15 (15.2%) with poorly differentiated carcinomas. Thirty-six patients (36.4%) had pT3 or pT4 tumors and 24 (24.2%) had lymph node metastases. The post-operative stage was I, II and III in 56 (50.5%), 41 (41.4%) and 8 patients (8.1%), respectively. The median follow-up duration was 80.4 months (range, 41.3-149.9 months).

Seventy (70.7%) patients received doxifluridine and 50 (71.4%) completed 1-year medication. Twenty-nine (29.3%) patients received FP chemotherapy, of whom 24 (82.7%) completed 6 cycles. Older patients and patients with positive

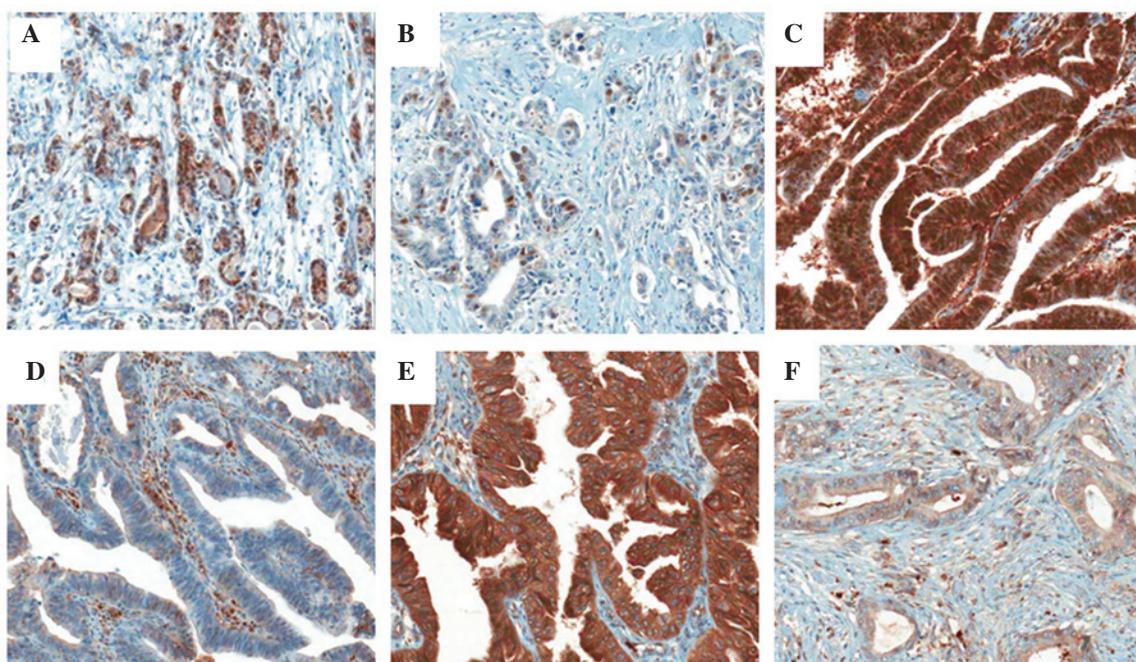


Figure 1. Expression of thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) by immunohistochemistry. (A and B) TS expression; (C and D) TP expression; (E and F) DPD expression. (A, C and E) High expression; (B, D and F) low expression (magnification, x200). Cancer cells of biliary tract origin exhibit cytoplasmic and/or nuclear brown-colored immunostaining.

lymphovascular invasion were more commonly administered doxifluridine ($P=0.003$ and 0.034 , respectively).

Expression of TS, TP and DPD. The expression rates of TS, TP and DPD were 25.3, 70.7 and 74.7%, respectively. TS, TP and DPD protein expression was observed in the cytoplasm as well as the nucleus (Fig. 1). The correlation between the clinicopathological findings and the expression of these proteins was analyzed (Table I). TS and TP expression was significantly correlated with cancer location ($P=0.044$ and 0.031 , respectively). TS and TP were more commonly expressed in extrahepatic bile duct cancer. A positive correlation was observed between the expression of TP and DPD and cancer stage ($P=0.026$ and 0.041 , respectively). Other parameters, such as age, gender, differentiation, lymphovascular invasion and resection margin status, were not associated with the expression of these proteins.

Association of TS, TP and DPD expression with clinical outcome. The univariate analysis of clinicopathological parameters and OS is presented in Table II. Age ($P=0.006$) and tumor location ($P<0.001$) were associated with OS. Patients with extrahepatic bile duct cancer exhibited shorter survival rates compared to those with cancer in other sites. T stage ($P<0.001$), lymph node metastasis ($P<0.001$), AJCC stage ($P<0.001$), differentiation ($P<0.001$), resection margin status ($P=0.001$) and type of chemotherapy regimen ($P=0.015$) were also significantly associated with OS. The patients who were administered FP exhibited improved survival rates compared to those who were administered doxifluridine.

The prognostic significance of TS, DPD and TP expression in BTC patients treated with adjuvant chemotherapy was then investigated (Table II). TS expression was not found to

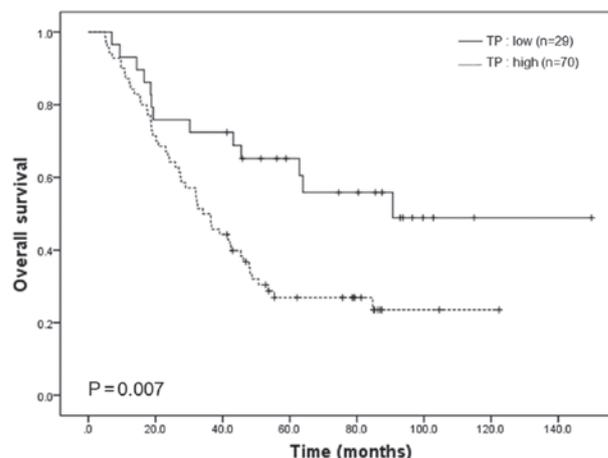


Figure 2. Overall survival curve according to the expression of thymidine phosphorylase (TP) ($P=0.007$).

be significantly correlated with OS ($P=0.222$). Patients with high TP-expressing cancers had a significantly shorter OS compared to those with low TP-expressing cancers ($P=0.007$, Fig. 2). DPD expression was of no prognostic significance in those patients ($P=0.192$).

To assess the independent prognostic value of these markers, a multivariate Cox proportional hazard analysis was used to control for other prognostic factors (Table III). Accordingly, age [hazard ratio (HR)=2.157; 95% confidence interval (CI): 1.217-3.822; $P=0.008$], stage (HR=2.234; 95% CI: 1.462-3.414; $P<0.001$), differentiation (HR=2.566; 95% CI: 1.818-3.620; $P<0.001$) and resection margin status (HR=2.748; 95% CI: 1.391-5.432; $P=0.004$), were independent prognostic factors for OS after controlling for the

Table II. Univariate analysis of OS according to clinicopathological parameters.

Clinicopathological parameters	5-year OS (%)	P-value
Age (years)		0.006
<65 (n=59)	44.3	
≥65 (n=40)	29.1	
Gender		0.838
Male (n=48)	36.0	
Female (n=51)	40.2	
Location		<0.001
Gallbladder (n=39)	58.3	
Extrahepatic bile duct (n=43)	13.8	
Ampulla (n=17)	52.9	
T stage		<0.001
1 (n=18)	77.4	
2 (n=45)	41.2	
3 (n=29)	13.8	
4 (n=7)	14.3	
N stage		<0.001
N0 (n=75)	47.7	
N1 (n=24)	8.3	
Stage ^a		<0.001
I (n=50)	61.0	
II (n=41)	12.4	
III (n=8)	25.0	
Differentiation		<0.001
High (n=56)	56.2	
Moderate (n=28)	21.2	
Poor (n=15)	0	
Lymphovascular invasion		0.354
- (n=83)	39.4	
+ (n=16)	25.0	
Resection margin		0.001
- (n=86)	42.9	
+ (n=13)	7.7	
Chemotherapy regimen		0.015
Oral 5-FU (n=70)	31.2	
5-FU + cisplatin (n=29)	54.5	
TS expression		0.222
Low (n=74)	35.1	
High (n=25)	46.8	
TP expression		0.007
Low (n=29)	65.2	
High (n=70)	26.9	
DPD expression		0.192
Low (n=25)	44.0	
High (n=74)	36.0	

^aAmerican Joint Committee on Cancer staging manual, 6th edition. OS, overall survival; FU, fluorouracil; TS, thymidylate synthase; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase.

other clinicopathological parameters. Expression of TP also exerted a significant effect on OS in the multivariate analysis (HR=2.014; 95% CI: 1.035-3.921; P=0.039).

Discussion

BTC is a heterogeneous disease and its prognosis varies according to tumor location (1,3). It is difficult to elucidate the efficacy of adjuvant chemotherapy for each type of tumor, due to the limited number of patients (4,5). To the best of our knowledge, there are currently only two RCTs available on adjuvant chemotherapy in BTC patients (6,7).

In a previous study by Takada *et al* (6), patients were randomly assigned to postoperative chemotherapy (mitomycin C and 5-FU) or surgery alone groups. The trial included 118 patients with BTC and 112 patients with gallbladder cancer. The results demonstrated that adjuvant chemotherapy did not significantly improve the 5-year survival in either the curative (41% chemotherapy arm vs. 28% surgery alone, P=0.48) or the non-curative resection patients (8% chemotherapy arm vs. 16% surgery alone, P=0.30). In the per-protocol analysis, the 5-year survival rate for gallbladder cancer patients was significantly higher with chemotherapy compared to the control arm (26 vs. 14%; P=0.0367). However, the intent-to-treat analysis identified no significant difference in the 5-year survival rate of gallbladder cancer patients between the chemotherapy and observation groups. In addition, the 5-year survival rate did not differ between bile duct and ampullary carcinoma patients.

The results of the ESPAC-3 periampullary cancer randomized trial, which was a multicenter RCT on adjuvant chemotherapy (5-FU plus folinic acid or gemcitabine) vs. observation in patients with ampullary cancer, were recently reported (7). The median survival time of the patients in the group who received chemotherapy after curative resection was not significantly different from that of the patients in the surgery alone group (43.1 vs. 35.2 months, respectively, P=0.25). However, the multivariate analysis adjusted for prognostic factors revealed a significant survival benefit associated with chemotherapy (HR=0.75; 95% CI: 0.57-0.98; P=0.03). It was concluded that adjuvant chemotherapy moderately improved survival in periampullary cancer patients. BTC patients were treated with FP or doxifluridine and a longer OS was achieved in the FP chemotherapy group (P=0.015). However, in the multivariate analysis, a regimen of chemotherapy was not an independent risk factor (HR=0.669, P=0.227), since elderly patients and lymphovascular invasion-positive patients were more commonly administered oral doxifluridine.

A previous meta-analysis revealed an insignificant benefit for adjuvant therapy in unselected BTC patients (4). However, in subgroups of high-risk patients, such as lymph node-positive disease (HR=0.49, P=0.004) and R1 disease (HR=0.36, P=0.002), postoperative adjuvant therapy appeared to be beneficial. Our data also demonstrated that TNM stage, differentiation and positive resection margin status were correlated with OS. Establishing predictive markers for chemosensitivity, other than the clinicopathological findings, may contribute to effective adjuvant chemotherapy administration in patients with a high risk of recurrence. Advances in molecular pharmacology have refined our understanding of the mechanisms of

Table III. Multivariate analysis of overall survival.

Variable	HR	95% CI	P-value
Age	2.157	1.217-3.822	0.008
Location	1.332	0.909-1.952	0.141
Stage	2.234	1.462-3.414	<0.001
Differentiation	2.566	1.818-3.620	<0.001
Resection margin	2.748	1.391-5.432	0.004
Thymidine phosphorylase	2.014	1.035-3.921	0.039
Chemotherapy regimen	0.669	0.349-1.284	0.227

HR, hazard ratio; CI, confidence interval.

action of 5-FU, as well as the mechanisms underlying resistance to chemotherapy (11). A previous study by Salonga *et al* (17) reported that TS, DPD and TP are independent predictive markers of 5-FU response and that the measurement of the three markers markedly enhanced the ability to predict tumor response to 5-FU-based chemotherapy.

The primary biochemical mechanism responsible for the cytotoxicity of 5-FU is the formation of 5-fluorouridine monophosphate (FdUMP), which binds closely to and inhibits TS in the presence of 5,10-methylene tetrahydrofolate. TS catalyzes the reductive methylation of deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP), which is the only pathway for the *de novo* synthesis of dTMP. Therefore, inhibition of TS by FdUMP disrupts the intracellular nucleotide pools necessary for DNA synthesis (11,21). Gene amplification of TS, with consequent increases in TS mRNA and protein expression, has been observed in cell lines that are resistant to 5-FU (22).

Previous clinical studies measured TS expression by immunohistochemistry and reverse-transcription PCR and demonstrated an improved clinical outcome with 5-FU-based therapy in patients with a low TS expression (23,24). A previous meta-analysis also reported an HR of 1.35 for a high TS expression in 2,610 patients with localized colorectal cancer (13). However, other studies reported discordant results in gastric cancer patients (14,15). TS expression in stage III-IV gastric cancer patients who received curative surgery followed by adjuvant chemotherapy was not associated with clinical outcomes (14). We also previously demonstrated that TS expression was not correlated with chemotherapeutic response or OS in metastatic gastric cancer patients (15) and that TS expression did not exhibit a statistically significant association with OS. Patients with lower TS expression levels exhibited a trend to correlate with a shorter OS compared to those with higher TS expression levels OS (P=0.222). Such conflicting findings may, in part, be due to the wide variation in immunohistochemical protocols and the use of different antibodies to detect p53.

The prognostic and predictive value of DPD was previously investigated. According to a previous study, low intratumoral DPD gene expression is associated with improved response to 5-FU (17). These findings presumably reflect a higher DPD-mediated degradation of 5-FU in these tumors. Findings of a previous study demonstrated that a low DPD expression was associated with better survival in stage III colorectal cancer

patients treated with 5-FU chemotherapy (10). However, the majority of published studies reported no significant association between DPD expression and prognosis (25). In this study, DPD expression was not found to be clinically significant in BTC.

TP converts 5'-DFUR to the active drug 5-FU by cleaving the 5-deoxyribose moiety, whereas, through the addition of 2'-deoxyribose-1-phosphate, TP may activate 5-FU to 5-fluoro-2'-deoxyuridine, a precursor of FdUMP, which inhibits TS, responsible for *de novo* thymidylate synthesis (11). It was previously demonstrated that high levels of TP affected 5-FU sensitivity and patients with a high TP expression exhibited higher survival rates (18). However, the elucidation of the role of TP in modulating 5-FU responsiveness has been challenging, due to contradictory preclinical and clinical data. According to previous findings, a high TP expression correlated with low sensitivity to 5-FU (17).

TP was found to possess angiogenic properties and to promote tumor growth. TP was found to be strongly angiogenic in a rat sponge and freeze-injured skin graft model, whereas the overexpression of TP in MCF-7 breast cancer cells markedly enhanced tumor growth *in vivo* (26). Consistent with the hypothesis that increased tumor vascularization leads to a greater metastatic propensity, higher TP levels were found to be associated with more aggressive bladder tumors (27). According to evidence reported in the literature, high TP expression levels are associated with a negative prognosis (25). According to a recent study, a low TP expression was associated with a trend for prolonged survival in stage III colorectal cancer treated with 5-FU, indicative of the response to chemotherapy in those patients (10). Therefore, a high TP expression may be a marker for a more invasive and aggressive tumor phenotype that is less responsive to chemotherapy. Our results have also demonstrated that TP expression was more common in extrahepatic bile duct cancer patients and there was a significant correlation between TP expression levels and OS in BTC cancer patients receiving postoperative FU-based adjuvant chemotherapy.

In conclusion, based on these findings, an immunohistochemical evaluation of TP expression may be beneficial in predicting the survival of BTC patients treated with 5-FU-based adjuvant chemotherapy. Confirmation of these results by a clinical trial with a larger patient sample may provide a promising selection tool for the most appropriate chemotherapeutic regimen in BTC patients.

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