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 [6-3H]FdUMP binding assay method, and DPD activity was analyzed by thin-layer chromatography (TLC). 5-FU IC50 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-Fluouracil — 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, 3

One of the oldest anti-cancer drugs, 5-fluorouracil (5-FU), first synthesized by Heidelberger et al. in 1957,4 is now widely used in digestive carcinoma chemotherapy, as well as in biliary tract carcinoma. 5-FU cellular activation occurs via several enzyme pathways5-8 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 toFdUMP, 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 experimental9, 10 and clinical studies.10 Dihydropyrimidine dehydrogenase (DPD) is the first rate-limiting enzyme of the chain of reactions that regulate 5-FU catabolism6 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 carcinoma19-22 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-128) and 3 gallbladder carcinomas (NOZ,29 Mz-ChA-2,30 TGBC-2TKB), 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
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 pancreatic 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 (5×10^4 cells/well) on 96-well plates and incubated at 37°C in an atmosphere containing 5% CO_2 in each well. The growth inhibition was assessed by means of the MTT test (Sigma, St Louis, MO) after 72 h of 5-FU exposure. The 5-FU concentration causing 50% growth inhibition as compared to the control (IC50) were determined for each cell line by using a curve of cell number plotted against 5-FU concentration.

### TS content
Cells harvested in the growth phase were washed with PBS twice and 3 volumes of homogenate buffer were added, consisting of 200 mM Tris-HCl buffer pH 7.4 with 20 mM 2-mercaptoethanol (2-ME), 15 mM cytidine 5'-monophosphate (5'-CMP), and 100 mM 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 000g for 60 min at 4°C. Fifty microliters of homogenized cell suspension with 50 µl of buffer, consisting of 600 mM NH_4HCO_3 buffer (pH 8.0) containing 100 mM 2-ME, 15 mM 5'-CMP, and 100 mM NaF, was incubated for 3 h at 0°C. The [6-3H]FdUMP binding assay was performed in a total volume of 175 µl, containing 50 µl of [6-3H]FdUMP (7.8 pmol), 2 mM tetrahydrofolic acid, 16 mM sodium ascorbate, 9 mM formaldehyde, 15 mM 5'-CMP, 20 mM β-mercaptoethanol, 100 mM NaF, 2% bovine serum albumin, and 50 mM KH_2PO_4 pH 7.4, with incubation for 20 min at 30°C. Excess [6-3H]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 mM NaH_2PO_4 pH 7.5, 2.5 mM MgCl_2, 0.25 M NADPH, and 20 µl [6-14C]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 [14C]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 IC50, 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-
ability among the cell lines, with 5-FU IC$_{50}$ values lying in a 1261-fold range from 20.9 (1.9) µM (COLO 320DM) to 211.2 (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-
The 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 IC50) ($R=0.700$, $P=0.003$); the greater the enzyme content, the higher 5-FU IC50 (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 IC50. 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.

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**REFERENCES**

1) Moon, Y., Dahlberg, W. K., Yu, Y., Ohno, T., Todoroki, T. and Little, J. B. Radiosensitivity of human biliary tract cancer cell lines in vitro. *Int. J. Oncol.*, 10, 545–551 (1997).

2) Harvey, J. H., Smith, F. P. and Schein, P. S. 5-Fluorouracil, mitomycin, doxorubicin (FAM) in carcinoma of the biliary tract, *J. Clin. Oncol.*, 2, 1245–1248 (1984).

3) Smith, G. W., Bukowski, R. M., Hewlett, J. S. and Groppe,
C. W. Hepatic artery infusion of 5-fluorouracil and mitomycin C in cholangiocarcinoma and gallbladder carcinoma. *Cancer*, **54**, 1513–1516 (1984).

4) Pitt, H. A., Nakeeb, A., Abrams, R. A., Coleman, J., Piantadosi, S., Yeo, C. J., Lillemoe, K. D. and Cameron, J. L. Preoperative cholangiocarcinoma: postoperative radiotherapy does not improve survival. *Ann. Surg.*, **221**, 788–798 (1995).

5) Heidelberger, C., Chaudhuri, N. K., Danenberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Pleven, E. and Scheiner, J. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature*, **179**, 663–666 (1957).

6) Diasio, R. B. and Harris, B. E. Clinical pharmacology of 5-fluorouracil. *Clin. Pharmacokinet.*, **16**, 215–237 (1989).

7) Parker, W. B. and Cheng, Y. C. Metabolism and mechanisms of action of 5-fluorouracil. *Pharmacol. Ther.*, **48**, 381–395 (1990).

8) Weckbecker, G. Biochemical pharmacology and analysis of fluoropyrimidine alone and in combination with modulators. *Pharmacol. Ther.*, **50**, 367–424 (1991).

9) Berger, S. H., Jehn, C. H., Johnson, L. F. and Berger, F. G. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol. Pharmacol.*, **28**, 461–467 (1985).

10) Okabe, H., Tsujimoto, H. and Fukushima, M. Preparation of the antibodies against recombinant human thymidylate synthase for the detection of its intratumoral levels and the application to sensitivity-study of 5-fluorouracil. *Oncol. Rep.*, **4**, 685–690 (1997).

11) Peters, G. J., Van der Wilt, C. L., Van Triest, B., Codacci-Pisanelli, G., Johnston, P. F., Van Groeningen, C. J. and Pinedo, H. M. Thymidylate synthase and drug resistance. *Eur. J. Cancer*, **31**, 1299–1305 (1995).

12) Heggie, G. D., Sommadossi, J. P., Cross, D. S., Huster, W. J. and Diasio, R. B. Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. *Cancer Res.*, **47**, 2203–2206 (1987).

13) Diasio, R. B., Beavers, T. L. and Carpenter, J. T. Familial deficiency of dihydropyrimidine dehydrogenase; biochemical basis for familial pyrimidinemia and severe 5-fluorouracil-induced toxicity. *J. Clin. Invest.*, **81**, 47–51 (1988).

14) Harris, B. E., Song, R., Soong, S. J. and Diasio, R. B. Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. *Cancer Res.*, **50**, 197–201 (1990).

15) Etienne, M. C., Cheradame, S., Fischel, J. L., Formento, P., Dassonville, O., Renee, N., Schneider, M., Thys, A., Demard, F. and Milano, G. Response to fluorouracil therapy in cancer patients: the role of tumoral dihydropyrimidine dehydrogenase activity. *J. Clin. Oncol.*, **13**, 1663–1670 (1995).

16) Beck, A., Etienne, M. C., Cheradame, S., Fischel, J. L., Formento, P., Renee, N. and Milano, G. A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumor sensitivity to fluorouracil. *Eur. J. Cancer*, **30**, 1517–1522 (1994).

17) Nita, M. E., Tominaga, O., Nagawa, H., Tsuruo, T. and Muto, T. Dihydropyrimidine dehydrogenase but not thymidylate synthase expression is associated with resistance to 5-fluorouracil in colorectal cancer. *Hepatogastroenterology*, **45**, 2117–2122 (1998).

18) Irinoda, T., Terashima, M., Kawamura, H. and Takagane, A. Prediction of 5-fluorouracil sensitivity by measurement of thymidylate synthase activity and dihydropyrimidine dehydrogenase activity against gastric cancer. *Jpn. J. Gastroenterol. Surg.*, **32**, 1955–1961 (1999).

19) Lenz, H. J., Leichman, C. G., Danenberg, K. D., Danenberg, P. V., Grosen, S., Cohen, H., Laine, L., Crookes, P., Silberman, H., Baranda, J., Garcia, Y., Li, J. and Leichman, L. Thymidylate synthase in mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. *J. Clin. Oncol.*, **14**, 176–182 (1996).

20) Kuniyasu, T., Nakamura, T., Tabuchi, Y. and Kuroda, Y. Immunohistochemical evaluation of thymidylate synthase in gastric carcinoma using a new polyclonal antibody. *Cancer*, **83**, 1300–1306 (1998).

21) Yeh, K. H., Shun, C. T., Chen, C. L., Lin, J. T., Lee, W. J., Lee, P. H., Chen, Y. C. and Cheng, A. L. High expression of thymidylate synthase is associated with the drug resistance of gastric carcinoma to high dose 5-fluorouracil-based systemic chemotherapy. *Cancer*, **82**, 1626–1631 (1998).

22) Ishikawa, Y., Kubota, T., Otani, Y., Watanabe, M., Teramoto, T., Kumai, K., Takechi, T., Okabe, H., Fukushima, M. and Kitajima, M. Dihydropyrimidine dehydrogenase and messenger RNA levels in gastric cancer: possible predictor for sensitivity to 5-fluorouracil. *Ipn. J. Cancer Res.*, **91**, 105–112 (2000).

23) Johnston, P. G., Fisher, E. R., Rockette, H. E., Fisher, B., Wolmark, N., Drake, J. C., Chabner, B. A. and Allegra, C. J. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J. Clin. Oncol.*, **12**, 2640–2647 (1994).

24) Kornmann, M., Link, K. H., Lenz, H. J., Pillasch, J., Metzger, R., Butzer, U., Leder, G. H., Weinidel, M., Saki, F., Danenberg, K. D., Beger, H. G. and Danenberg, P. V. Thymidylate synthase is a predictor for response and resistance in hepatic infusion chemotherapy. *Cancer Lett.*, **118**, 29–35 (1997).

25) Lenz, H. J., Hayashi, K., Salonga, D., Danenberg, K. D., Danenberg, P. V., Metzger, R., Banerjee, D., Berti, J. R., Grosen, S., Leichman, L. P. and Leichman, C. G. p53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. *Clin. Cancer Res.*, **4**, 1243–1250 (1998).

26) Yamachika, T., Nakanishi, H., Inada, K., Tsukamoto, T., Kato, T., Fukushima, M., Inoue, M. and Tatematsu, M. A new prognostic factor for colorectal carcinoma, thymidylate
synthase, and its therapeutic significance. *Cancer, 82*, 70–77 (1998).

27) Salonga, D., Danenberg, K. D., Johnson, M., Metzger, R., Groshen, S., Tsao Wei D. D., Lenz, H. J., Leichman, C. G., Leichman, L., Diasio, R. B. and Danenberg, P. V. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin. Cancer Res.*, 6, 1322–1327 (2000).

28) Knuth, A., Gabbert, H., Dippold, W., Klein, O., Sachsse, W., Bitter-Suermann, D., Prellwitz, W. and Meyer zum Büschenfelde, K. H. Biliary adenocarcinoma characterisation of three new human tumor cell lines. *J. Hepatol.*, 1, 579–596 (1985).

29) Homma, S., Hasumura, S., Nagamori, S. and Kameda, H. Establishment and characterization of a human gall bladder carcinoma cell line NOZ. *Hum. Cell*, 1, 95–97 (1988).

30) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65, 55–63 (1983).