The SMAD4 Protein and Prognosis of Pancreatic Ductal Adenocarcinoma


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ABSTRACT

Purpose: SMAD4 (also called Dpc4) is a tumor suppressor in the TGF-β signaling pathway that is genetically inactivated in ~55% of all pancreatic adenocarcinomas. We investigated whether prognosis after surgical resection for invasive pancreatic adenocarcinoma is influenced by SMAD4 status.

Experimental Design: Using immunohistochemistry, we characterized the SMAD4 protein status of 249 pancreatic adenocarcinomas resected from patients who underwent pancreaticoduodenectomy (Whipple resection) at The Johns Hopkins Hospital, Baltimore, MD, between 1990 and 1997. The SMAD4 gene status of 56 of 249 (22%) pancreatic carcinomas was also determined. A multivariate Cox proportional hazards model assessed the relative risk of mortality associated with SMAD4 status, adjusting for known prognostic variables.

Results: Patients with pancreatic adenocarcinomas with SMAD4 protein expression had significantly longer survival (unadjusted median survival was 19.2 months as compared with 14.7 months in patients with pancreatic cancers lacking SMAD4 protein expression; P = 0.03). This SMAD4 survival benefit persisted after adjustment for prognostic factors including tumor size, margins, lymph node status, pathological stage, blood loss, and use of adjuvant chemoradiotherapy. The relative hazard of mortality for cancers lacking SMAD4 after adjusting for other prognostic factors was 1.36 (95% confidence interval, 1.01–1.83; P = 0.04).

Conclusion: Patients undergoing Whipple resection for pancreatic adenocarcinoma survive longer if their cancers express SMAD4.

INTRODUCTION

Pancreatic carcinoma is a deadly disease with a 5-year survival of 3–5% (1). Currently, long-term survival from pancreatic cancer is best achieved through surgical resection, which is associated with 5-year survival rates of 15–20% (range, 7–24%; Refs. 2, 3). Survival is best among resected patients with small carcinomas (<3 cm), negative lymph nodes, and negative resection margins; this subgroup of patients has a 5-year survival rate approaching 40% (1, 4). Adjuvant chemoradiotherapy seems to further improve survival, although the optimal regimens are still under investigation (2, 5–10). Despite recent improvements in survival, most patients who undergo surgical resection ultimately die of their disease. Current prognostic indicators such as tumor size, margin status, and lymph node involvement do not accurately predict responses to treatment (1, 4, 5, 11). Apart from DNA index (12, 13), molecular alterations common to pancreatic carcinoma have not yet been shown to independently predict prognosis after surgical resection once established prognostic markers are taken into account (14–18). In addition to improving prognostication, new molecular prognostic markers would help to more accurately estimate responses to investigative treatments and may identify the biological factors within pancreatic carcinomas that most influence survival.

Pancreatic carcinoma is a genetic disease characterized by somatic mutations of multiple genes, including the K-ras oncogene and the tumor suppressor genes p16, p53, and SMAD4 (19). SMAD4 is a tumor suppressor gene that is inactivated in ~55% of pancreatic adenocarcinomas, either by the intragenic mutation of one allele in combination with the loss of the other allele or by homozygous deletion of both alleles (20). In the cytoplasm, SMAD4 protein mediates signals from a family of TGF-β ligands and their transmembrane receptors through phosphorylation of SMAD proteins, which heterodimerize with SMAD4. This SMAD4/SMAD complex transmits upstream signals by translocating to the nucleus, binding to specific DNA sequences, and activating gene transcription (see Fig. 1). Many of the functions of TGF-β and its related ligands, such as growth suppression and apoptosis, are abrogated by inactivation of SMAD4 (21).

We hypothesized that pancreatic adenocarcinomas with inactivation of SMAD4 would behave more aggressively than those with intact SMAD4. To evaluate the clinical significance...
of SMAD4 inactivation in pancreatic adenocarcinoma, we evaluated the SMAD4 status of pancreatic adenocarcinomas in 249 patients who underwent potentially curative Whipple resection between 1990 and 1997.

PATIENTS AND METHODS

Patients and Tumor Specimens. Two hundred and fifty-three formalin-fixed, paraffin-embedded pancreatic adenocarcinomas from patients who underwent Whipple resection were obtained from the archival tumor banks of The Johns Hopkins Hospital, Baltimore, MD, from 1990 to 1997. The clinical and pathological data from this patient population were readily available from pathology reports and a regularly updated clinical database. Patients are being monitored by reviewing the patients’ records and contacting the patients, or by their physicians annually, with 94% follow-up until July 15, 2000.

Patients with mucinous cystic and medullary adenocarcinomas and IPMNs were not included in this series because IPMNs with adenocarcinoma have a better prognosis and much lower rates of SMAD4 loss than ductal adenocarcinomas, and initial reports of medullary carcinoma suggested an improved prognosis and a lower rate of SMAD4 inactivation in pancreatic adenocarcinoma with a known homozygous deletion which account for 30% of the genetic alterations of SMAD4. Homozygous deletions are usually not detectable in the primary adenocarcinoma because cancer cells are typically intimately intermixed with nonneoplastic stroma. The 56 cancers characterized for SMAD4 alterations included all cases for which xenograft DNA was available. The results of SMAD4 gene status on 23 of these 56 pancreatic adenocarcinomas have been reported previously (20). Genetic and immunolabeling assays of SMAD4 were performed independently of each other with investigators blinded to the results of the complementary test.

Immunohistochemical Analysis. SMAD4 immunolabeling was performed as reported previously (24). H&E-stained slides from each case were screened by light microscopy for blocks containing adenocarcinoma and adjacent normal pancreas. Antigen retrieval was achieved by steaming at 80°C for 20 min, after which the slides were allowed to cool for 5 min. A 1:100 dilution of monoclonal antibody to SMAD4 protein (clone B8, Santa Cruz Biotechnology, Santa Cruz, CA) was then applied using the Bio Tek-Mate 1000 automated stainer (Ventana Bio Tek Solutions). The anti-SMAD4 antibody was detected by adding biotinylated secondary antibodies, avidin-biotin complex and 3,3’-diaminobenzidine chromagens. Hematoxylin was used to counterstain the nuclei. A pancreatic ductal adenocarcinoma with a known homozygous deletion of SMAD4 served as a negative control. Normal pancreas in the selected tissue specimens served as an internal positive control. The SMAD4 immunolabeling concurs with >90% concordance with the genetic status of SMAD4 (24).

Immunohistochemical Evaluation. Immunohistochemical labeling of SMAD4 was simultaneously evaluated by four of the authors (M. T., R. H. H., G. J. O., and R. W.) with agreement in all cases examined. The labeling in each case was scored as “negative” when absolutely no cytoplasmic nor nuclear staining in the neoplastic cells was visible; as “trace positive” when neoplastic cells showed a very weak, barely perceptible labeling that at low-power magnification appeared negative, and only on very close inspection at high power was faint labeling seen; as “positive” when neoplastic cells were clearly positive with a staining intensity comparable with the surrounding normal pancreas; and as “focally positive” when the tumor contained two distinct populations of cells, those that labeled with the antibody to SMAD4 and those that did not.

We interpreted a barely detectable level of immunoreactivity (trace positive) for SMAD4 when compared with surrounding normal pancreas to indicate a virtual lack of SMAD4 protein expression. The absolute level of other SMAD proteins has a bearing on the transcription of downstream genes (25); hence, these adenocarcinomas were grouped together as negative with those that showed an absence of SMAD4 immunolabeling. The few pancreatic adenocarcinomas with focally positive SMAD4 expression were also categorized as negative for prognostic purposes, because we hypothesized that its more abnormal clone (the one lacking SMAD4 expression) would dictate the prognosis of such cancers.

Genetic Analysis of SMAD4. The SMAD4 gene status was characterized in 56 of 249 pancreatic adenocarcinomas by homozygous deletion analysis and cycle sequencing of PCR products on pancreatic cancer xenograft DNA generated by implantation of primary adenocarcinoma into athymic nude mice as reported previously (20). The enrichment for neoplastic cells achieved with xenografting allows one to detect homozygous deletions which account for ~30% of the genetic alterations of SMAD4. Homozygous deletions are usually not detectable in the primary adenocarcinoma because cancer cells are typically intimately intermixed with nonneoplastic stroma. The 56 cancers characterized for SMAD4 alterations included all cases for which xenograft DNA was available. The results of SMAD4 gene status on 23 of these 56 pancreatic adenocarcinomas have been reported previously (20). Genetic and immunolabeling assays of SMAD4 were performed independently of each other with investigators blinded to the results of the complementary test.

Statistical Analysis. The main end point for this study was overall survival from the date of surgery to the time of the last follow-up or death. Data on survival were censored if the patient was still alive at the time of the last follow-up. Kaplan-Meier survival curves compared cumulative probability of survival on the basis of SMAD4 status and a log-rank test provided the P. A Cox proportional hazards logistic regression model (13) assessed the simultaneous contribution of the following baseline covariates to the relative risk of mortality: (a) tumor size (≥3.0 cm versus <3.0 cm); (b) resection margin status (positive mar-
RESULTS

Characteristics of the Patients. Table 1 lists the clinical characteristics of the 249 patients whose pancreatic adenocarcinomas were analyzed immunohistochemically. Demographic factors and tumor factors were not different by SMAD4 expression status (Table 1). The median duration of follow-up was 17 months. One or more of the clinical characteristics used in the Cox proportional hazards model (see below) to identify prognostic markers were not available for 29 of 249 patients. Hence, the multivariate regression results are based on complete data from 220 of the 249 patients in the series.

Immunohistochemical Labeling. One hundred and nine (43%) of pancreatic adenocarcinomas completely lacked SMAD4 protein expression by immunohistochemistry, 25 (10%) were graded trace positive, 4 (2%) were graded focal positive, 111 (45%) were graded positive (see Fig. 2). Twenty-five pancreatic adenocarcinomas were immunolabeled in duplicate with 100% concordance between results. Thus, a total of 138 (55%) of the adenocarcinomas were categorized as negative and 111 (45%) as positive.

Genetic Analysis of SMAD4 and Correlation with Immunohistochemistry. Fifty-six of 249 pancreatic adenocarcinomas were sequenced for mutations and homozygous deletions in the SMAD4 gene. A detailed description of the mutations is not presented here.3 The results of immunolabeling and genetic analysis were concordant (SMAD4 expression in-...
detection of SMAD4 (24) by observing that SMAD4 is lost in cancers with a wide variety of SMAD4 mutations, including missense mutations.

Factors Associated with Prognosis in Univariate and Multivariate Analysis. In univariate analysis, SMAD4-expressing pancreatic adenocarcinomas were associated with a significantly improved prognosis (Fig. 3). The hazard ratio for survival was $1.36$ (95% CI, 1.01–1.83; $P = 0.042$). The median survival of pancreatic adenocarcinoma patients with intact SMAD4 protein expression was 19.2 months compared with 14.7 months among patients with pancreatic adenocarcinomas lacking SMAD4. This survival advantage corresponds to a cumulative 5-year survival after surgery of 20.5% for patients with SMAD4-intact tumors compared with 13.7% of patients with SMAD4-negative tumors. In addition, crude relative hazards for tumor size, margin status, differentiation, lymph node status, amount of intraoperative blood loss, and treatment with chemoradiotherapy were all significant prognostic factors (Table 2). The tumor size that was most prognostic in single-factor analysis was a tumor size of <3.0 versus ≥3.0 cm, therefore tumor size was stratified at 3 cm. Lymph node status was stratified into two groups: those with 0–1 resected nodes involved by cancer or those with >1 node positive, as this stratification provided the most prognostic information by univariate analysis.

In the multivariate Cox model, only SMAD4 status, margin status, tumor size (<3 or >3 cm), the amount of intraoperative blood loss, tumor differentiation, and treatment with adjuvant chemoradiotherapy were independently prognostic (Table 3). Although the presence of lymph node metastases was prognostic, it was not independently prognostic and did not confound the relationship between SMAD4 status and survival. SMAD4 status was also independently associated with prognosis if the effect of adjuvant treatment was excluded from the model [hazard ratio, 1.35 (95% CI, 1.02–1.80; $P = 0.035$)]. There was no evidence for an interaction between SMAD4 status and response to chemoradiotherapy, suggesting that SMAD4 status does not influence the response to standard adjuvant chemoradiotherapy. Most patients in this series treated with chemoradiotherapy received 4000–5000 cGy of postoperative radiotherapy in divided doses to the tumor bed with cycles of 5-fluorouracil/leukovorin (5, 26).

Among the 30 patients (17 with SMAD4-expressing cancers) with the earliest stage pancreatic adenocarcinomas (cancers 3 cm or less, with negative margins, and lymph node negative), 5-year survival was 37% (95% CI, 17.2%, 57.1%) among patients whose cancers lacked SMAD4, and 46.8% (95% CI, 24.7%, 66.2%) among patients with cancers expressing SMAD4.

**DISCUSSION**

In this study we report that among patients undergoing surgical resection of their pancreatic adenocarcinoma, the presence of SMAD4 expression in their cancer independently predicts a better outcome. The median survival of patients with intact SMAD4 expression in their pancreatic cancers was 19.2 months, compared with 14.7 months among those lacking SMAD4 expression in their cancers. This survival advantage persisted 5 years after surgery, with 20.5% of patients with SMAD4-intact tumors surviving to this milestone compared with 13.7% of patients with SMAD4-negative tumors. SMAD4 expression provides additional prognostic information even
after adjusting for resection margins, tumor size, lymph nodes, differentiation, and operative blood loss. Furthermore, the SMAD4 status of pancreatic adenocarcinomas also remained significant after adjustment for the effect of chemoradiotherapy. In our prognostic model, receipt of adjuvant chemoradiotherapy was the strongest predictor of survival. In a large nonrandomized series from our institution, patients treated with adjuvant chemoradiotherapy (4000–5000 cGy) and 5-fluorouracil/leukovorin had a significantly prolonged survival after pancreaticoduodenectomy (5). It is not clear whether treatment with adjuvant chemoradiotherapy is actually greatly effective, or whether there is a substantial selection bias which favors those patients fit enough to receive such therapy. But those patients who receive such adjuvant chemoradiotherapy survive longer than those who do not. Among patients with the earliest stage pancreatic adenocarcinomas (cancers ≤3 cm, with negative margins, and lymph node negative), 5-year survival was 47% among patients with cancers expressing SMAD4. The impressive survival of patients with "early" pancreatic carcinomas suggests that survival from pancreatic adenocarcinoma could be improved if such cancers could be detected earlier. They also provide a rationale for investigating early detection strategies among individuals at increased risk of developing pancreatic adenocarcinoma (27).

Although several molecular markers have been investigated for their prognostic significance, including p53 gene status (28), type of K-ras oncogene mutation (17, 28, 18), expression of bcl-2 (14, 15), bax (14), and the expression of TGF-β1 (29), only DNA index has been consistently shown to provide prognostic information independent of standard pathological prognostic indicators (12, 13). Although the immunohistochemical analysis of the protein products of tumor suppressor genes is an attractive approach to use in clinical practice for understanding the molecular profile of cancers, it has limitations. Because some cancers will inactivate tumor suppressor genes by missense mutations that still permit antibody binding to the mutant protein, evidence of tumor suppressor gene inactivation often cannot be reliably inferred by immunohistochemical evidence for protein loss. This is not true for SMAD4 immunolabeling. Because many mutant forms of SMAD4 protein undergo degradation through the ubiquitination pathway, most mutations of SMAD4 result in a loss of protein (30). In this study, we observed almost 100% concordance between the genetic status of SMAD4 and immunohistochemical detection of SMAD4 protein, making SMAD4 immunolabeling an ideal marker in pancreatic carcinoma.

SMAD4 inactivation seems to result in a biologically more aggressive form of pancreatic adenocarcinoma. These results highlight the molecular heterogeneity of histologically indistinguishable forms of pancreatic adenocarcinoma. Previously we have shown that SMAD4 gene inactivation is a late event in pancreatic neoplastic progression, suggesting that other genetic events must occur in a developing neoplasm before loss of SMAD4 function provides a selective advantage (31). It is not certain which of the biological effects of SMAD4 is most important in suppressing cancer growth. Although much is known about how TGF-β signals through SMAD4 to mediate transcription, the cancer suppressive functions that have been attributed to SMAD4 are those that reflect its mediation of TGF-β signals, although other upstream pathways also signal through SMAD4 (32). Activation of the TGFβ/SMAD4 pathway under certain conditions may result in apoptosis or growth arrest in the G1 phase of the cell cycle (33, 34). In addition, inactivation of the SMAD4 gene within an evolving neoplasm may indirectly influence the extracellular matrix to promote neoplastic growth. Experimental SMAD4 restoration to human pancreatic adenocarcinoma cells transplanted into nude mice suggests that SMAD4 can inhibit angiogenesis by decreasing vascular endothelial growth factor and by increasing thrombospondin-1 (35).

For a disease with such a poor prognosis, the median survival advantage of SMAD4-expressing pancreatic adenocarcinomas of ~5 months is clinically significant. Because the survival benefits for patients treated with chemoradiotherapy for pancreatic adenocarcinoma are usually measured in months, the SMAD4 status of pancreatic adenocarcinomas could be used to improve the stratification of patients enrolled in clinical trials for the treatment of this disease. The prognostic impact of inactivating SMAD4 raises the possibility that in the future novel therapeutics that replace the loss of SMAD4 function may have a therapeutic impact in pancreatic adenocarcinoma (36).

**ACKNOWLEDGMENTS**

We thank Jennifer Parsons for creating the TGFβ/DPC4 pathway illustration.

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**Table 2** Unadjusted Cox proportional hazards estimates of selected variables in 249 pancreatic cancer patients evaluated for DPC4

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative hazard of mortality (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dpc4 status (negative)</td>
<td>1.36 (1.03–1.81)</td>
<td>0.030</td>
</tr>
<tr>
<td>Tumor grade (poor differentiation)</td>
<td>1.76 (1.32–2.35)</td>
<td>&lt;0.001</td>
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<tr>
<td>Positive tumor margins</td>
<td>1.48 (1.11–1.98)</td>
<td>0.007</td>
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<tr>
<td>Tumor size (≤3.0 cm)</td>
<td>1.63 (1.23–2.17)</td>
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<tr>
<td>Received chemo-radiotherapy</td>
<td>0.54 (0.38–0.75)</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Intraoperative blood loss (per liter)</td>
<td>1.15 (1.05–1.26)</td>
<td>0.002</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>1.27 (0.95–1.69)</td>
<td>0.100</td>
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</tbody>
</table>

*These estimates are not adjusted for other variables.

**Table 3** Cox proportional hazards estimates of selected variables in 249 pancreatic cancer patients evaluated for DPC4

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative hazard of mortality (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPC4 Status (negative)</td>
<td>1.36 (1.01–1.83)</td>
<td>0.042</td>
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<tr>
<td>Received chemo-radiotherapy</td>
<td>0.54 (0.38–0.75)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive tumor margins</td>
<td>1.32 (0.96–1.82)</td>
<td>0.085</td>
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<tr>
<td>Tumor size (≤3.0 cm)</td>
<td>1.31 (0.96–1.78)</td>
<td>0.084</td>
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<tr>
<td>Tumor grade (poor differentiation)</td>
<td>1.70 (1.26–2.31)</td>
<td>0.001</td>
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<tr>
<td>Intraoperative blood loss</td>
<td>1.14 (1.04–1.25)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*Each estimate is adjusted for all other estimates in the model.
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