SMAD4 Gene Mutations Are Associated with Poor Prognosis in Pancreatic Cancer

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Abstract

Purpose: Recently, the majority of protein coding genes were sequenced in a collection of pancreatic cancers, providing an unprecedented opportunity to identify genetic markers of prognosis for patients with adenocarcinoma of the pancreas.

Experimental Design: We previously sequenced more than 750 million base pairs of DNA from 23,219 transcripts in a series of 24 adenocarcinomas of the pancreas. In addition, 39 genes that were mutated in more than one of these 24 cancers were sequenced in a separate panel of 90 well-characterized adenocarcinomas of the pancreas. Of these 114 patients, 89 underwent pancreatectoduodenectomy, and the somatic mutations in these cancers were correlated with patient outcome.

Results: When adjusted for age, lymph node status, margin status, and tumor size, SMAD4 gene inactivation was significantly associated with shorter overall survival (hazard ratio, 1.92; 95% confidence interval, 1.20-3.05; P = 0.006). Patients with SMAD4 gene inactivation survived a median of 11.5 months, compared with 14.2 months for patients without SMAD4 inactivation. By contrast, mutations in CDKN2A or TP53 or the presence of multiple (≥4) mutations or homozygous deletions among the 39 most frequently mutated genes were not associated with survival.

Conclusions: SMAD4 gene inactivation is associated with poorer prognosis in patients with surgically resected adenocarcinoma of the pancreas.

Pancreatic cancer is the fourth leading cause of cancer death in the United States (1). Worldwide pancreatic cancer is responsible for more than 213,000 deaths each year (2). The 5-year overall survival rate for all patients diagnosed with pancreatic cancer is less than 4% (3). Surgical resection offers the best hope for long-term survival, with a 17% 5-year survival rate in most surgical series (4–10). A number of pathologic features have been shown to correlate with outcome following surgery (4, 11–14). For example, the completeness of resection (margin status), size of the cancer, degree of differentiation, vascular invasion, lymph node status, and tumor stage are all independent prognostic indicators following pancreatectoduodenectomy for pancreatic cancer (4, 11–16). These factors have been useful guides in the clinical management of patients with pancreatic cancer. Genetic markers that could be used as prognostic indicators of outcome would be useful in establishing an individualized treatment plan for a patient. For example, a more aggressive surgical approach, such as vascular resection and reconstruction, may be considered for a patient with a reduced risk of systemic recurrence.

In an attempt to establish such genetic markers, we took advantage of the recently completed mutational analysis of the pancreatic cancer coding genome (17). The results of this “pancreatic cancer genome project” provide a unique opportunity to determine if any genes with somatic changes correlate with patient outcome following surgical resection (17). This previous study included the sequencing of the protein-coding exons from 20,661 genes in 24 advanced adenocarcinomas of the pancreas as well as copy number analyses using high-density oligonucleotide arrays (17). In addition, 39 of the genes mutated in two or more of the 24 cancers were sequenced in an
Translational Relevance

The identification of somatic genetic alterations that are associated with patient outcome may lead to useful clinical tools to guide the treatment of patients with pancreatic cancer, and may provide insight into alterations underlying the aggressive behavior of this cancer.

Materials and Methods

This study was approved by the Johns Hopkins Institutional Review Board.

Patients. Our previous sequencing study included 114 patients (24 in the Discovery Screen and 90 from the Validation Screen) treated between February 1989 and May 2007 (11, 17, 18). Ninety-one of these 114 patients underwent a pancreatoduodenectomy (Whipple procedure), 12 a distal pancreatectomy, and 3 a total pancreatectomy, whereas 8 were autopsied. The current study considered only the 91 patients from this previous study who underwent a pancreatoduodenectomy (11, 18). There were two perioperative deaths in this group, defined as death during the patient’s initial hospitalization or within 30 d of surgery. The 89 remaining pancreatoduodenectomy patients were included in the current study.

As reported previously, most of the patients included in this study were evaluated by a multidisciplinary group (surgery, medical oncology, radiation oncology, and pathology) and postoperative chemoradiation therapy was encouraged (4, 11). The majority of the patients received standard chemoradiation protocols consisting of 4,000 to 5,000 cGy of external beam radiation to the tumor bed given by weekly bolus of 5-FU for 4 additional months (4, 11). Other therapies included more intensive 5-FU plus leucovorin-based chemoradiation, gemcitabine-based chemotherapy, and chemoradiation including 5-FU, mitomycin C, leucovorin, and dipyridamole (4, 11, 19). Overall survival rates have been reported to be similar for all forms of adjuvant chemoradiation.

Somatic mutations. The “Discovery Screen” of the previous sequencing study included the sequencing of 20,661 protein coding genes in a series of 24 adenocarcinomas of the pancreas (17). In the “Validation Screen,” 39 genes that were mutated more than once in the Discovery Screen were sequenced in an additional panel of 90 well-characterized adenocarcinomas of the pancreas (17). In the current study focused on the 39 genes included in both the Discovery and Validation Screens and on the 89 patients who underwent a pancreatoduodenectomy and survived at least 30 d.

Deletions were identified in the Discovery Screen using high-density oligonucleotide arrays as previously described (17). High-density oligonucleotide array analysis was not done on the Validation Screen samples. Therefore, for purposes of analysis in this study, deletions in the samples of the Validation Screen were determined by patterns of sequencing failure. For the sequencing in the Validation Screen, 11 sets of primers were used to amplify SMAD4, 9 sets for TGFβR2 and SMAD3, 8 sets for TGFβR1, and 1 set for CDKN2A. For SMAD4, TGFβR2, SMAD3, and TGFβR1, we defined a homozygous deletion as having failed sequencing reactions (with <20% of the region of interest having a phred score <20) for three or more consecutive amplicons. For CDKN2A, we defined a homozygous deletion as having a failed sequencing reaction (with <20% of the region of interest having a phred score <20) for the single amplicon that was analyzed. When these criteria were applied to the 90 cancers included in the Validation Screen, 33% of the cancers were categorized as having a CDKN2A deletion, 19% a SMAD4 deletion, 1% a TGFβR2 deletion, 1% a TGFβR1 deletion, and 1% a SMAD3 deletion. These percentages are similar to those observed with the high-density oligonucleotide arrays in the Discovery Phase (17). In addition, when these criteria were used, we did not observe cancers with both a homozygous deletion and an inactivating intragenic somatic mutation.

Statistical analyses. Survival curves for the 89 patients who underwent a pancreatoduodenectomy and survived at least 30 d after surgery were constructed using the Kaplan-Meier method. Although 39 genes were sequenced in the Validation phase, the majority of the genes were altered in only one or two patients. Consequently, several approaches were used to examine if genetic alterations affected patient outcome. First, a set of genes hypothesized to differentiate survival, SMAD4, TP53, CDKN2A, and members of the transforming growth factor β (TGFβ) signaling pathway, were each correlated with survival. Next, in a subanalysis, the members of the TGFβ pathway were combined, and patients were grouped by whether they had a mutation or a homozygous deletion in any gene in the pathway. Finally, all 39 genes sequenced in the Validation phase were combined, and patients were grouped by whether they had <4 or ≥4 mutations and/or homozygous deletions among the 39 genes.

Survival curves were compared between groups of patients, using a log-rank statistic, for descriptive purposes. Coefficients from Cox proportional hazards models were used to test for the effect of genetic alterations on survival while adjusting for age, tumor size, margins, lymph nodes, and grade. We considered P < 0.01 to be statistically significant, adjusting for the multiple survival comparisons done in this study.

Table 1. Characteristics of patients who underwent a pancreatoduodenectomy and survived at least 30 days after surgery

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients, N = 89</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>65.3 (10.5)</td>
</tr>
<tr>
<td>Gender, no. (%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>43 (48.3)</td>
</tr>
<tr>
<td>Female</td>
<td>46 (51.7)</td>
</tr>
<tr>
<td>Race, no. (%)</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>80 (89.9)</td>
</tr>
<tr>
<td>Other race</td>
<td>9 (10.1)</td>
</tr>
<tr>
<td>Grade, no. (%)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>34 (38.2)</td>
</tr>
<tr>
<td>Moderate/well</td>
<td>55 (61.8)</td>
</tr>
<tr>
<td>Tumor size, no. (%)</td>
<td></td>
</tr>
<tr>
<td>&lt;3 cm</td>
<td>32 (36.0)</td>
</tr>
<tr>
<td>3-5 cm</td>
<td>44 (49.4)</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>12 (13.5)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Margin, no. (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>58 (65.2)</td>
</tr>
<tr>
<td>Positive</td>
<td>31 (34.8)</td>
</tr>
<tr>
<td>Positive lymph nodes, no. (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>71 (79.8)</td>
</tr>
<tr>
<td>No</td>
<td>18 (20.2)</td>
</tr>
<tr>
<td>No. of positive lymph nodes, mean (SD)</td>
<td>3 (3.8)</td>
</tr>
<tr>
<td>No. of lymph nodes examined, mean (SD)</td>
<td>15 (8.5)</td>
</tr>
<tr>
<td>Median (range) follow-up (mo)</td>
<td>12.9 (3.1, 56.0)</td>
</tr>
</tbody>
</table>
Results

Patients. The mean (SD) age of the 89 patients was 65.3 (10.5) years (range, 36-85 years; Table 1). There were 43 males (48%) and 46 females (52%), with the racial distribution being 80 White (90%) and 9 of other races (10%). The mean (SD) size of the tumors was 3.6 (1.7) cm. Seventy-one patients (80%) had lymph node metastases. Fifty-five of the carcinomas were well or moderately differentiated and 34 were poorly differentiated. Fifty-eight of the 89 patients had negative margins and 31 had positive margins.

Follow-up was available on all 89 patients, ranging from 3.1 to 56.0 months, with a median of 12.9 months. Follow-up was extensive, with 80 of the 89 (90%) patients included in this study followed until the time of death. The 5-year survival rate for all 89 patients in this series (Fig. 1) is less than the 17% to 20% 5-year survival rate we have previously reported for all patients treated by pancreaticoduodenectomy for adenocarcinoma of the pancreas at our institution (4–6, 11, 18, 20). This may reflect the reported trend for clinically more aggressive malignancies to successfully grow as xenografts and cell lines (21, 22).

SMAD4 and the TGFβ pathway versus survival. The survival curves of patients with and without SMAD4 gene inactivation were significantly different (log-rank P = 0.04). When adjusted for lymph node status, tumor grade, margin status, tumor size, and age, the difference remained nonsignificant [hazard ratio, 1.21 (95% CI, 0.76-1.93); P = 0.42]. Similarly, TP53 gene mutation status did not correlate with survival (log-rank P = 0.21), even when adjusted for lymph node status, tumor grade, margin status, tumor size, and age [Fig. 3; hazard ratio, 1.41 (95% CI, 0.69-2.85); P = 0.34]. Virtually all of the cancers (98.9%) harbored a KRAS gene mutation, excluding the possibility of a relationship between KRAS gene status and prognosis.

CDKN2A, TP53, and KRAS versus survival. There were no significant differences in survival between patients with and without CDKN2A gene inactivation (log-rank P = 0.63). When adjusted for lymph node status, tumor grade, margin status, tumor size, and age, the difference remained nonsignificant [hazard ratio, 1.21 (95% CI, 0.76-1.93); P = 0.42]. Similarly, TP53 gene mutation status did not correlate with survival (log-rank P = 0.21), even when adjusted for lymph node status, tumor grade, margin status, tumor size, and age [Fig. 3; hazard ratio, 1.41 (95% CI, 0.69-2.85); P = 0.34]. Virtually all of the cancers (98.9%) harbored a KRAS gene mutation, excluding the possibility of a relationship between KRAS gene status and prognosis.

Because the SMAD4 gene encodes for a protein that is an important member of the TGFβ pathway, we examined all of the members of this pathway (SMAD3, SMAD4, TGFβR1, and TGFβR2) individually and together for additional alterations in these tumors. Patients were grouped by whether they had a mutation or deletion in any of these four genes. Inactivation of a member of the (TGFβ) signaling pathway did not correlate with survival when adjusted for other factors [hazard ratio, 1.56 (95% CI, 0.96-2.52); P = 0.07]. We then examined each gene in the TGFβ signaling pathway individually. One patient had a mutation on SMAD3 and another had a homozygous deletion on SMAD3. There were no patients with mutations or deletions on TGFβR1. However, there were 11 patients (9 with mutations, 2 with homozygous deletions) with alterations on the TGFβR2 gene by itself, and although the numbers are relatively small, there was no difference in survival for the TGFβR2 mutant versus TGFβR2 wild-type cases [Fig. 2; hazard ratio, 0.94 (95% CI, 0.45-1.96); P = 0.87]. This observation highlights the conclusion that not all of the inactivating mutations in members of a pathway have the same effect on survival.

CDKN2A, TP53, and KRAS versus survival. There were no significant differences in survival between patients with and without CDKN2A gene inactivation (log-rank P = 0.63). When adjusted for lymph node status, tumor grade, margin status, tumor size, and age, the difference remained nonsignificant [hazard ratio, 1.21 (95% CI, 0.76-1.93); P = 0.42]. Similarly, TP53 gene mutation status did not correlate with survival (log-rank P = 0.21), even when adjusted for lymph node status, tumor grade, margin status, tumor size, and age [Fig. 3; hazard ratio, 1.41 (95% CI, 0.69-2.85); P = 0.34]. Virtually all of the cancers (98.9%) harbored a KRAS gene mutation, excluding the possibility of a relationship between KRAS gene status and prognosis.

Fig. 1. Kaplan-Meier curves of survival probability for patients with and without mutation or deletion in the SMAD4 gene.

Fig. 2. Kaplan-Meier curves of survival probability for patients with and without mutation or deletion in the TGFβR2 gene.
Finally, we examined the relationship between multiple gene mutations (an intragenic mutation or homozygous deletion in < 4 versus 4 or more of the 39 genes) and survival. The survival curves for these two groups of patients were not significantly different (log-rank \( P = 0.28 \)), and the difference remained nonsignificant even when adjusted for lymph node status, tumor grade, margin status, tumor size, and age [Fig. 4; hazard ratio, 1.28 (95% CI, 0.78-2.12); \( P = 0.34 \)].

The hazard ratios for all variables included in the multivariate models are presented in Table 2. For all models, increasing age was the only factor that was significantly associated with shorter survival, apart from \( SMAD4 \) gene status (see Table 2).

**Discussion**

Pancreatic cancer is often thought of as a homogeneous disease in which all patients develop early metastases and rapidly progress to death (23). Recent comprehensive studies have shown that molecularly defined subgroups of pancreatic cancer can identify patients with distinct clinical features, including prognosis and response to therapy (24–29). For example, Franko et al. (26) found that high levels of allelic loss in ductal adenocarcinomas correlate with poorer prognosis following resection, and Sato et al. (27) reported that aberrant methylation of Reprimo, a gene involved in p53-induced G2 cell cycle arrest, is associated with significantly worse prognosis. Whereas efforts to identify genetic alterations of prognostic significance have examined one gene at a time, the recent sequencing of the coding regions of the pancreatic cancer genome provided the opportunity to correlate the mutational status of a large number of genes with patient survival after surgery (17).

We correlated the mutational status of 6 the 39 genes included in both the Discovery and Validation Screen phases of the pancreatic cancer genome initiative with the survival of 89 patients in this previous study who underwent a pancreateco-duodenectomy (17). We found that patients whose cancers had \( SMAD4 \) gene inactivation, by either intragenic mutation or homozygous deletion, had significantly worse survival than patients with intact \( SMAD4 \) (Fig. 1). This result builds on previous observations that the loss of expression of the smad4 protein by immunolabeling is associated with poor prognosis (30, 31). Tascilar et al. (31) immunolabeled 249 surgically resected ductal adenocarcinomas of the pancreas for the smad4 protein and found that patients with cancers with intact smad4 protein expression survived significantly longer than did patients with cancers lacking smad4 (median survival, 19.2 versus 14.7 months; \( P = 0.03 \)). We also noted that whereas \( SMAD4 \) gene inactivation was strongly correlated with worse survival, inactivation of the \( TGF \beta R2 \) gene, another member of the \( TGF \beta \) signaling pathway, did not show an association with survival. \( TGF \beta \) signaling is mediated by the canonical pathway, for which smad4 is a central mediator, and noncanonical pathways such as the extracellular signal–regulated kinase, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase pathways, among others (32). Whereas further work is needed to investigate the role of \( TGF \) downstream pathways on patient outcome, our observation of a relationship between shorter survival and \( SMAD4 \) gene inactivation suggests a role for canonical \( TGF \beta \) signaling in pancreatic cancer.

The association of \( SMAD4 \) gene inactivation with poorer prognosis may relate to an increased propensity of pancreatic cancers with inactivated \( SMAD4 \) to metastasize widely. Iacobuzio-Donahue et al. (33) recently studied the patterns of metastases in a series of 76 patients with pancreatic cancer who underwent a rapid autopsy. Remarkably, 12% of these patients

**Fig. 3.** Kaplan-Meier curves of survival probability for patients with and without mutation or deletion in the \( TP53 \) gene.

**Fig. 4.** Kaplan-Meier curves of survival probability for patients with <4 mutations or homozygous deletions and with \( \geq 4 \) mutations or homozygous deletions.
had no evidence of metastatic disease, but instead died of complications of locally advanced pancreatic cancer (33). Among patients with metastases, there was a significant range of metastatic disease, with some patients having only a few metastases (1 to 10), whereas others had hundreds to thousands (33). The patterns of failure found at autopsy (local versus metastatic) correlated with the smad4 immunolabeling pattern of the patient’s primary carcinoma, with loss of smad4 expression correlating with widespread metastases (33). Similarly, Maitra et al. (34) showed that loss of smad4 expression correlates with progression to metastasis in colorectal carcinoma. In this latter study, none of the adenomas or stage I colorectal adenocarcinomas showed loss of smad4 expression, whereas 5 of 23 (22%) stage IV cancers showed loss of smad4 expression (34).

The association of SMAD4 gene inactivation with poorer prognosis and an increased propensity to metastasize has direct clinical implications. Some patients with pancreatic cancer have “borderline” resectable tumors—they have resectable pancreatic head cancers that are at high risk for a margin-positive resection (35). Whereas further work is needed, our results, combined with those previously reported in the literature, suggest that patients with borderline resectable pancreatic cancers and SMAD4 gene inactivation might be spared the risk of surgery because their cancer is more likely to metastasize, whereas patients with borderline resectable pancreatic cancers and intact SMAD4 may benefit from the local control provided by neoadjuvant therapy and surgical resection.

In addition to the SMAD4 gene, we correlated the mutational status of four other genes individually and a summary of all 39 genes with survival (17). The mutational status of these four other genes did not correlate with survival (Figs. 2 – 4). Whereas previous reports have suggested that the mutational status of KRAS, TP53, and CDKN2A each correlate with patient survival, we found no such correlations (36 – 48). These results are perhaps not surprising because the KRAS gene is almost universally activated, and the CDKN2A gene, if one includes methylation, is also nearly universally inactivated in pancreatic cancer (17, 49).

In summary, we correlated the mutational status of the most frequently mutated genes in pancreatic carcinoma with survival and found that only SMAD4 gene mutational status significantly correlated with outcome.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### References

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