Thymidylate Synthase Expression Predicts the Response to 5-Fluorouracil-based Adjuvant Therapy in Pancreatic Cancer

Ying Chuan Hu, Richard A. Komorowski, Shannon Graewin, Galen Hostetter, Olli-P. Kallioniemi,1 Henry A. Pitt, and Steven A. Ahrendt2

Department of Surgery, University of Rochester, Rochester, New York 14642 [Y. C. H., S. A. A.]; Departments of Pathology [R. A. K.] and Surgery [S. G., H. A. P.], Medical College of Wisconsin, Milwaukee, Wisconsin 53226; and Cancer Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, Maryland 20892 [G. H., O.-P. K.]

ABSTRACT

Purpose: Thymidylate synthase (TS) is the target enzyme for 5-fluorouracil (5-FU), and TS expression may determine clinical response and survival after therapy with 5-FU in colorectal cancer. 5-FU is also widely used in the adjuvant therapy of pancreatic cancer. Therefore, we explored the hypothesis that TS expression was associated with patient prognosis and the response to adjuvant therapy in pancreatic cancer.

Experimental Design: Cylindrical tissue cores from a large retrospective, nonrandomized series covering 132 resected patients were used to build a pancreatic cancer tissue microarray. TS expression was determined using immunohistochemistry.

Results: High intratumoral TS expression and low intratumoral TS expression were present in 83 of 132 (63%) and 49 of 132 (37%) tumors, respectively. Median survival among patients with low intratumoral TS expression (18 months) was longer than that among patients with high TS expression (12 months). In multivariate analysis, more advanced pathological stage [risk ratio (RR) = 1.70; P = 0.015], poorly differentiated histology (RR = 1.71; P = 0.015), management with adjuvant therapy (RR = 0.49; P = 0.011), and high TS expression [RR = 1.66; 95% confidence interval (CI) = 1.05–2.63; P = 0.029] were independent predictors of mortality. The risk of death was significantly reduced by any adjuvant therapy (RR = 0.40; 95% CI = 0.18–0.90; P = 0.001) among patients with high TS expression. This difference in survival among patients with low- and high- TS-expressing tumors became more significant when the analysis was restricted to the 73 patients receiving 5-FU-based adjuvant therapy (RR = 0.37; 95% CI = 0.16–0.86; P = 0.0006). In contrast, 5-FU-based adjuvant therapy did not influence survival among patients with low-TS-expressing pancreatic cancer.

Conclusions: High TS expression is a marker of poor prognosis in resected pancreatic cancer. Patients with high intratumoral TS expression benefit from adjuvant therapy.

INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer death in the United States, with an overall 5-year survival after diagnosis of 4% (1). For the 15–20% of patients with localized pancreatic cancer, resection offers the only chance for long-term survival. However, the vast majority of patients with resected pancreatic cancer will develop disseminated disease, and the 5-year survival in resected patients with pancreatic cancer remains only 17–24% (2, 3). Thus, a large percentage of patients receive chemotherapy and/or radiotherapy before or after surgical resection to reduce the high risk of treatment failure seen with surgery alone. Several pathological factors have consistently been associated with improved survival after resection, including small tumor size, the absence of regional lymph node metastases, early pathological stage, negative resection margins, low tumor grade, and the absence of aneuploidy (3–5). However, these prognostic factors have not proven useful in predicting the response to adjuvant therapy.

A survival benefit for adjuvant 5-FU3-based chemoradiation for pancreatic cancer has yet to be definitively established (6, 7). A small randomized trial published in 1987 by the Gastrointestinal Tumor Study Group demonstrated improved median and long-term survival in patients receiving adjuvant 5-FU and external beam radiation (8). A larger nonrandomized series confirmed these findings with a significant increase in median survival from 14 to 20 months in patients receiving adjuvant chemotherapy (3). However, this improved survival may represent the administration of adjuvant therapy to better-risk patients. A small prospective randomized trial of adjuvant chemoradiation using infusional 5-FU versus observation also demonstrated an increase in median survival with chemoradiation from 13 to 17 months (2). However, this increase failed to reach statistical significance (P = 0.099) in this underpowered trial (2). More recently, a large, prospective randomized trial failed to demonstrate any survival benefit for adjuvant chemoradiotherapy but did demonstrate a potential benefit for adjuvant chemotherapy with 5-FU (5).

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1 Present address: VTT Technical Research Centre of Finland, Turku, Finland.
2 To whom requests for reprints should be addressed, at Department of Surgery, Box SURG, University of Rochester, 601 Elmwood Avenue, Rochester, NY 14642. Phone: (716) 275-2147; Fax: (716) 275-8513; E-mail: steven_ahrendt@urmc.rochester.edu.

3 The abbreviations used are: 5-FU, 5-fluourouracil; TS, thymidylate synthase; CI, confidence interval; RR, risk ratio; TNM, tumor-node-metastasis.
Recent technological advances have continued to expand our understanding of the molecular biology of pancreatic cancer. However, this information has yet to be translated into useful prognostic or predictive markers, which guide clinical decision-making. TS is a critical enzyme in the de novo synthesis of dTMP. dTMP is required for DNA synthesis and is the cellular target for 5-FU. To further distinguish which patients are most likely to benefit from 5-FU-based adjuvant therapy, we examined the effect of TS expression on the response to adjuvant therapy in patients with pancreatic cancer.

PATIENTS AND METHODS

Patient Characteristics. Formalin-fixed, paraffin-embedded tumor tissue was collected retrospectively from 138 patients, who underwent pancreatic resection for pancreatic ductal adenocarcinoma at Strong Memorial Hospital/University of Rochester (n = 79) or Froedtert Memorial Lutheran Hospital/Medical College of Wisconsin (n = 59) between January 1994 and February 2002. Distal bile duct, ampullary, and duodenal adenocarcinomas as well as other pancreatic neoplasms (mucinous cystic adenocarcinoma and intraductal papillary mucinous tumors with adenocarcinoma) were excluded from this study. Any patient who received preoperative therapy (neoadjuvant chemotherapy and radiotherapy) was not eligible to participate. All pathology reports were reviewed, and TNM stage and grade were assigned using American Joint Committee on Cancer criteria (9). Surgical margins were considered positive if infiltrating adenocarcinoma was present at the uncinate process, retroperitoneal soft tissue, or final pancreatic neck margin. Four patients died within 30 days of surgery from perioperative complications and were excluded from analysis.

Clinical information was obtained from a review of hospital and physician charts or from the respective hospital tumor registry. Patient follow-up was obtained through the review of hospital and physician records, direct patient contact, and the Social Security Death Index. Two patients were lost to follow-up before the completion of the study. This research protocol was reviewed and approved by the University of Rochester Research Subjects Review Board.

Construction of Pancreatic Cancer Tissue Microarray. H&E-stained standard slides were reviewed from each pancreatic cancer, and a representative tumor region and the corresponding formalin-fixed paraffin tissue block were selected for use in the tissue microarray (10). Two discrete histomorphologically representative regions were selected from each tissue block. Three 0.6-mm tissue cores were taken from each region using an automated custom-built tissue arrayer and transferred to three individual recipient blocks at defined array coordinates (6 cores/tumor). In addition, tissue cores were also selected from histologically normal pancreatic acini, pancreatic ducts, and duodenal mucosa for use as controls. Five-μm sections were cut from each recipient tissue microarray block using an adhesive-coated tape system (Instrumedics, Hackensack, NJ; Ref. 10). Sections were stained with H&E to confirm the presence of pancreatic cancer within each tissue core and for immunohistochemical analysis.

TS Immunohistochemistry. Tissue sections from the pancreatic cancer tissue microarray were deparaffinized, rehydrated through graded alcohols, washed with Tris-buffered saline, and processed using the streptavidin-biotin-peroxidase complex method. Briefly, antigen retrieval was performed by microwave heating sections in 10 mm sodium citrate buffer (pH 6) for 10 min. After endogenous peroxidase activity was quenched and nonspecific binding was blocked, monoclonal antibody TS106 anti-TS (NeoMarkers Inc., Fremont, CA) was incubated at 4°C overnight in a 1:15 dilution (11). The secondary antibody was biotinylated rabbit antimouse antibody (DAKO, Carpinteria, CA) used at a dilution of 1:200 for 30 min at 37°C. After further washing with Tris-buffered saline, sections were incubated with StrepABCComplex/horseradish peroxidase (1:100 dilution; DAKO) for 30 min at 37°C. Immunolocalization was performed by immersion in 0.05% 3,3′-diaminobenzidine tetrahydrochloride as chromagen. A colon carcinoma with known TS positivity served as a positive control. Negative control was performed by replacing the primary antibody by normal serum. Slides were counterstained with hematoxylin before dehydration and mounting.

All sections were reviewed independently by two pathologists (Y. C. H. and R. A. K.) blinded to all clinical and pathological information. The intensity of TS staining was graded from 0 to 3 as described previously (11). The highest staining intensity among six cores was used to classify each tumor. Scores of 0 and 1 were assigned low TS expression, and scores of 2 and 3 were classified as high TS expression (11). In cases of disagreement, consensus was reached by joint review. Normal pancreatic acini and ducts and duodenal mucosa were included as controls and exhibited low TS expression. Two cases contained insufficient cancer cells to be accurately scored and were excluded from further analysis.

Statistical Analysis. The association between TS expression and individual clinical and pathological variables (age, gender, race, tumor size, pathological stage, pathological grade, margin status, and operative procedure) was assessed using Fisher’s exact test or χ² (categorical variables) or Wilcoxon’s rank-sum test (continuous variables).

The associations between individual clinical and pathological variables (age, gender, race, T stage, N stage, pathological stage, pathological grade, margin status, operative procedure, and TS expression) and survival were assessed using the Cox proportional hazards regression model. A stepwise variable selection procedure was used to build a Cox proportional hazards multiple regression model for time to death; a significance level of 0.20 was used to determine whether a variable could be entered into or removed from the regression model. Associations were quantified using hazard ratios and their 95% CIs.

Survival time was determined as the time from resection to death. For survivors, survival times were censored on the last date that patients were known to be alive. Survival probabilities were estimated using the method of Kaplan and Meier. Log-rank tests were used to compare survival curves among the various subgroups of patients. All statistical tests were two-tailed.

4 J. Kakareka, G. Hostetter, O-P. Kallioniemi, unpublished data.
RESULTS

Patient Characteristics and TS Immunohistochemistry. One hundred and thirty-two patients were evaluable for TS staining. TS staining was localized in the cytoplasm of tumor cells. High and low intratumoral TS expression was present in 83 of 132 (63%) and 49 of 132 (37%) tumors, respectively. Patient clinical and pathological characteristics are depicted in Table 1. No statistically significant associations between TS expression and any clinical or pathological characteristic were observed.

A history of adjuvant therapy was available from 121 of the 132 (92%) patients. Ninety-seven (80%) of these patients received some form of adjuvant radiation and/or chemotherapy. Eighty-nine (74%) patients received external beam radiation, and 94 (78%) patients received postoperative chemotherapy. Eighty-six of the 121 patients (71%) received combined adjuvant chemoradiation. Overall, 73 patients received 5-FU as one component of their adjuvant therapy, and 33 patients received gemcitabine as one component of their adjuvant therapy. Twenty-two patients participated in the Radiation Therapy Oncology Group Phase III trial (97-04) of pre- and postchemoradiation 5-FU versus pre- and postchemoradiation gemcitabine for postoperative adjuvant treatment of resected pancreatic adenocarcinoma. Patients receiving adjuvant therapy were younger than patients not receiving adjuvant therapy (64 ± 11 years versus 73 ± 11 years; \(P = 0.0009\)). No differences in gender, stage, histological grade, or margin status were present among patients receiving adjuvant therapy and patients not receiving adjuvant therapy.

Survival Analysis. Median follow-up among all patients was 15 months, and median follow-up among surviving patients was 29 months. Actuarial 1-, 2-, 3-, and 5-year survival rates for this population of patients with resected pancreatic cancer were 64%, 34%, 20%, and 10%, respectively. The role of TS and other clinical and pathological variables in predicting prognosis in pancreatic cancer was evaluated using single-variable Cox proportional hazards regression analysis (Table 2). High TS expression among patients with low-TS-expressing tumors was longer than that among patients with high-TS-expressing tumors (18.3 versus 12.0 months). Tumor N stage, overall pathological stage, and tumor grade were also predictive of overall patient survival. Overall survival was also significantly increased in patients receiving adjuvant therapy (RR = 0.55; 95% CI = 0.32–0.92; \(P = 0.023\) versus no adjuvant therapy). Age, gender, race, T stage, tumor size, operative procedure (proximal versus distal versus total pancreatectomy), and surgical margin status did not significantly influence survival in this group of patients.

Multiple regression analysis was performed using a Cox proportional hazards model to determine whether TS expression independently predicted survival in patients with resected pancreatic cancer (Table 3). Pathological (TNM) stage, gender, tumor grade, margin status, adjuvant therapy, and TS expression...
were included in the stepwise model selection process. Pathological stage, tumor grade, adjuvant therapy, and TS expression were each predictive of patient outcome. With the use of the Cox model, high TS expression was a significant ($P = 0.029$) independent predictor of death in resected pancreatic cancer with a RR of 1.66 (95% CI = 1.05–2.60).

**TS Expression and Response to Adjuvant Therapy.**
The influence of TS expression on the response to adjuvant therapy was further analyzed in resected pancreatic cancer. In patients with high-TS-expressing tumors, adjuvant therapy significantly improved overall survival (RR = 0.40; 95% CI = 0.18–0.90; $P = 0.001$). Overall actuarial survival 1 and 2 years after resection in patients with high-TS-expressing tumors was 64% and 40%, respectively, with adjuvant therapy versus 43% and 0%, respectively, without adjuvant therapy (Fig. 2A). In patients with low-TS-expressing tumors, no significant difference in overall survival was observed among patients managed with resection versus resection plus adjuvant therapy (Fig. 2B). Median survival among adjuvantly treated patients with high-TS-expressing tumors was similar to the median survival in both treated and untreated patients with low-TS-expressing tumors (Table 4).

The influence of TS expression on survival after adjuvant therapy was also examined in the 73 patients receiving a 5-FU-based regimen. Among patients with high-TS-expressing tumors, 5-FU-based adjuvant therapy significantly reduced the risk of death (RR = 0.37; 95% CI = 0.16–0.86; $P = 0.0006$). 5-FU-based adjuvant therapy did not influence survival among patients with low-TS-expressing pancreatic cancer.

**DISCUSSION**
High TS expression was an independent negative prognostic factor in this retrospective analysis of patients with resected pancreatic cancer. Adjuvant chemotherapy and/or radiation therapy were also associated with a 50% increase in median survival. This improvement in survival was only observed in patients with tumors exhibiting high TS expression. Adjuvant therapy did not appear to improve survival among patients with low-TS-expressing pancreatic cancer. The survival advantage observed with adjuvant therapy in high-TS-expressing tumors was most marked in patients receiving 5-FU-based adjuvant therapy.

The value of TS as a prognostic factor has been most widely studied in colorectal cancer. Johnston et al. (11) determined the level of TS protein expression immunohistochemically in the primary rectal cancers of 294 patients enrolled in National Surgical Adjuvant Breast and Bowel Project Protocol R-01. High TS expression was associated with a significant decrease in overall and disease-free survival independent of Dukes’ stage (11). Since this study, 11 of 13 studies have confirmed the stage-independent negative prognostic effect of TS in colorectal cancer (12). High TS expression determined using immunohistochemistry has also been associated with negative independent prognostic effect in breast, esophageal, head and neck, and gastric cancer (13–16). An increase in TS expression is common in most malignancies (compared with corresponding normal tissues) and is determined in part by an inherited polymorphism in the promoter of the TS gene (17). The homozygous presence of a triple 28-bp repeat polymorphism is common (30% of Caucasians and 42% of Hispanics) and has been associated with increased TS expression and decreased survival in rectal cancer and acute lymphoblastic leukemia (18, 19).

The role of TS expression in pancreatic cancer has not been well studied. Using a polyclonal anti-TS antibody, Takamura et al. (20) demonstrated improved survival in patients with high-TS-expressing tumors, although TS did not have independent prognostic capability. The monoclonal antibody used in the present study has been used in the vast majority of the studies that have demonstrated the negative prognostic effect of elevated TS expression (11, 14–16). A close correlation between TS mRNA expression measured by reverse transcription-PCR, the level of TS protein detected by Western blot, and the results of immunohistochemistry using this antibody (TS106) has been demonstrated previously (21). Our results suggest that TS expression provides additional prognostic information beyond the traditional clinical and pathological prognostic markers (nodal status, resection markers, tumor differentiation, tumor size, and pathological stage) in pancreatic cancer.

The ability of TS to predict therapeutic responses to 5-FU-based chemotherapy is far less clear (12). Several studies in patients with measurable advanced colorectal and head and neck cancer treated with 5-FU-based chemotherapy have demon-

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Table 3  Multiple regression analysis of prognostic factors in resected pancreatic cancer

<table>
<thead>
<tr>
<th>Pathological stage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RR (95% CI)</th>
<th>$P^*$</th>
</tr>
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<tbody>
<tr>
<td>Tumor grade&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.71 (1.11–2.63)</td>
<td>0.015</td>
</tr>
<tr>
<td>Received adjuvant therapy</td>
<td>0.49 (0.28–0.85)</td>
<td>0.011</td>
</tr>
<tr>
<td>High TS expression</td>
<td>1.66 (1.05–2.63)</td>
<td>0.029</td>
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<sup>a</sup>Cox proportional hazards multiple regression analysis.

<sup>b</sup>RR for death for stage III and IVa versus stage I and II.

<sup>c</sup>RR for death for poorly differentiated tumors versus well and moderately differentiated tumors.
adjuvant therapy for colorectal cancer is limited to patients with low-TS-expressing tumors (25). Two additional studies have also demonstrated that improved survival with 5-FU-based chemotherapy has correlated with TS levels (11). Recently, Edler et al. reported a similar increase in survival versus patients treated with resection alone. A, effect of adjuvant therapy in patients with high-TS-expressing pancreatic cancer. No statistically significant difference in survival was observed among patients managed with resection alone and patients managed with resection plus adjuvant therapy.

Table 4 Effect of adjuvant therapy and TS expression on median survival (months) in patients with resected pancreatic cancer

<table>
<thead>
<tr>
<th>Adjuvant therapy</th>
<th>High TS expression</th>
<th>Low TS expression</th>
</tr>
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<tbody>
<tr>
<td>Any adjuvant therapy</td>
<td>18.9 (P = 0.0006)</td>
<td>18.9 (P = 0.92)</td>
</tr>
<tr>
<td>No adjuvant therapy</td>
<td>11.2</td>
<td>11.2</td>
</tr>
<tr>
<td>Any 5-FU-based adjuvant therapy</td>
<td>18.9 (P = 0.0006)</td>
<td>25.0 (P = 0.98)</td>
</tr>
<tr>
<td>No adjuvant therapy</td>
<td>11.2</td>
<td>18.9</td>
</tr>
</tbody>
</table>

* Log-rank test.

Fig. 2 A, effect of adjuvant therapy in patients with high-TS-expressing pancreatic cancer. Adjuvant therapy was associated with a significant (P = 0.001) increase in survival versus patients treated with resection alone. B, effect of adjuvant therapy in patients with low-TS-expressing pancreatic cancer. No statistically significant difference in survival was observed among patients managed with resection alone and patients managed with resection plus adjuvant therapy.

Several limitations should be kept in mind when interpreting the results of this retrospective study. Multiple adjuvant therapy regimens were used at the two institutions over the 8-year study period. Although the majority of patients received 5-FU, the drug was not administered in standardized fashion throughout the duration of the study. No other agents were used with sufficient frequency to draw any meaningful conclusions. A difference in age was also noted among patients receiving and patients not receiving adjuvant therapy. Patients receiving adjuvant therapy were younger than patients not treated with adjuvant therapy, although age was not a predictor of survival in this group of patients. Our bias has been to treat patients with resected pancreatic cancer with adjuvant therapy. This practice has resulted in a small untreated group of patients that may not be comparable with the adjuvant-treated patients and also limits the statistical power of comparisons among patients receiving and patients not receiving adjuvant therapy. In addition, the use of the tissue microarray could have underestimated the true frequency of high-TS-expressing tumors, particularly in patients with focal staining. However, to compensate for this, we used three distinct arrays, each sampling two different sites within the tissue microarray.

strated greater response rates in patients with low TS activity (21–24). However, in these studies, all patients received chemotherapy, making it impossible to define whether TS expression was able to predict patients experiencing a survival benefit with 5-FU. TS expression has also been used to evaluate survival in patients receiving 5-FU in the adjuvant setting. In the National Surgical Adjuvant Breast and Bowel Project R-01 trial of surgery versus surgery plus lomustine, 5-FU, and vincristine for rectal cancer, both disease-free (17% to 38%) and overall survival (31% to 54%) were significantly improved in patients with high TS levels who received chemotherapy when compared with patients managed with surgery alone (11). In contrast, no difference was noted among treatment arms in patients with low TS levels (11). Recently, Edler et al. (25) reported a similar improvement in survival in patients with high-TS-expressing colorectal cancer, who received 5-FU-based adjuvant therapy. Unexpectedly, adjuvant therapy negatively impacted survival in patients with low-TS-expressing tumors (25). Two additional studies have also demonstrated that improved survival with adjuvant therapy for colorectal cancer is limited to patients with high TS expression (26, 27). Furthermore, treatment of node-positive breast cancer with cyclophosphamide, 5-FU, and methotrexate produced a greater survival benefit in patients with high-TS-expressing tumors (15). However, other large studies have not observed a predictive role for TS in the adjuvant therapy of colorectal cancer (28).

Although our results are consistent with these other studies suggesting that the benefit of adjuvant therapy is limited to patients with high-TS-expressing tumors, the basis for this observation remains unclear. Improved survival with adjuvant therapy implies the eradication of micrometastatic cancer, which may differ in molecular characteristics from the primary cancer. For example, the response of colorectal liver metastases to 5-FU-based chemotherapy has correlated with TS levels in the metastatic tumor but not with TS levels in the primary tumor (29). TS levels have also been shown to correlate with the activity of certain cell cycle-regulatory proteins (p21WAF-1), which may also influence responses to chemotherapy and radiation in pancreatic cancer (30, 31). p53 mutant colorectal cancer and pancreatic cancer cell lines also express higher levels of TS mRNA (32, 33). Recent *in vitro* studies have demonstrated that pancreatic cancer lacking functional p53 also demonstrates enhanced radiosensitivity with 5-FU (33).
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each tumor. In previous studies of other tumors, even single-site sampling or sampling from 2–4 sites from each tumor has been proven to be an accurate means of assessing cancer biomarkers (34–36). Finally, one would expect only homogenous TS staining to have any significance for cancer therapy response.

In summary, TS represents a candidate prognostic and predictive marker for patients with resectable pancreatic cancer. The largest prospective randomized trial of postoperative adjuvant therapy for pancreatic cancer has demonstrated a modest survival benefit with 5-FU as a single agent versus no treatment (5). Furthermore, a novel chemoradiation regimen including 5-FU, cisplatinum, and IFN- 


REFERENCES


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