Jpn. J. Cancer Res. **92**, 1127–1132, October 2001

High Expression of Thymidylate Synthase Leads to Resistance to 5-Fluorouracil in Biliary Tract Carcinoma *in vitro*

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To evaluate the effect of chemotherapy of 5-fluorouracil (5-FU) in human biliary tract carcinoma, we studied 5-FU sensitivity, thymidylate synthase (TS) content, and dihydropyrimidine dehydrogenase (DPD) activity in 4 human biliary tract carcinoma cell lines compared to 12 various digestive carcinoma cell lines of human organs *in vitro*. 5-FU sensitivity in the cell lines was analyzed by MTT assay. TS content was analyzed by the [6-^{3}H]FdUMP binding assay method, and DPD activity was analyzed by thin-layer chromatography (TLC). 5-FU IC_{50} values of biliary tract carcinoma cell lines were significantly higher than those of the carcinoma cell lines of the other digestive organs: 97, 45, 119, and 194 times the concentration of the other digestive, pancreas, colon, and gastric carcinoma cell lines, respectively. TS content of biliary tract carcinoma cell lines was also significantly greater than that of the carcinoma cell lines of the other organs. No difference in DPD activity, however, was recognized between the carcinoma cell lines of each organ. TS content in the cell lines significantly correlated with 5-FU sensitivity, but DPD activity did not. Therefore, in the present study, TS expression was concluded to influence the high resistance to 5-FU of biliary tract carcinoma in comparison with the carcinomas of the other digestive organs.

Key words: Thymidylate synthase — Biliary tract carcinoma — 5-Fluorouracil — Dihydropyrimidine dehydrogenase

Biliary tract carcinomas are infrequent tumors with a dismal prognosis. Although surgery is the most effective therapy for these tumors, in many cases they are unresectable because of invasion into adjacent major blood vessels, the presence of extensive intraductal spread, peritoneal seeding, or distant lymph node metastasis.[1)] In addition, recurrence rates are very high even if curative surgical resection has been achieved.[1)] Therefore, the role of chemotherapy or radiotherapy for these tumors is necessarily important, yet there are only a few published trials of chemotherapy or radiotherapy, and there is little evidence of effective response.[2–4)]

One of the oldest anti-cancer drugs, 5-fluorouracil (5-FU), first synthesized by Heidelberger *et al.* in 1957,[5)] is now widely used in digestive carcinoma chemotherapy, as well as in biliary tract carcinoma. 5-FU cellular activation occurs via several enzyme pathways[6–8)] leading to at least three well-identified cellular targets, i.e., thymidylate synthase (TS), RNA, and DNA. TS is the target enzyme inhibiting DNA biosynthesis when 5-FU is converted in tumor cells to FdUMP, which forms a tight-binding covalent ternary complex with TS in the presence of the folate cofactor 5,10-methylene tetrahydrofolate.[6–8)] The clinical importance of TS has been established by experimental[9, 10)] and clinical studies.[11)] Dihydropyrimidine dehydrogenase (DPD) is the first rate-limiting enzyme of the chain of reactions that regulate 5-FU catabolism[6)] and plays an important role in determining tissue levels of 5-FU.[12–15)] The importance of catabolism and, particularly, DPD in 5-FU chemotherapy has been demonstrated in a recent report of a patient with a complete deficiency of DPD activity.[13)] Overproduction of TS or DPD has been shown to correlate with 5-FU resistance.[9, 15)] Analyses of these enzymes' expression are valuable for the prediction of 5-FU effectiveness *in vitro*.[10, 16–18)] It has been shown that tumoral TS or DPD expression is not only linked to 5-FU treatment response, but also to patient survival in gastric carcinoma[19–22)] and colorectal carcinoma.[23–27)] Thus, TS and DPD expression may influence tumoral 5-FU sensitivity and be predictive of the effectiveness of 5-FU-based chemotherapy.

Given that in biliary tract carcinoma the overall clinical response rate to 5-FU is still low,[2)] and there has not yet been any theoretical study of the relation between TS and DPD expression and sensitivity to 5-FU, we studied TS and DPD expression and 5-FU sensitivity in human biliary tract carcinoma cell lines *in vitro*.

MATERIALS AND METHODS

Cell lines Sixteen human digestive carcinoma cell lines, including 4 biliary tract carcinomas; 1 bile duct carcinoma (SK-ChA-1[28)]) and 3 gallbladder carcinomas (NOZ,[29)] Mz-ChA-2,[28)] TGBC-2TKB[1)]), 4 pancreas carcinomas (MIA-PaCa-2, PANC-1, AsPC-1, BxPC-3), 5 colon carcinomas (Caco-2, COLO 320DM, DLD-1, HCT-15, NCI-H747), and 3 gastric carcinomas (MKN-28, MKN45, MKN74), were examined. SK-ChA-1, Mz-ChA-2, and TGBC-2TKB

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Table I. Characteristics of Biliary Tract Carcinoma Cell Lines

Cell lines	Origin	Histology
SK-ChA-1	metastatic bile duct cancer (ascites)	poorly differentiated adenocarcinoma
NOZ	metastatic gallbladder cancer (ascites)	moderately differentiated adenocarcinoma
Mz-ChA-2	metastatic gallbladder cancer (liver)	moderately differentiated adenocarcinoma
TGBC-2TKB	metastatic gallbladder cancer (lymph node)	poorly differentiated adenocarcinoma

were kind gifts from Dr. Todoroki (Tsukuba University School, Ibaraki). NOZ was a kind gift from Dr. Nagamori (Jikei University School of Medicine, Tokyo). Three gastric carcinoma cell lines were purchased from Japanese Cancer Research Resources Bank (Tokyo). Four pancreas carcinoma cell lines and 5 colon carcinoma cell lines were purchased from American Type Culture Collection (Manassas, VA). Four biliary tract carcinoma cell lines and 2 pancreas carcinoma cell lines (MIAPaCa-2, PANC-1) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Two pancreas carcinoma cell lines (AsPC-1, BxPC-3), 5 colon carcinoma cell lines, and 3 gastric carcinoma cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The characteristics of the biliary tract carcinoma cell lines are summarized in Table I.

5-FU sensitivity Equal numbers of cells (5×10^4 cells/ml) harvested during the exponential part of their growth phase from each cell line were plated in 100 μl per well (5×10^3 cells/well) on 96-well plates and incubated at 37°C in an atmosphere containing 5% CO_2 in each medium with 10% fetal bovine serum. After 24 h, various concentrations of 5-FU (2^{14}–2^{-3} μM) were added to each well. The growth inhibition was assessed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT test; Sigma, St Louis, MO)[30] 72 h after 5-FU exposure. The 5-FU concentration causing 50% growth inhibition as compared to the control (IC_{50}) were determined for each cell line by using a curve of cell number versus logarithm of drug concentration. The 5-FU IC_{50} was evaluated at least 4 times for each cell line.

TS content Cells harvested in the growth phase were washed with PBS twice and 3 volumes of homogenate buffer were added, consisting of 200 m*M* Tris-HCl buffer pH 7.4 with 20 m*M* 2-mercaptoethanol (2-ME), 15 m*M* cytidine 5′-monophosphate (5′-CMP), and 100 n*M* NaF pH 7.4. After sonication on ice and immediate centrifugation at 3000 rpm for 15 min at 4°C, the cell suspension was centrifuged at 105 000*g* for 60 min at 4°C. Fifty microliters of homogenized cell suspension with 50 μl of buffer, consisting of 600 m*M* NH_4HCO_3 buffer (pH 8.0) containing 100 m*M* 2-ME, 15 m*M* 5′-CMP, and 100 n*M* NaF, was incubated for 3 h at 0°C. The [6-^{3}H]FdUMP binding assay was performed in a total volume of 175 μl, containing 50 μl of [6-^{3}H]FdUMP (7.8 pmol), 2 m*M* tetrahydrofolic acid, 16 m*M* sodium ascorbate, 9 m*M* formaldehyde, 15 m*M* 5′-CMP, 20 m*M* β-mercaptoethanol, 100 m*M* NaF, 2% bovine serum albumin, and 50 m*M* KH_2PO_4 pH 7.4, with incubation for 20 min at 30°C. Excess [6-^{3}H]FdUMP was removed by adding 2 ml of 0.5 *N* $HClO_4$, sonication, and centrifugation at 3000 rpm for 15 min at 4°C twice. The precipitate was solubilized with 0.5 ml of HCOOH and the residual radioactivity in the supernatant, representing enzyme-bound FdUMP, was counted by liquid scintigraphy. The results of all assays were standardized for cytosolic protein and expressed in picomols per milligram of cytosolic protein.

DPD activity Twenty-five microliters of reaction mixture, consisting of 35 m*M* NaH_2PO_4 pH 7.5, 2.5 m*M* $MgCl_2$, 0.25 *M* NADPH, and 20 μl [6-^{14}C]5-FU after preincubation at 37°C for 1 min, was added to 25 μl of the cell suspension obtained after homogenization and gel-filtration of cells or tumor tissues. The duration of incubation was 15 min at 37°C. The reaction was stopped by letting the mixture stand for 2 min in boiled water at 90°C and adding 25 μl of 0.36 *M* KOH. The samples with 25 μl of 0.36 *M* $HClO_4$, after standing for 30 min at room temperature, were centrifuged at 15 000 rpm for 5 min at 4°C. The supernatant (5 μl) was applied to a thin-layer chromatography (TLC) plate (Silica gel 60 F_{254}, Merck, Darmstadt, Germany). DPD activity was calculated by measuring radioactivity with a scintillation counter (BAS 2,000, Fujix, Tokyo). All assay results were standardized for reaction time (15 min) and cytosolic protein, and the results of DPD activity were expressed in picomols of [^{14}C]5-FU catabolized per minute and per milligram of cytosolic protein.

Statistics Linear regressions and Student's *t* tests were performed on Microsoft Excel 2000 for Windows Millennium Edition. Analyzed variables were 5-FU IC_{50}, TS content, and DPD activity in the cell lines. *P* values less than 0.05 were considered significant.

RESULTS

The 5-FU sensitivity, TS content, and DPD activity of the 16 cell lines are summarized in Table II.

5-FU sensitivity 5-FU sensitivity showed marked vari-

Table II. 5-FU IC_{50}, TS Content, and DPD Activity in the Cell Lines

Cell lines	5-FU IC_{50} [log 2 (μM)]	TS content [pmol/mg protein]	DPD activity [pmol/min/mg protein]
Biliary tract (n=4)	9.8±1.3	0.567±0.318	35.20±67.23
SK-ChA-1	9.6±3.1	0.571	135.98
NOZ	11.2±0.8	1.006	4.80
Mz-ChA-2	8.1±1.4	0.264	ND[d]
TGBC-2TKB	10.1±1.1	0.427	ND
Others (n=12)	3.2±1.5[a]	0.183±0.076[b]	26.31±59.02
Pancreas (n=4)	4.3±1.1[a]	0.182±0.057[b]	61.78±97.10
MIAPaCa-2	3.7±0.9	0.160	205.37
PANC-1	5.6±1.6	0.138	5.02
AsPC-1	4.8±2.3	0.265	36.73
BxPC-3	3.2±1.6	0.164	ND
Stomach (n=3)	2.2±0.8[a]	0.228±0.097[c]	21.07±29.32
MKN28	1.4±0.6	0.326	54.55
MKN45	2.1±0.6	0.227	ND
MKN74	3.0±1.6	0.132	8.65
Colon (n=5)	2.9±1.7[a]	0.157±0.079[b]	1.09±2.43
Caco-2	3.2±1.0	0.114	ND
COLO 320DM	0.9±1.1	0.276	ND
DLD-1	2.1±1.4	0.195	ND
HCT-15	2.8±1.2	0.121	ND
NCI-H747	5.4±1.9	0.079	5.43

a) $P<0.001$ compared to biliary tract carcinoma cell lines.
b) $P<0.05$ compared to biliary tract carcinoma cell lines.
c) $P<0.060$ compared to biliary tract carcinoma cell lines.
d) ND: not detectable because catabolic reaction compounds by DPD amounted to less than 5 pmol.

ability among the cell lines, with 5-FU IC_{50} values lying in a 1261-fold range from $2^{0.9}$ (1.9) μM (COLO 320DM) to $2^{11.2}$ (2352) μM (NOZ). 5-FU sensitivity, measured in terms of 5-FU IC_{50}, of biliary tract carcinoma cell lines was significantly poorer than that of the carcinoma cell lines of the other digestive organs. 5-FU IC_{50} of biliary tract carcinoma cell lines was 97 times higher than that of the other digestive carcinoma cell lines ($P<0.001$), 45 times higher than that of pancreas carcinoma cell lines ($P<0.001$), 119 times higher than that of colon carcinoma cell lines ($P<0.001$), and 194 times higher than that of gastric carcinoma cell lines ($P<0.001$).

TS content TS content was measurable in all cell lines and varied over a 12.7-fold range from 0.079 pmol/mg protein (NCI-H747) to 1.006 pmol/mg protein (NOZ). TS content of biliary tract carcinoma cell lines was significantly greater than those of the carcinoma cell lines of the other digestive organs; 3.10 times greater than that of the other digestive carcinoma cell lines ($P<0.05$), 3.12 times greater than that of pancreas carcinoma cell lines ($P<0.05$), 3.61 times greater than that of colon carcinoma cell lines ($P<0.05$), and 2.49 times greater than that of gastric carcinoma cell lines ($P=0.060$).

DPD activity DPD activity was detected in only 8 cell lines and showed a 41-fold range of variation from 4.80 pmol/min/mg protein (NOZ) to 205.37 pmol/min/mg protein (MIAPaCa-2). In the other 8 cell lines, DPD activity could not be detected because catabolic reaction compounds amounted to less than 5 pmol. The difference in DPD activity between the cell lines of biliary tract carci-

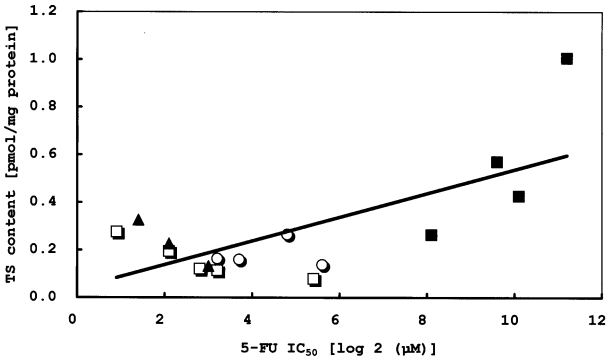

Fig. 1. Correlation of 5-FU IC_{50} and TS content in the cell lines. 5-FU IC_{50} was significantly correlated with TS content. $R=0.700$, $P=0.003$, $y=0.038+0.050x$. ■ biliary tract (4), ○ pancreas (4), □ colon (5), ▲ stomach (3).

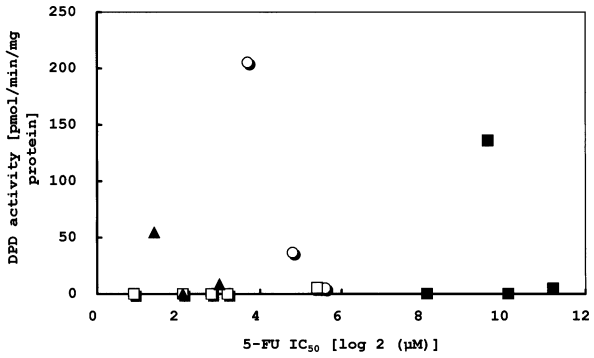

Fig. 2. Correlation of 5-FU IC_{50} and DPD activity in the cell lines. 5-FU IC_{50} was not correlated with DPD activity. R=0.086, P=0.571. ■ biliary tract (4), ○ pancreas (4), □ colon (5), ▲ stomach (3).

noma and the other organs was not statistically significant. **Correlation of 5-FU sensitivity, TS content, and DPD activity** Simple linear regression analysis showed that TS content of the cell lines was significantly correlated to 5-FU sensitivity (log 2 5-FU IC_{50}) (R=0.700, P=0.003); the greater the enzyme content, the higher 5-FU IC_{50} (Fig. 1). However, DPD activity was not correlated to 5-FU sensitivity (R=0.086, P=0.571) (Fig. 2). Nor was any correlation of TS content and DPD activity observed (R=0.088, P=0.747).

DISCUSSION

Chemotherapy of biliary tract carcinoma represents an unexplored field of clinical investigation. The major reasons for the lack of development in chemotherapy include the rarity, delayed presentation, and difficulty of curative resection of these tumors.[1, 4] There have been only a few controlled studies on chemotherapy of biliary tract carcinoma.[2, 3] The drugs 5-FU, adriamycin, or mitomycin C alone or in combination have been mainly used for the chemotherapy of biliary tract carcinoma, but the administration of those drugs only showed limited effectiveness.[3] This fact has been well recognized, but the reasons for it remain unclear. The primary reasons for this insufficient progress in the chemotherapy of these tumors are the difficulty of establishing cell lines of these tumors for experimental study and the difficulty of accurate preoperative diagnosis.[28] Therefore, the present study represents an indispensable step in evaluating the effectiveness of 5-FU-based chemotherapy in biliary tract carcinoma.

A significant finding in the present study was that the 5-FU sensitivity of biliary tract carcinoma cell lines was significantly poorer than those of other digestive organs, directly indicating that 5-FU based chemotherapy promises limited effectiveness in biliary tract carcinoma. Further, the TS content of biliary tract carcinoma cell lines was significantly greater than that of the carcinoma cell lines of the other digestive organs, and TS content was significantly correlated to 5-FU sensitivity; the greater the enzyme content, the higher 5-FU IC_{50}. It can thus be concluded that poor 5-FU sensitivity may be strongly influenced by TS content in biliary tract carcinoma cell lines. However, a difference in DPD activity was not recognized between the carcinoma cell lines of different organs, and DPD activity in the cell lines was not correlated to 5-FU sensitivity. These results *in vitro* suggested that DPD activity might not be associated with resistance to 5-FU. Several recent reports of studies *in vivo* or in the human body have indicated that analyses of DPD expression were useful in the prediction of the effectiveness of 5-FU-based chemotherapy.[17, 18, 22, 27] Our results failed to confirm the findings of these previous reports, and therefore this discrepancy should bring into question the appropriateness of their evaluation of DPD expression *in vitro*.

In conclusion, in the present study *in vitro*, human biliary tract carcinoma showed distinctly different characteristics from carcinoma of other organs, displaying significantly greater resistance to 5-FU, with TS expression as a major factor. Therefore, in clinical terms simple administration of 5-FU in cases of human biliary tract carcinoma may be nearly meaningless. For the purpose of establishing an effective chemotherapy of biliary tract carcinoma, treatment with newly developed agents or combined administration of drugs, or the examination of the 5-FU-metabolizing enzymes by use of DNA arrays, should be the subjects of further studies.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Nagayama and Mr. Ikeda (Taiho Pharmaceutical Co., Ltd., Tokushima) for their technical assistance.

(Received May 10, 2001/Revised July 17, 2001/Accepted July 24, 2001)

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