Expression of S100A2 Calcium-Binding Protein Predicts Response to Pancreatectomy for Pancreatic Cancer

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BACKGROUND & AIMS: Current methods of preoperative staging and predicting outcome following pancreatectomy for pancreatic cancer (PC) are inadequate. We evaluated the utility of multiple biomarkers from distinct biologic pathways as potential predictive markers of response to pancreatectomy and patient survival. **METHODS:** We assessed the relationship of candidate biomarkers known, or suspected, to be aberrantly expressed in PC, with disease-specific survival and response to therapy in a cohort of 601 patients. **RESULTS:** Of the 17 candidate biomarkers examined, only elevated expression of S100A2 was an independent predictor of survival in both the training (n = 162) and validation sets (n = 439; hazard ratio [HR], 2.19; 95% confidence interval [CI]: 1.48–3.25; $P < .0001$) when assessed in a multivariate model with clinical variables. Patients with high S100A2 expressing tumors had no survival benefit with pancreatectomy compared with those with locally advanced disease, whereas those without high S100A2 expression had a survival advantage of 10.6 months (19.4 vs 8.8 months, respectively) and a HR of 3.23 (95% CI: 2.39–4.33; $P < .0001$). Of significance, patients with S100A2-negative tumors had a significant survival benefit from pancreatectomy even in the presence of involved surgical margins (median, 15.7 months; $P = .0007$) or lymph node metastases (median, 17.4 months; $P = .0002$). **CONCLUSIONS: S100A2 expression is a good predictor of response to pancreatectomy for PC and suggests that high S100A2 expression may be a marker of a metastatic phenotype. Prospective measurement of S100A2 expression in diagnostic biopsy samples has potential clinical utility as a predictive marker of response to pancreatectomy and other therapies that target locoregional disease.**

Stratification and, ultimately, individualization of therapy for cancer are current major challenges in oncology. Pancreatectomy remains the only potentially curative treatment option for pancreatic cancer and, with adjuvant chemotherapy, is associated with a 5-year survival rate of ~20%.[1,2] Preoperative staging, which governs decisions concerning the appropriateness of pancreatectomy, is currently based purely on imaging criteria, with major prognostic factors not determined until after the resected specimen has been examined microscopically. Despite clear resection margins, up to one third of patients succumb within 12 months of resection, suggesting that occult metastatic disease was present at the time of surgery.[1,3–9] Furthermore, patients with involved resection margins and/or lymph node metastases often survive longer than those who have clear margins and no evidence of lymph node metastases. An improved ability to predict individual tumor behavior and response to surgery preoperatively would reduce morbidity for patients who would not benefit from pancreatectomy, while justifying more aggressive approaches for those with a high probability of response. Although numerous candidate molecules have been examined as potential biomarkers of prognosis in pancreatic cancer (PC), few studies have assessed the utility of multiple candidate markers or performed validation studies of their predictive value in independent cohorts.[10] To address this issue, we examined the relationship of multiple potential prognostic and predictive markers from distinct biologic pathways known, or suspected, to play a role in pancreatic cancer with disease outcome and response to pancreatectomy in a large cohort of patients.

Patients and Methods

Patients and Tissue Specimens

Detailed clinicopathologic and outcome data for a total of 601 patients with a diagnosis of pancreatic ductal adenocarcinoma who underwent pancreatic resec-

Abbreviation used in this paper: PC, pancreatic cancer.

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tion or biopsy were obtained from teaching hospitals associated with the NSW Pancreatic Cancer Network (www.pancreaticcancer.net.au), Sydney, Australia (Table 1). This cohort was the combination of a training cohort of 162 patients (76 resections, which consisted of 62 Whipple pancreaticoduodenectomies and 14 left-sided pancreatectomies, and 86 intraoperative biopsies), which has previously been described,[11] and an independent validation cohort of 439 patients (296 resections, which consisted of 239 Whipple pancreaticoduodenectomies and 57 left-sided pancreatectomies, 110 intraoperative biopsies, and 33 percutaneous core biopsies). The overall survival for the training set was a median of 7.9 months with a 3- and 5-year survival of 7.5% and 2.8%, respectively. The overall survival for the validation set was a median of 13.2 months with a 3- and 5-year survival of 17.7% and 8.8%, respectively. In the majority of cases, intraoperative biopsies were performed because the tumor was assessed to be unresectable at the time of surgery (n = 66). In 44 cases, biopsy samples were taken when unsuspected metastatic disease was found at the time of operation or as part of a planned biliary bypass procedure. The training set was accrued from 4 hospitals and consisted of all patients with PC at those hospitals. They were treated with surgery only because, prior to 1998, adjuvant chemotherapy for pancreatic cancer was not used in Australia. Patients who composed the validation set were accrued from all patients with PC from 8 hospitals within the NSW Pancreatic Cancer Network who were treated between 1998 and 2007, with 30% of resected patients having received adjuvant chemotherapy (gemcitabine or 5-fluorouracil). Both cohorts displayed clinical and pathologic features that are consistent with the expected clinical behavior of pancreatic cancer and are similar to published pancreatic cancer cohorts worldwide[1,3–9] (Table 1, Supplementary Figures 1–4). Both the training and validation cohorts included patients who had intraoperative biopsies but did not undergo resection. This group included patients who had surgery with biopsy of their tumor but did not undergo pancreatectomy because of locally advanced disease. These patients provided a comparison group to allow assessment of the response to pancreatectomy compared with those with locally advanced disease and no evidence of distant metastases at surgery. Ethical approval for the acquisition of data and biologic material was obtained from the Human Research Ethics Committee at each participating institution. The diagnosis and pathologic stage were reviewed centrally by a single histopathologist (J.G.K.), and date and cause of death were obtained from the NSW Cancer Registry and treating clinicians.

Assay Development

Assays that could be readily applied in routine pathology laboratories were developed and predominantly utilized immunohistochemistry using methodology that has been previously described,[11–16] except in the case of RAI3, where in situ hybridization was used. Tissue microarrays were constructed with each resected specimen represented by a minimum of 3 × 1-mm tissue cores. Antigen was retrieved using DAKO S2367 solution (Dako Corporation, Carpenteria, CA) in a pressure cooker for 5 minutes. Immunostaining was performed using the Dako Auto-stainer (Dako Corporation). The microarrays were treated with 3% Peroxidase Block (K4011; Dako Corporation) for 5 minutes then with Protein Block (X0909; Dako Corporation) for 10 minutes. Slides were then incubated with anti-S100A2 mouse monoclonal antibody, 1:50 dilution, for 60 minutes (clone DAK-S100A2/1; Dako Corporation). The primary antibody was visualized using the Dako Envision+ secondary detection system (K4003; Dako Corporation) followed by color development using 3,3-diaminobenzidine (K3468; Dako Corporation). Sections were counterstained using hematoxylin. Staining was assessed by 2 independent observers for each assay, at least one of whom was a specialist histopathologist. Standardization of scoring was achieved by comparison of scores between observers and by conferencing, which resolved any discrepancies by consensus. Scores were dichotomized with cut points determined based on the distribution of scores, reproducibility, or as previously described.[11–15] High S100A2 expression was defined as cytoplasmic staining with intensity 3+ in >30% of cells; moderate-high staining was defined as intensity 2+ in >30% of cytoplasmic staining, up to intensity 3+ in 30% of cells. Low expression was defined as any staining with 1+ intensity up to intensity 2+ in 30% of cells.

Statistical Analysis

Median survival was estimated using the Kaplan-Meier method, and hazard ratios (HR) were derived using the univariate and multivariate Cox proportional hazard model. Statistical analysis was performed using StatView 5.0 Software (Abacus Systems, Berkeley, CA). A P value of $< .05$ was accepted as statistically significant. Disease-specific survival (DSS) was used as the primary end point. Analysis was performed sequentially on all patients and then on a subgroup of patients who underwent operative resection from both the training set and the validation set.

Results

Gene Expression and Survival

Seventeen candidate biomarkers were selected based on their potential role in pancreatic cancer and from global analysis of gene expression of pancreatic cancers performed by our group[15] and others[17–19] (Table 2 and Supplementary Data). Some of these genes (DPC4/Smad4, LMO4, sFRP4, β-catenin, cyclin E1, HOXB2) have been investigated previously as potential biomarkers

Table 1. Clinicopathologic Parameters and Outcome for all Patients[f]

	Training set						Validation set					
	All patients			Resected			All patients			Resected		
Parameter	n = 162 No. (%)	Median DSS (mo)	*P* value (log rank)	n = 76 No. (%)	Median DSS (mo)	*P* value (log rank)	n = 439 No. (%)	Median DSS (mo)	*P* value (log rank)	n = 296 No. (%)	Median DSS (mo)	*P* value (log rank)
Sex												
Female	75 (46.3)			31 (40.8)			222 (50.6)			148 (50)		
Male	87 (53.7)			45 (59.2)			217 (49.4)			148 (50)		
Age, *y*												
Mean	64.6			62.3			66.5			66.8		
Median	66.5		.5425	65.0		.1405	68.7		.1887[a]	69.0		.2631[a]
Range	34.4–85.7			34–82			28–87			28–87		
Specimen												
Resection	76 (46.9)	12.2					296 (67.4)	17.9				
Biopsy	79 (48.8)	4.8	<.0001				143 (32.6)	7.5	<.0001			
Postmortem	7 (4.3)											
Outcome												
Follow-up (mo)	0–124.0			0.3–124			0–168			0–168		
Median follow-up	7.3			12.2			11.9			16.1		
30-day mortality	2 (1.2)			2 (2.6)			21 (4.8)			13 (4.4)		
Death PC	154 (95.1)			69 (90.9)			371 (84.5)			221 (74.6)		
Death other	2 (1.2)			2 (2.6)			10 (2.3)			10 (3.4)		
Alive	3 (1.9)			3 (3.9)			54 (12.3)			52 (17.6)		
Lost to follow-up	1 (0.6)			0			4 (0.9)			0		
Stage[b]	160[c]						437[c]					
I	8 (5.0)			8 (10.5)	18.6		28 (6.4)			28 (9.5)	27.9	
II	68 (42.5)	12.2		68 (89.5)	11.5	.9187	268 (61.3)	17.9		268 (90.5)	17.1	.0166
III	62 (38.8)						57 (13.0)					
IV	22 (13.7)	4.8	<.0001				84 (19.3)	7.5	<.0001			
Differentiation[d]												
Well	11 (6.8)			7 (9.2)			43 (9.8)			25 (8.4)		
Moderate	90 (55.6)	9.5		44 (57.9)	14.8		269 (61.3)	13.5		198 (66.9)	18.1	
Poor	61 (37.7)	5.8	.0026	25 (32.9)	10.1	.0480	127 (28.9)	11.9	.1065[e]	73 (24.7)	17.5	.8257
Tumor location[f]												
Head				62 (81.6)	15.6					239 (80.7)	19.4	
Body/tail				14 (18.4)	7.9	.0002				57 (19.3)	13.2	.0201
Tumor size[g]												
≤20 mm				15 (19.7)	17.1					69 (23.3)	31.0	
>20 mm				61 (80.3)	11.2	.2325				227 (76.7)	16.2	<.0001
Margins												
Clear				42 (55.2)	19.7					184 (62.2)	23.3	
Involved				34 (44.8)	9.5	<.0001				112 (37.8)	13.2	<.0001
Lymph nodes				75								
Negative				35 (46.7)	19.7					110 (37.2)	23.3	
Positive				40 (53.3)	10.1	.0007				186 (62.8)	16.2	.0056
Subtype												
Tubular/papillary				69 (90.8)	14.2					282 (95.3)	17.8	
Other				7 (9.2)	10.8	.0937				14 (4.7)	36.4	.1235
Perineural invasion												
Negative				30 (39.5)	12.2					81 (27.4)	26.0	
Positive				46 (60.5)	12.2	.0818				215 (72.6)	16.9	.0060
Vascular invasion												
Negative				46 (60.5)	14.8					162 (54.7)	19.6	
Positive				30 (39.5)	9.2	.0270				134 (45.3)	16.7	.0700
Chemotherapy[h]												
No therapy				65 (85.5)	12.2					178 (60.8)	15.1	
Any therapy[i]				11 (14.5)	18.6	.3103				115 (39.2)	26.0	.0191
Chemotherapy												
No adjuvant										208 (71.0)	16.0	
Adjuvant										85 (29.0)	27.5	.0071
Chemotherapy												
No Adj or <3 cycles										238 (81.2)	16.2	
Adjuvant (≥3 cycles)[j]										55 (18.8)	34.3	.0007
Radiotherapy												
No radiotherapy										275 (92.9)	17.8	
Any radiotherapy[k]										21 (7.1)	22.4	.4742

NOTE. N = 601.

[a]Analyzed as a continuous variable.

[b]Stage I and II tumors vs stage III and IV for survival analysis; stage information was not available for 2 patients.

[c]Clinical stage.

[d]Well and moderately differentiated tumors grouped together for survival analysis.

[e]Heterogeneity with 3 variables, *P* = .1080.

[f]Patients with tumors located in the head of the pancreas underwent Whipple pancreaticoduodenectomies, and those with tumors of the body/tail had left-sided pancreatectomies.

[g]Tumor size was prognostic as a continuous variable (*P* = .0021), >30 mm (*P* = .0012), and >40 mm (*P* = .0008) in the validation set.

[h]Gemcitabine or 5-FU: Adjuvant (n = 85), Nil chemotherapy at any time (n = 178), palliative (n = 27), neoadjuvant (n = 3), unknown (n = 3).

[i]Patients who received any form of chemotherapy at any time (note that, in the training set, chemotherapy was only given for palliation of symptoms).

[j]Analysis compares those patients who received ≥3 cycles of chemotherapy vs those who received less or no therapy.

[k]Analysis compares those patients who received radiotherapy at any time to all others.

Table 2. Gene Expression and Survival

Gene	All patients, No. (%)	Median survival, mo	*P* value (log rank)	Resected, No. (%)	Median survival, mo	*P* value (log rank)
Training set			n = 162			n = 76
Regulators of transcription						
HOXB2						
Negative	79 (62.7)	11.2		60 (78.9)	16.2	
Positive[a]	47 (37.3)	5.8	<.0001	16 (21.1)	7.5	<.0001
LMO4						
Negative	20 (16.7)	4.7		9 (12.0)	8.4	
Positive	100 (83.3)	9.5	.0227	66 (88.0)	13.6	.1635
S100 Calcium binding proteins						
S100A2						
Negative	86 (74.8)	9.8		54 (73.0)	14.2	
Positive[b]	29 (25.2)	7.9	.0182	20 (27.0)	9.5	.0073
S100A6						
Negative	56 (45.5)	5.8		17 (23.0)	11.5	
Positive	67 (54.5)	11.9	<.0001	57 (77.0)	13.6	.3509
S100P						
Negative	59 (47.6)	6.8		20 (26.7)	11.9	
Positive	65 (52.4)	12.2	.0005	55 (73.3)	14.2	.4708
Cell cycle regulation						
Cyclin E1						
Negative	85 (68.5)	10.8		51 (68.0)	16.8	
Positive	39 (31.5)	7.2	.0071	24 (32.0)	9.1	.0355
Cyclin D1						
Negative	60 (53.6)	7.0		24 (53.3)	14.2	
Positive	52 (46.4)	6.4	.3253	21 (46.7)	9.5	.3481
$p16^{INK4A}$						
Negative	53 (59.6)	6.2		19 (59.4)	12.2	
Positive	36 (40.4)	6.4	.9926	13 (40.6)	8.7	.4111
$p21^{WAF1/CIP1}$						
Negative	59 (50.9)	7.9		30 (65.2)	12.2	
Positive	57 (49.1)	6.4	.0898	16 (34.8)	9.2	.5468
$p27^{Kip1}$						
Negative	49 (40.5)	9.7		25 (34.7)	16.3	
Positive	72 (59.5)	8.4	.9797	47 (65.3)	11.9	.9922
p53						
Negative	56 (48.3)	7.0		23 (50.0)	8.6	
Positive	60 (51.7)	6.8	.8510	23 (50.0)	14.2	.1873
Receptor signaling						
EGFR						
Negative	72 (54.1)	9.2		46 (62.2)	11.2	
Positive	61 (45.9)	7.9	.6110	28 (37.8)	15.6	.4470
RAI3						
Negative	33 (29.2)	8.0		9 (12.2)	12.2	
Positive	80 (70.8)	10.1	.0541	65 (87.8)	12.2	.6851
DPC4/Smad4						
Negative	60 (51.7)	6.4		12 (26.1)	7.5	
Positive	56 (48.3)	9.5	.0215	34 (73.9)	14.8	.2507
sFRP4						
Negative	66 (56.9)	6.1		32 (45.1)	9.1	
Positive	50 (43.1)	12.9	.0146	39 (54.9)	16.3	.1228
β-catenin^{cytoplasm}						
Negative	55 (40.4)	6.0		22 (29.7)	10.5	
Positive	81 (59.6)	8.7	.0224	52 (70.3)	15.0	.2250
β-catenin^{nuclear}						
Negative	119 (87.5)	6.6		61 (82.4)	12.2	
Positive	17 (12.5)	9.5	.0362	13 (17.6)	18.6	.3766
CRBP1						
Negative	62 (69.7)	10.4		34 (63.0)	10.8	
Positive	27 (30.3)	13.2	.3720	20 (37.0)	16.3	.3175
Validation set			n = 439			n = 296
S100A2 (high expression)[c]						
Negative	308 (85.3)	15.5		224 (84.8)	19.4	
Positive	53 (14.7)	11.2	.0015	40 (15.2)	11.1	<.0001

Table 2. (Continued)

Gene	All patients, No. (%)	Median survival, mo	*P* value (log rank)	Resected, No. (%)	Median survival, mo	*P* value (log rank)
S100A2 (mod/high expression)[d]						
Negative	260 (72.0)	15.8		193 (73.1)	19.6	
Positive	101 (28.0)	11.3	.0011	71 (26.9)	13.2	.0008
S100A2 (stratified)						
Negative	205 (56.8)	16.2		150 (56.8)	22.3	
Low/moderate[e]	103 (28.5)	13.2	.0007	74 (28.0)	16.1	<.0001
High	53 (14.7)	11.2		40 (15.2)	11.1	
HOXB2 (mod/high expression)						
Negative				189 (71.9)	17.9	
Positive				74 (28.1)	17.5	.8922
HOXB2 (high expression)				n = 263		
Negative				246 (93.5)	17.9	
Positive				17 (6.5)	15.1	.2246

NOTE. Dichotomization of gene expression based on characteristics of expression pattern of individual genes with positive denoting high expression and negative low/absent expression. For detailed methodology, please refer to Supplementary Material Table 3.
[a]Cut point of homogeneous intensity 2+ nuclear staining in >20% of cells.[15]
[b]Cut point of homogeneous intensity 2+ cytoplasmic staining in >30% of cells (mod/high expression) was used.
[c]High expression of S100A2 was defined as intensity 3+ in >30% of cells.
[d]Mod/high expression was defined as for the training set (intensity 2+ cytoplasmic staining in >30% of cells).
[e]Low/moderate expression was defined as cells with cytoplasmic staining of intensity 1+ or more in >30% of cells but no greater than 3+ in 30% of cells, which was the high expression cut point.

in the training cohort prior to its maturation to reach a follow-up of over 5 years.[11–15] The results of univariate analyses showed that expression of 9 biomarkers correlated with disease-specific survival in all patients in the training set (Table 2). However, the majority were not independent prognostic factors in multivariate models incorporating clinicopathologic variables (Supplementary Table 1). Results of the Cox multivariate regression analysis showed that only high expression of HOXB2 and cyclin E1, moderate/high expression of S100A2, and low/absent expression of LMO4 remained independent markers of poor disease-specific survival (Table 3, heading A).

Patients Who Underwent Pancreatectomy

To investigate the potential predictive value of candidate biomarkers of response to operative resection, we next assessed the 17 candidate markers in the subset of patients in the training cohort who underwent operative resection (n = 76). Kaplan–Meier estimates of cumulative disease-specific survival showed that high expression of HOXB2 and cyclin E1 and moderate/high expression of S100A2 were associated with poor survival (Table 2, Figure 1*A* and *B*, Supplementary Figure 2*J*). In addition, S100A2 expression was inversely related to survival when modeled as a continuous variable using a simplified H-score[20] (intensity × percentage of staining cells, $P < .0001$). Multivariate analysis of all factors that were associated with outcome on univariate analysis identified that margin involvement by tumor, high HOXB2 expression, and moderate/high S100A2 expression were the only independent poor prognostic factors (Table 3, heading B, Supplementary Table 1, heading B to I).

Expression of HOXB2 and S100A2 and Response to Pancreatectomy

To assess whether expression of these genes cosegregated with a differential response to pancreatectomy, we made comparisons with patients who had locally advanced disease who had intraoperative biopsies but were not resected. Patients with cancers that were S100A2 and/or HOXB2 positive compared with patients with locally advanced disease (ie, intraoperative assessment and biopsy with no evidence of macroscopic peritoneal or hepatic metastases) had a median disease-specific survival benefit of only 3.8 months (HR, 1.77; 95% confidence interval [CI]: 1.11–2.82; $P = .0142$), with no survivors beyond 22 months, whereas those that were negative for both S100A2 and HOXB2 had a benefit of 11.9 months (HR, 4.33; 95% CI: 2.70–6.94; $P < .0001$, Figure 1*C*). Only 3 patients had high expression of both HOXB2 and S100A2, and all 3 died of their disease by 7.5 months.

This finding has potential clinical significance and utility because gene expression can be assessed preoperatively using endoscopic ultrasound (EUS) guided fine-needle aspiration biopsy (FNAB) and could facilitate decision making regarding operative resection. Thirty-nine percent (39%) of patients who underwent Whipple pancreaticoduodenectomy had negative margins and were negative for HOXB2 and S100A2. These patients had a substantially better outcome than the remaining patients (Figure 1*D*), with a median disease-specific survival for patients who were treated with surgery alone of 31.1 months, a 3-year survival of 50%, a 5-year survival of 28%, and a 10-year survival of 21%. This suggests that pancre-

Table 3. Multivariate Analysis

	Variable	Hazard ratio (95% CI)	*P* value
Training set			
A. PC (n = 98)	Treatment (biopsy only)	3.00 (1.65–5.45)	.0003
	Stage (III and IV)	2.36 (1.37–4.06)	.0020
	HOXB2 expression (positive)	2.00 (1.15–3.49)	.0138
	LMO4 expression (negative)	2.82 (1.25–6.41)	.0130
	S100A2 expression (positive)	1.98 (1.19–3.27)	.0081
	Cyclin E1 (positive)	1.62 (1.04–2.54)	.0343
B. Resected PC (n = 72)	Margin involvement (positive)	2.45 (1.41–4.28)	.0016
	HOXB2 expression (positive)	3.15 (1.66–5.96)	.0004
	S100A2 expression (positive)	2.00 (1.11–3.61)	.0216
C. Whipple resection (n = 59)	Margin involvement (positive)	2.77 (1.51–5.06)	.0010
	HOXB2 expression (positive)	5.01 (2.36–10.6)	<.0001
	S100A2 expression (positive)	3.23 (1.58–6.62)	.0014
Validation set			
D. PC (n = 439)	Treatment (biopsy only)	3.66 (2.80–4.78)	<.0001
	Stage (III and IV)	1.41 (1.10–1.81)	.0063
	S100A2 expression (high)	1.45 (1.12–1.89)	.0053
E. Resected PC (n = 296)	Tumor size (≥20 mm)	1.60 (1.13–2.25)	.0076
	Tumor location (body/tail)	1.43 (1.02–2.01)	.0405
	Lymph node metastases (positive)	1.54 (1.15–2.04)	.0033
	Margin involvement (positive)	1.73 (1.31–2.28)	<.0001
	Vascular invasion (positive)	1.32 (1.01–1.73)	.0446
	Adjuvant chemotherapy (≥3 cycles)	0.53 (0.37–0.78)	.0011
F. Resected PC (n = 296)	Tumor size (≥20 mm)	1.59 (1.11–2.29)	.0122
	Lymph node metastases (positive)	1.50 (1.11–2.03)	.0080
	Margin involvement (positive)	1.68 (1.25–2.25)	.0005
	Adjuvant chemotherapy (≥3 cycles)	0.58 (0.39–0.87)	.0079
	S100A2 expression (high)	1.87 (1.25–2.81)	.0024

NOTE. Heading A is the resolved model for all patients in the training set. Heading B is the resolved model for resected patients. Heading C is the resolved model for the subgroup of patients who underwent Whipple pancreaticoduodenectomy. Heading D is the resolved model for all patients in the validation set. Heading E is the resolved model for clinicopathologic factors in resected patients. Heading F is the resolved model for clinicopathologic factors and biomarkers and shows that high S100A2 expression is an independent prognostic factor.

atectomy in patients with S100A2- and HOXB2-negative tumors, particularly if clear margins are achieved, is associated with significant long-term survival. Limitations in available tissue in biopsy samples restricted the number of patients assessed for individual gene expression, precluding analysis of this subgroup in isolation. Expression of HOXB2 and S100A2 were independent poor prognostic factors in the training set and thus progressed to assessment in the validation set. There was no correlation between S100A2 and HOXB2 in the training cohort using χ^2 analysis of proportions (all patients, $\chi^2 = 0.060$; $P = .8061$ and resected patients, $\chi^2 = 2.214$; $P = .1368$).

Analysis of HOXB2 and S100A2 Expression in the Validation Set

High expression of S100A2, but not HOXB2, cosegregated with a poor disease-specific survival in patients who underwent pancreatectomy in the validation set. Immunohistochemical detection of S100A2 expression was consistent and reproducible with those with high expression segregating into a discrete group. Scores were assessed for both intensity of cytoplasmic staining, and the percentage of cells that were positive (Figure 2*A–D*). Low-intensity staining was seen in only occasional cells in the normal pancreas (Figure 2*E*). There was no correlation seen between HOXB2 and S100A2 expression in the validation cohort (resected patients, $\chi^2 = .000379$; $P = .9845$).

S100A2 expression using a simplified H-score[19] (HR, 1.003 per increment of 1, range: 0–300, 95% CI: 1.001–1.004; $P < .0001$) or dichotomized to differentiate those with moderate/high expression (cut point used in the training set, HR, 1.68; 95% CI: 1.24–2.29; $P = .0009$) or with high expression of S100A2 (HR, 2.19; 95% CI: 1.48–3.25; $P < .0001$) was an independent marker of poor outcome in patients who underwent pancreatectomy (Table 3, heading F; Supplementary Table 2, heading J; Figure 3*A*; and Supplementary Figure 5*A*). The association between S100A2 expression and disease-specific survival was robust over several cut points either side of those presented and had an expression dependent relationship with survival (Figure 3*B*). Those patients who had no detectable expression of S100A2 had the best outcome, those with high expression had the worst, and those with low to moderate expression had an intermediate outcome. High S100A2 expression was associated with poor differentiation ($P = .0029$) and increased tumor size ($P = .0110$) but was not associated with lymph node metastases ($P = .62$) or any of the other clinicopath-

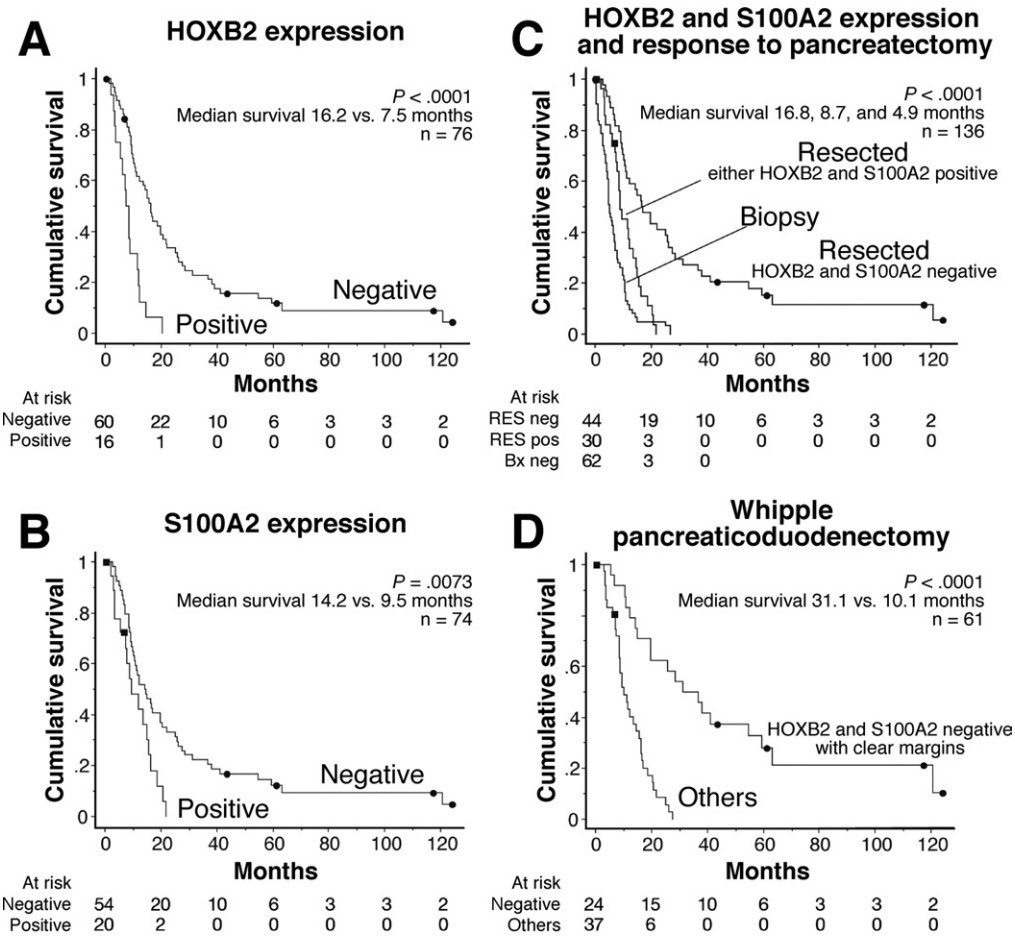

Figure 1. Kaplan–Meier survival curves (training set) for (*A*) HOXB2 expression, (*B*) moderate/high S100A2 expression, (*C*) HOXB2 and S100A2 expression, and response to pancreatectomy. Patients whose tumors were HOXB2 and S100A2 negative had a superior response to pancreatectomy with a median survival advantage of 11.9 months over equivalent stage tumors that were not resected, compared with those who are positive for HOXB2 and/or S100A2 where the median survival advantage was only 3.8 months. (*D*) Patients who underwent Whipple pancreaticoduodenectomy showing that those who were HOXB2 and S100A2 negative and resected with clear margins had a better outcome with a median survival of 31.1 months, a 3-year survival of 50%, a 5-year survival of 28%, and a 10-year survival of 21%.

ologic parameters. Neither S100A2 nor HOXB2 was associated with a differential response to adjuvant chemotherapy.

Expression of S100A2 Cosegregates With Response to Pancreatectomy

Patients with tumors that demonstrated high expression of S100A2 had a median survival of 11.1 months, which was not significantly different from patients with locally advanced disease irrespective of S100A2 expression who only had biopsies (median survival, 8.8 months; HR, 1.49; 95% CI: 0.96–2.30; $P = .0725$). In contrast, patients with tumors that did not demonstrate high S100A2 expression had a median survival of 19.4 months, which was significantly better than

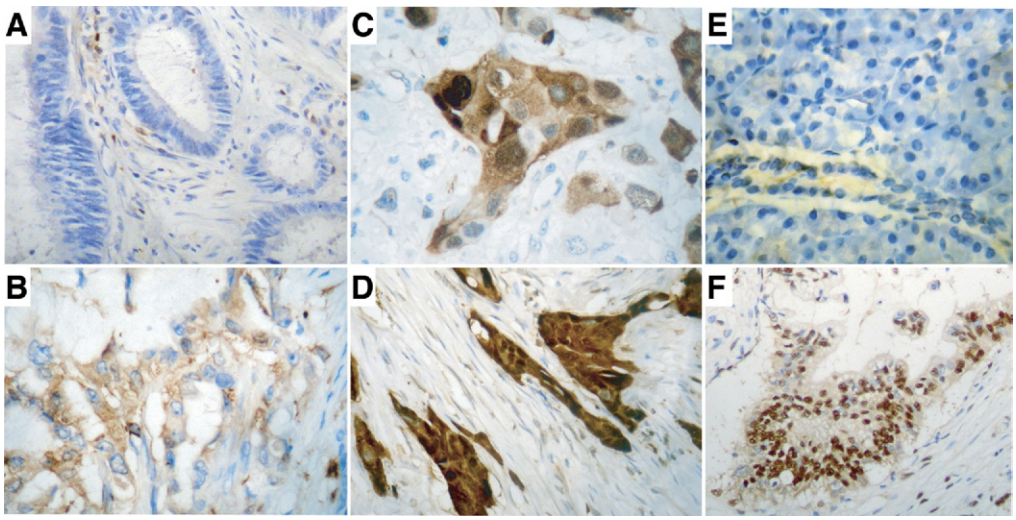

Figure 2. Photomicrographs showing examples of PC with no S100A2 expression (*A*), low S100A2 expression (*B*), moderate S100A2 expression (*C*), and high S100A2 expression (*D*). *E* shows that normal pancreas has no significant S100A2 expression and (*F*) HOXB2 expression (positive) in pancreatic cancer.

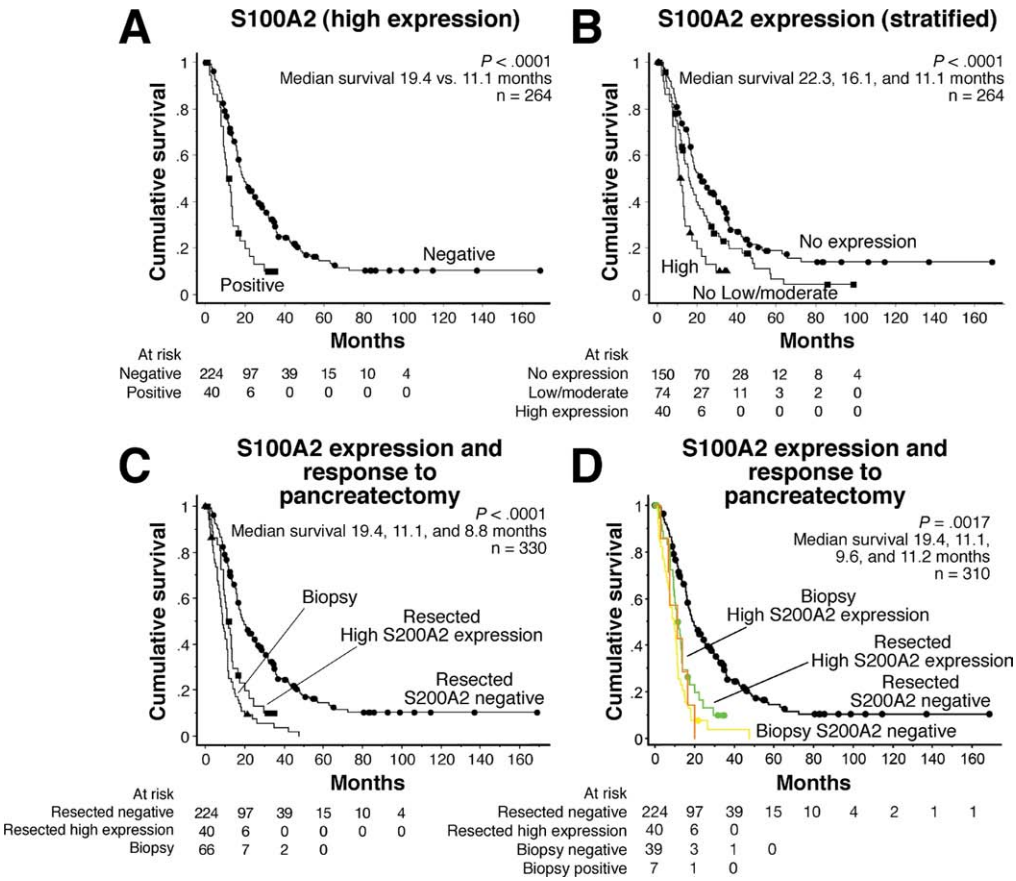

Figure 3. Kaplan–Meier survival curves (validation set) for (*A*) high S100A2 expression, (*B*) dose-dependent relationship with survival and S100A2 expression, (*C*) patients with tumors that demonstrated high S100A2 expression did not have a statistically significant survival advantage with resection compared with locally advanced tumors that did not undergo resection, and (*D*) showing survival curves of resection vs biopsy stratified by S100A2 status.

biopsy-only patients (HR, 3.23, 95% CI: 2.39–4.33; $P < .0001$, Figure 3*C*).

A key finding was that patients who had S100A2-negative tumors, even those with involved surgical margins and lymph node metastases, had a significant survival benefit with pancreatectomy (median survival, 15.7 months; HR, 2.48; 95% CI: 1.47–4.17; $P = .0007$ in margin-positive patients, Figure 4*A*, and a median survival of 17.4 months, HR, 2.22; 95% CI: 1.47–3.37; $P = .0002$ in patients with lymph node metastases, Figure 4*B*). In the subgroup of patients who had resections with clear surgical margins (n = 167), those whose tumors demonstrated high or moderate/high expression of S100A2 cosegregated with a poor outcome ($P = .0058$ and $P = .0060$, respectively, Figure 4*C* and Supplementary Figure 5*B*), suggesting that occult distant metastatic disease may have been present that generated the hypothesis that S100A2 was either a surrogate marker of, or plays a key role in the metastatic process. Further analysis was restricted by small numbers in the subgroup of interest. However, 66 patients had clear resection margins and no lymph node metastases, and, in this group, high S100A2 expression (n = 11) was a poor prognostic factor of borderline statistical significance (HR, 2.11; 95% CI: 0.96–4.67; $P = .0575$, Figure 4*D*). S100A2 expression did not cosegregate with survival in patients who only underwent biopsy ($P = .7419$) suggesting that S100A2 expression is a predictive marker of response to pancreatectomy rather than a prognostic factor in pancreatic cancer. Conversely, in the subgroup of patients who had positive resection margins and lymph node metastases (n = 80), patients with S100A2-negative tumors had a median survival of 14.2 months, compared with those who were positive who had a median survival of only 8.3 months ($P = .0015$).

Discussion

Substantial improvements in outcomes have been achieved in some cancers, eg, breast cancer, through defining phenotypic subgroups using molecular markers of outcome and therapeutic responsiveness.[21] The ability to forecast an individual patient's response to specific therapies using biomarkers stratifies patients to appropriate therapeutic regimens and facilitates ongoing investigation of treatment-resistant subgroups to identify novel, more effective therapies. This study identifies that high expression of S100A2 is associated with a poor response to pancreatectomy for pancreatic cancer and may either play a key role in, or be a surrogate marker of, the development of a metastatic phenotype. S100A2 expression is currently the only predictive biomarker that has been validated in pancreatic cancer and has significant potential utility in facilitating clinical decision making

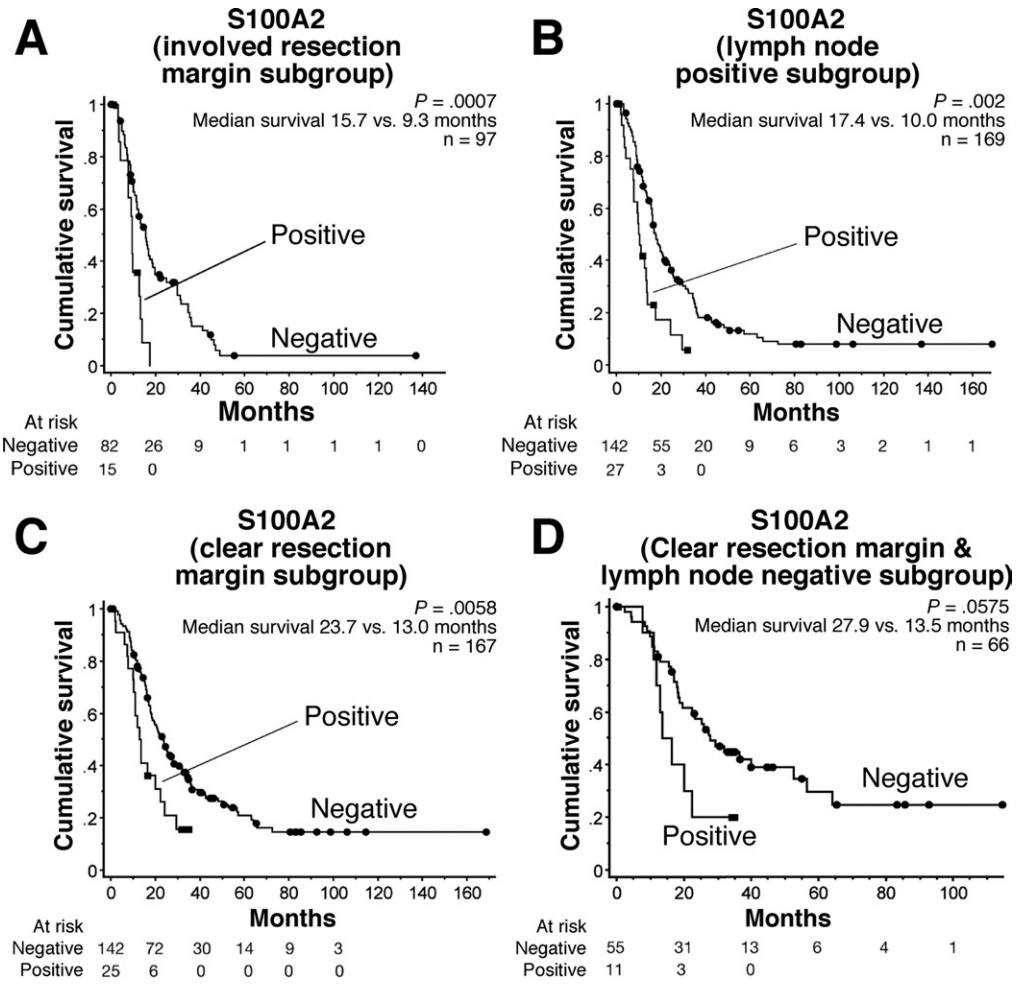

Figure 4. Kaplan–Meier survival curves of patients with tumors that did not demonstrate high S100A2 expression had significant survival benefits from pancreatectomy even in the presence of involved resection margins (*A*) and lymph node metastases (*B*). High S100A2 expression cosegregated with poor survival in patients who underwent pancreatectomy and had either (*C*) clear resection margins or (*D*) clear resection margins and no lymph node metastasis.

with regard to operative resection and other therapies that target locoregional disease in pancreatic cancer.

High S100A2 expression was an independent poor prognostic factor in resected pancreatic cancer both in a training set of patients with long-term follow-up who were treated with surgery alone and a validation set that was treated with more contemporary approaches, which included adjuvant chemotherapy, strongly supporting and extending a recent report that identified high S100A2 expression as a poor prognostic factor in a cohort of 24 patients.[22] In addition, high expression of S100A2 cosegregated with a poor response to pancreatectomy, and these patients did not have a significant survival advantage over patients with locally advanced tumors who only underwent intraoperative biopsy. Conversely, patients with involved surgical margins and lymph node metastases who had S100A2-negative tumors still had substantial benefit from pancreatectomy with median survivals in excess of 15 months.

The potential clinical significance of these findings is that knowledge of S100A2 expression preoperatively could guide decisions regarding selection of patients for pancreatectomy. Substantial morbidity and impact on quality of life for 3 to 6 months after surgery[23] could be avoided for those with high S100A2 expressing tumors, and patients could be directed to more appropriate therapeutic modalities without delay. More aggressive approaches would be warranted in those with S100A2-negative tumors because of greater confidence in providing a benefit with pancreatectomy. Novel surgical techniques and radiotherapy strategies to treat locoregional disease, which do not appear to be beneficial overall, or associated with adverse effects, could be investigated and assessed in subgroups of patients who have S100A2-negative tumors based on the hypothesis that they have a lower prevalence of occult metastatic disease. As a consequence, including assessment of S100A2 expression in future clinical trials of therapeutic approaches for pancreatic cancer would further define its clinical utility as a biomarker and could potentially identify subgroups of patients with a differential response to a specific therapy. Approximately 40% of patients have clinically localized disease at diagnosis; stratification of therapies based on S100A2 expression may thus increase overall resection rates because only 15% of these patients demonstrate high S100A2 expression. Patient selection

using biomarkers that better define nonmetastatic phenotypes, particularly in clinical trials of novel therapies directed at locoregional disease, has the potential to improve overall outcomes for pancreatic cancer.

These data suggest that high S100A2 expression may be a potential marker of occult distant metastatic disease at the time of surgery because even in patients with tumors that had clear surgical margins in the validation cohort, high expression of S100A2 remained a poor prognostic factor. Furthermore, the significant survival benefit with pancreatectomy in patients with S100A2-negative tumors even with involved surgical margins and lymph node metastases suggests that occult distant metastatic disease was less prevalent in this subgroup. In addition, cell lines derived from metastatic sites, particularly from peritoneal metastases, demonstrate markedly higher expression of S100A2 compared with those derived from primary tumors,[22] and S100A2 directly represses expression of BNIP3, a proapoptotic member of the Bcl-2 family of apoptosis-regulating proteins.[24] Recent data based on a xenograft model of NSCLC suggest that S100A2 may have an integral role in the metastatic process.[25] Other S100 calcium-binding proteins such as S100A4 are also thought to play a key role in the development of metastatic disease in pancreatic cancer.[26,27] Further experimentation is necessary to determine whether S100A2 plays a key role in tumor progression and metastasis in pancreatic cancer and therefore whether it is an attractive target for the development of novel therapies or whether it is merely a surrogate marker of other molecular mechanisms involved in these processes.

From the initial analysis of 17 candidate biomarkers of poor survival from pancreatic cancer presented here, only S100A2 was validated to have potential predictive value in forecasting response to pancreatectomy for pancreatic cancer. This result further highlights the necessity for validation of the utility of candidate markers in independent cohorts. The limitations of retrospectively accrued cohorts, which include this study, may lead to conflicting results,[28] and greater insights would be gained from prospective clinical trials. We have designed a prospective clinical trial to assess S100A2 expression in FNAB samples obtained preoperatively using EUS as a predictor of response to pancreatectomy for pancreatic cancer. Using modifications of existing methodologies,[29,30] we determined that assessment of messenger RNA expression in these samples is feasible and reproducible. If the association of S100A2 expression with response to pancreatectomy is prospectively validated in the setting of a clinical trial, clinical assessment of S100A2 in preoperative specimens could significantly influence patient management decisions in the treatment of pancreatic cancer by resecting those cancers that are S100A2 negative and directing those with S100A2-positive cancers to alternative treatment, eg, neoadjuvant pathways.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.04.009.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Data

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Candidate Biomarker Identification

Initially, candidate genes were selected based on their known or perceived role in cancer in general and pancreatic cancer in particular. Those that demonstrated aberrant expression in a significant proportion of pancreatic cancer (PC) included cell cycle regulatory molecules (cyclins D1 and E1, $p21^{CIP1}$, $p27^{KIP1}$, $p16^{INK4A}$, p53) and molecules involved in receptor-mediated signaling (EGFR and *DPC4*/Smad4).

Subsequently, a gene discovery approach using Affymetrix Genechip HGU133 (Santa Clara, CA) microarrays[1] was used to identify novel candidates that were selected based on degree of differential expression in PC compared with normal pancreas, potential functional importance in pancreatic carcinogenesis, and potential clinical utility. Genes that demonstrated differential expression in a significant proportion of PC compared with normal pancreas included molecules involved in retinoic acid signaling (RAI3 [26-fold], CRBP1 [0.2-fold]), those involved in *wnt* signalling (sFRP4 [5.7-fold], β-catenin [2.0 fold]), members of the S100 family of calcium-binding proteins (S100A2 [23-fold], S100A6 [15-fold], and S100P [152-fold]), and transcriptional regulators HOXB2 (6.7-fold) and LMO4 (2.0-fold). Detailed methodology concerning global analysis of gene expression using Affymetrix Genechip oligonucleotide microarrays has been described previously.[1]

Immunohistochemistry

Tissue microarrays were cut at 4 microns, deparaffinized, and rehydrated before unmasking in target retrieval solution (Dako Corporation, Carpentaria, CA). Using a Dako autostainer, endogenous peroxidase activity was quenched in 3% hydrogen peroxide in methanol, followed by avidin/biotin and serum-free protein blocks (Dako Corporation).

A streptavidin-biotin peroxidase detection system (ABC Vectstain Elite, or LSAB label + link kit; Dako Corporation) or a labelled polymer horseradish peroxidase secondary antibody detection system (Envision +, Dakocytomation; Dako Corporation) was used according to the manufacturer's instructions with 3,3′-diaminobenzidine as a substrate. Counterstaining was performed with Mayer hematoxylin. Appropriate positive and negative human cell line and tissue controls where protein expression of the particular gene was known using other methodologies were used in each case as well as a no primary antibody control.

In Situ Hybridization

Complementary DNA was prepared using the Expand Reverse Transcriptase System (Roche Diagnostics, Mannheim, Germany) followed by a polymerase chain reaction using the Expand High Fidelity PCR system (Roche Diagnostics) and RAI3 primers (forward, 5′-TTA AGT GGG AGT CTC AGG CA-3′; reverse, 5′-GAG GCA GCA CTA GAG AGA TGA-3′). Polymerase chain reaction (PCR) product was purified using Roche HighPure RNA Isolation Kit (Roche Diagnostics), according to the manufacturer's instructions. Template for the in vitro transcription reaction was constructed by ligation of the T7 promoter to 25 ng of the PCR product using a T4 DNA

ligase and the T7 Promoter Adapter in the Lig'nScribe Kit (Ambion, Austin, TX). Amplification of the IVT templates was achieved using the RAI3 in situ primers, T7 adapter primers (provided in the Lig'nScribe kit) using the Expand High Fidelity PCR system (Roche Diagnostics). IVT templates were sequenced (Australian Genome Research Facility, Brisbane, QLD, Australia), and the IVT reaction was performed using T7 RNA polymerase reaction followed by DNase1 digestion using the DIG RNA Labelling Mix (Roche Diagnostics) as per manufacturer's instructions. The IVT reaction was purified using ethanol precipitation in the presence of LiCl, and the resultant pellet was dissolved in RNase inhibitor and stored at −70°C and quantified by serial dilution of the probe and controlled DIG-labelled RNA (Roche Diagnostics) and spotted onto a Transblot Transfer Nitrocellulose Membrane (Bio-Rad Laboratories, Hercules, CA).

In situ hybridization was performed using the Discovery automated machine (Ventana Medical Systems, Tucson, AZ), the RiboMap Kit for tissue preparation, and the BlueMap Kit for detection (Ventana Medical Systems). Briefly, sections were pretreated with Riboprep for 30 minutes at 37°C, RiboClear for 10 minutes at 37°C, Mild Cell Conditioning Solution No. 2 for 2 minutes, and Protease III for 2 minutes at 37°C (all from Ventana Medical Systems). Hybridization was performed by wet application of 10 ng of in situ probe in Liquid Cover Slip, denaturation for 10 minutes at 70°C, and hybridization for 8 hours at 60°C followed by 2 washes with Ribowash for 6 minutes at 75°C and posttreatment fixation for 6 minutes at 37°C with Ribofix (all products from Ventana Medical Systems). Signal detection was achieved using a monoclonal antidigoxin biotin conjugate antibody (Sigma Chemical Co, St. Louis, MO) for 30 minutes at 37°C with the substrate provided by the BlueMap Detection Kit for 6 hours. Sections were counterstained using Nuclear Fast Red (Dako Corporation).

Scoring

Staining was assessed by 2 independent observers for each assay, at least one of whom was a specialist anatomical pathologist. Standardization of scoring was achieved by comparison of scores between observers and by conferencing, which resolved any discrepancies by consensus. Scores were dichotomized with cut points determined based on the distribution of scores, the known or suspected functional attributes of the protein and their relevance to carcinogenesis, reproducibility, or as previously described[1–5] and presented in Supplementary Table 3.

Statistical Evaluation

Statistical evaluation was performed using Kaplan-Meier survival for univariate analysis and the Cox proportional hazards model for multivariate analysis using StatView 5.0 software (Abacus Systems, Berkeley, CA). A P value of $< .05$ was accepted as statistically significant. Disease-specific survival was used as the end point. Those factors that were prognostic on univariate analysis were assessed in multivariate models to distinguish those factors that were independently prognostic from those that were the result of confounding. This analysis was performed sequentially on all patients and then on a subgroup of patients who underwent operative resection in both the training set and the validation set.

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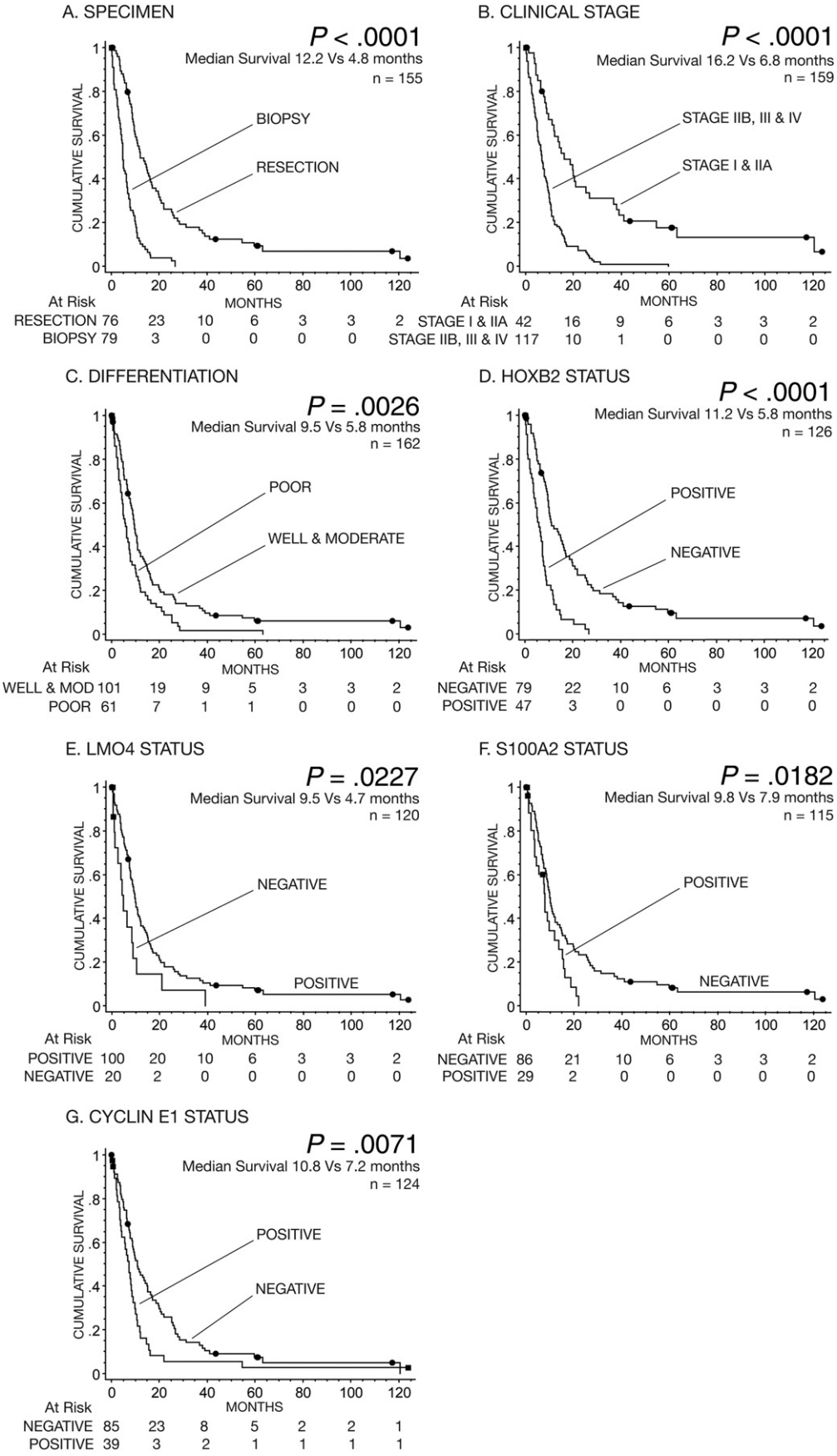
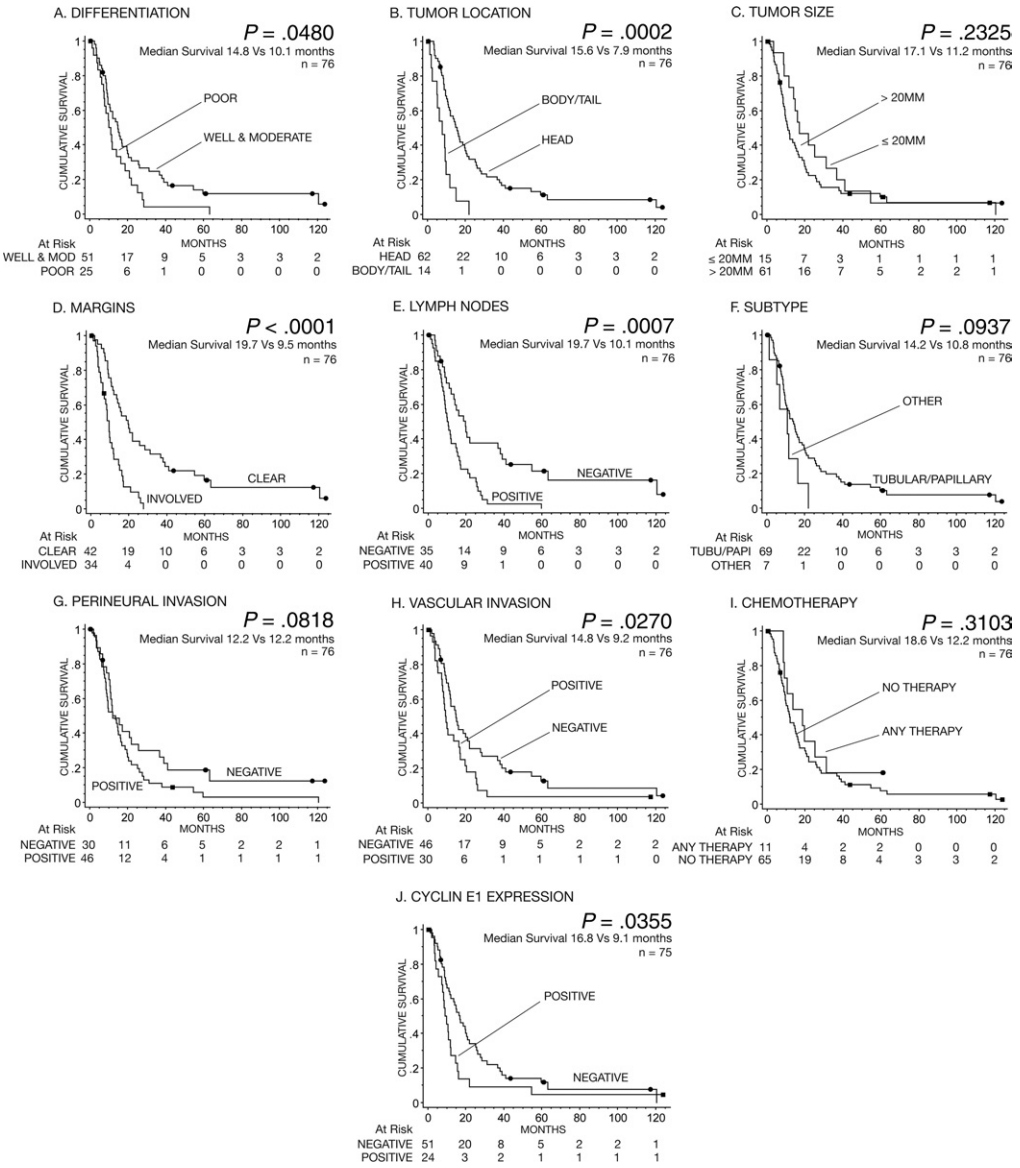
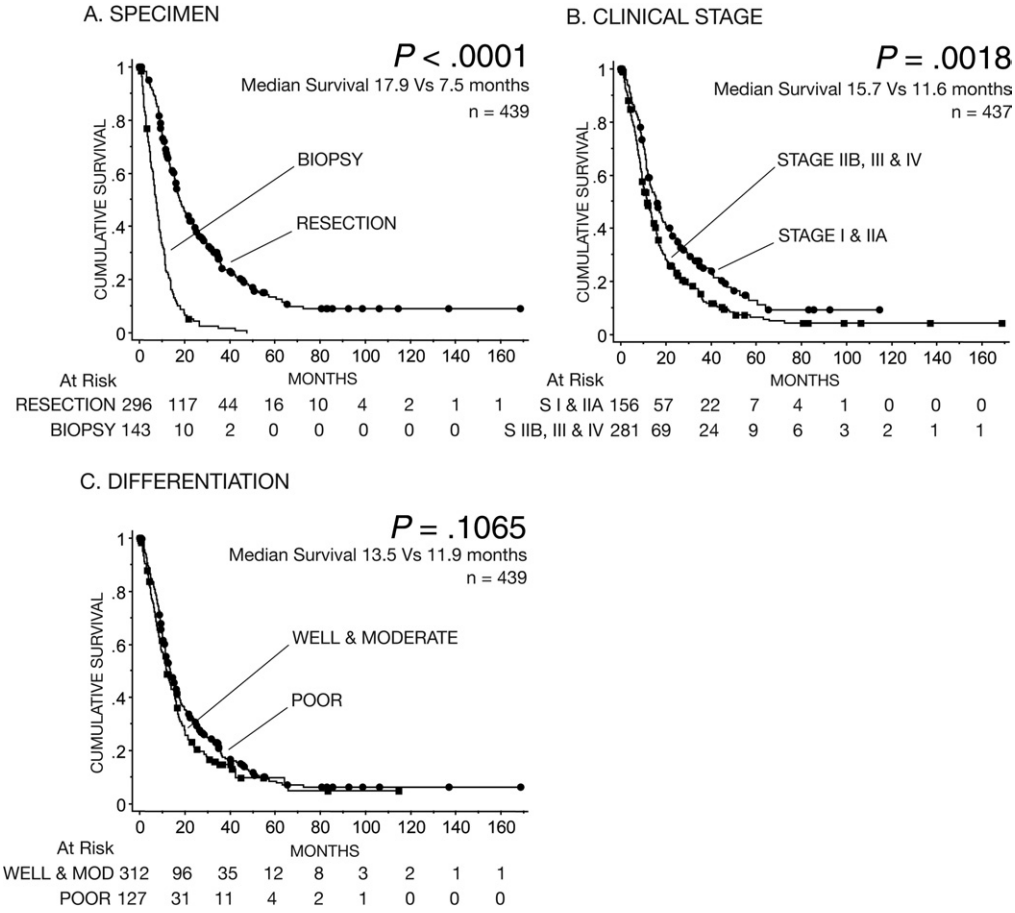


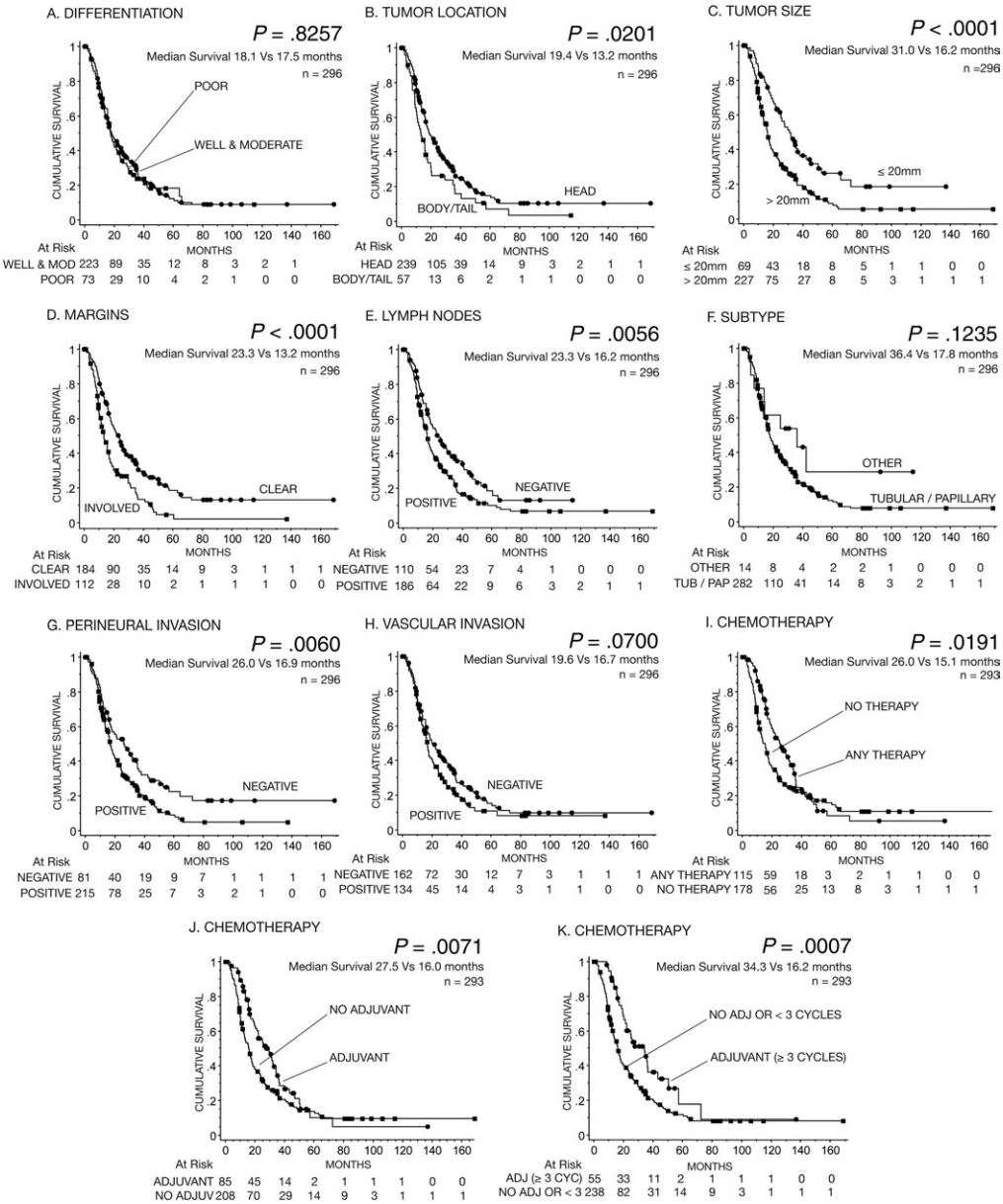
Supplementary Figure 1. KM survival curves of clinicopathologic and biomarker variables in the training set.



Supplementary Figure 2. KM survival curves of patients who underwent pancreatectomy in the training set.



Supplementary Figure 3. KM survival curves of clinicopathologic variables in the validation set.



Supplementary Figure 4. KM survival curves of clinicopathologic variables of patients who underwent pancreatectomy in the validation set.

Supplementary Table 1. Multivariate Analysis of Training Set

	Variable	Hazard ratio (95% CI)	*P* value
A. PC (n = 152)	Treatment (biopsy only)	2.70 (1.85–3.94)	<.0001
(final model)	Differentiation (poor)	1.59 (1.13–2.24)	.0083
	Stage (III and IV)	2.23 (1.43–3.48)	.0004
B. PC (n = 98)	Treatment (biopsy only)	2.95 (1.62–5.38)	.0004
Clinicopathology and biomarkers	Differentiation (poor)	1.22 (0.77–1.90)	.3968
	Stage (III and IV)	2.33 (1.35–4.01)	.0024
	HOXB2 expression (positive)	2.00 (1.15–3.48)	.0144
	LMO4 expression (negative)	2.68 (1.17–6.13)	.0198
	S100A2 expression (positive)	1.92 (1.15–3.20)	.0126
	Cyclin E1 (positive)	1.60 (1.02–2.51)	.0423
C. PC (n = 98)	Treatment (biopsy only)	3.00 (1.65–5.45)	.0003
Clinicopathology and biomarkers (final model)	Stage (III and IV)	2.36 (1.37–4.06)	.0020
	HOXB2 expression (positive)	2.00 (1.15–3.49)	.0138
	LMO4 expression (negative)	2.82 (1.25–6.41)	.0130
	S100A2 expression (positive)	1.98 (1.19–3.27)	.0081
	Cyclin E1 (positive)	1.62 (1.04–2.54)	.0343
D. Resected PC (n = 72)	Resection type (distal)	2.00 (0.88–4.52)	.0976
Clinicopathology and biomarkers	Differentiation (poor)	1.16 (0.64–2.11)	.6190
	Lymph node invasion (positive)	1.24 (0.61–2.53)	.5592
	Margin involvement (positive)	1.70 (0.85–3.43)	.1347
	Vascular invasion (positive)	1.79 (1.00–3.22)	.0513
	HOXB2 expression (positive)	2.79 (1.42–5.49)	.0030
	S100A2 expression (positive)	1.62 (0.77–3.42)	.2052
	Cyclin E1 expression (positive)	1.38 (0.73–2.60)	.3176
E. Resected PC (n = 72)	Resection type (distal)	1.96 (0.87–4.42)	.1032
Clinicopathology and biomarkers	Lymph node invasion (positive)	1.28 (0.63–2.58)	.4989
	Margin involvement (positive)	1.76 (0.88–3.50)	.1095
	Vascular invasion (positive)	1.75 (0.98–3.13)	.0582
	HOXB2 expression (positive)	2.77 (1.40–5.45)	.0033
	S100A2 expression (positive)	1.70 (0.83–3.51)	.1494
	Cyclin E1 expression (positive)	1.38 (0.73–2.61)	.3149
F. Resected PC (n = 72)	Resection type (distal)	1.91 (0.85–4.29)	.1153
Clinicopathology and biomarkers	Margin involvement (positive)	2.08 (1.16–3.72)	.0138
	Vascular invasion (positive)	1.77 (1.01–3.11)	.0461
	HOXB2 expression (positive)	2.68 (1.37–5.25)	.0040
	S100A2 expression (positive)	1.70 (0.83–3.48)	.1449
	Cyclin E1 expression (positive)	1.40 (0.76–2.60)	.2837
G. Resected PC (n = 72)	Resection type (distal)	1.94 (0.88–4.27)	.1010
Clinicopathology and biomarkers	Margin involvement (positive)	2.13 (1.20–3.77)	.0097
	Vascular invasion (positive)	1.66 (0.97–2.82)	.0626
	HOXB2 expression (positive)	3.00 (1.59–5.66)	.0007
	S100A2 expression (positive)	1.79 (0.90–3.54)	.0969
H. Resected PC (n = 72)	Margin involvement (positive)	2.34 (1.35–4.04)	.0024
Clinicopathology and biomarkers	Vascular invasion (positive)	1.57 (0.93–2.66)	.0892
	HOXB2 expression (positive)	3.23 (1.72–6.06)	.0003
	S100A2 expression (positive)	2.23 (1.22–4.10)	.0096
I. Resected PC (n = 72)	Margin involvement (positive)	2.45 (1.41–4.28)	.0016
Clinicopathology and biomarkers (final model)	HOXB2 expression (positive)	3.15 (1.66–5.96)	.0004
	S100A2 expression (positive)	2.00 (1.11–3.61)	.0216
J. Whipple resection (n = 59)	Differentiation (poor)	1.33 (0.67–2.65)	.4119
Clinicopathology and biomarkers	Lymph node invasion (positive)	1.25 (0.57–2.73)	.5721
	Margin involvement (positive)	2.19 (1.04–4.60)	.0397
	Vascular invasion (positive)	1.72 (0.90–3.29)	.1022
	HOXB2 expression (positive)	4.71 (2.12–10.5)	.0001
	S100A2 expression (positive)	3.21 (1.41–7.34)	.0056
	Cyclin E1 expression (positive)	1.46 (0.72–2.98)	.2967
K. Whipple resection (n = 59)	Differentiation (poor)	1.34 (0.69–2.58)	.3919
Clinicopathology and biomarkers	Margin involvement (positive)	2.56 (1.35–4.84)	.0038
	Vascular invasion (positive)	1.69 (0.90–3.18)	.1013
	HOXB2 expression (positive)	4.49 (2.05–9.84)	.0002
	S100A2 expression (positive)	3.13 (1.39–7.08)	.0060
	Cyclin E1 expression (positive)	1.48 (0.75–2.93)	.2550

Supplementary Table 1. (Continued)

	Variable	Hazard ratio (95% CI)	*P* value
L. Whipple resection (n = 59)	Margin involvement (positive)	2.75 (1.49–5.09)	.0013
Clinicopathology and biomarkers	Vascular invasion (positive)	1.65 (0.88–3.10)	.1169
	HOXB2 expression (positive)	4.38 (2.00–9.61)	.0002
	S100A2 expression (positive)	3.54 (1.64–7.64)	.0013
	Cyclin E1 expression (positive)	1.40 (0.71–2.74)	.3312
M. Whipple resection (n = 59)	Margin involvement (positive)	2.74 (1.50–5.00)	.0010
Clinicopathology and biomarkers	Vascular invasion (positive)	1.49 (0.84–2.65)	.1773
	HOXB2 expression (positive)	5.07 (2.42–10.7)	<.0001
	S100A2 expression (positive)	3.52 (1.70–7.29)	.0007
N. Whipple resection (n = 59)	Margin involvement (positive)	2.77 (1.51–5.06)	.0010
Clinicopathology and biomarkers (final model)	HOXB2 expression (positive)	5.01 (2.36–10.6)	<.0001
	S100A2 expression (positive)	3.23 (1.58–6.62)	.0014

NOTE. Multivariate Cox proportional hazards models showing initial model for all patients (heading A), with complete resolution of model using both stepwise removal of redundant variables and addition of variables to base model (heading B) to generate the final model (heading C), where HOXB2, LMO4, S100A2, and Cyclin E1 expression are independent prognostic factors. Headings D to I show relevant models for patients who underwent operative resection of their tumor. Sections J and N show similar models for those who underwent Whipple pancreaticoduodenectomy. Note that, in those who underwent operative resection, only margin status and biomarker expression (HOXB2 and S100A2) were independent prognostic factors, potentially eliminating the requirement for clinicopathological variables apart from margin status, to estimate prognosis.

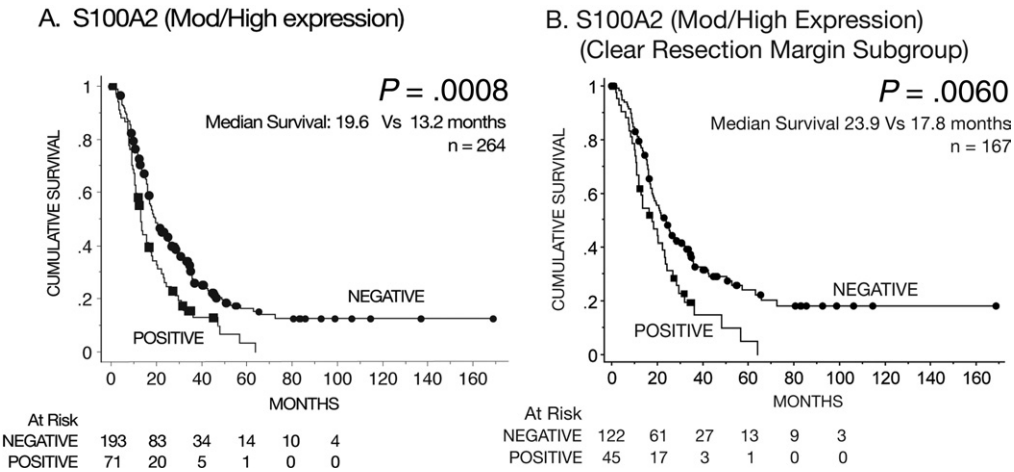
Supplementary Table 2. Multivariate Analysis of Validation Set

	Variable	Hazard ratio (95% CI)	*P* value
A. PC (n = 439)	Treatment (biopsy only)	3.66 (2.80–4.78)	<.0001
(final model)	Stage (III and IV)	1.41 (1.10–1.81)	.0063
	S100A2 expression (positive)	1.45 (1.12–1.89)	.0053
B. Resected PC (n = 296)	Tumor size (≥20 mm)	1.55 (1.10–2.19)	.0124
Clinicopathologic variables only	Tumor location (body/tail)	1.41 (1.00–1.98)	.0484
	Lymph node metastases (positive)	1.54 (1.15–2.05)	.0032
	Margin involvement (positive)	1.70 (1.29–2.24)	.0002
	Perineural invasion (positive)	1.29 (0.94–1.78)	.1155
	Vascular invasion (positive)	1.29 (0.99–1.70)	.0630
	Adjuvant chemotherapy (≥3 cycles)	0.53 (0.36–0.78)	.0011
C. Resected PC (n = 296)	Tumor size (≥20 mm)	1.60 (1.13–2.25)	.0076
Clinicopathologic variables only	Tumor location (body/tail)	1.43 (1.02–2.01)	.0405
(final model)			
	Lymph node metastases (positive)	1.54 (1.15–2.04)	.0033
	Margin involvement (positive)	1.73 (1.31–2.28)	<.0001
	Vascular invasion (positive)	1.32 (1.01–1.73)	.0446
	Adjuvant chemotherapy (≥3 cycles)	0.53 (0.37–0.78)	.0011
D. Resected PC (n = 296)	Tumor size (≥20 mm)	1.51 (1.05–2.17)	.0283
	Tumor location (body/tail)	1.33 (0.92–1.93)	.1268
	Lymph node metastases (positive)	1.48 (1.09–2.00)	.0111
	Margin involvement (positive)	1.65 (1.23–2.22)	.0008
	Perineural invasion (positive)	1.30 (0.92–1.82)	.1377
	Vascular invasion (positive)	1.15 (0.86–1.53)	.3461
	Adjuvant chemotherapy (≥3 cycles)	0.56 (0.37–0.83)	.0042
	S100A2 expression (high)	1.68 (1.10–2.54)	.0154
E. Resected PC (n = 296)	Tumor size (≥20 mm)	1.52 (1.06–2.19)	.0238
	Tumor location (body/tail)	1.33 (0.92–1.93)	.1257
	Lymph node metastases (positive)	1.48 (1.09–2.00)	.0110
	Margin involvement (positive)	1.63 (1.22–2.19)	.0011
	Perineural invasion (positive)	1.32 (0.94–1.85)	.1135
	Adjuvant chemotherapy (≥3 cycles)	0.56 (0.38–0.84)	.0049
	S100A2 expression (high)	1.70 (1.12–2.58)	.0118
F. Resected PC (n = 296)	Tumor size (≥20 mm)	1.54 (1.07–2.22)	.0198
	Lymph node metastases (positive)	1.47 (1.09–1.99)	.0119
	Margin involvement (positive)	1.64 (1.23–2.20)	.0008
	Perineural invasion (positive)	1.34 (0.96–1.88)	.0894
	Adjuvant chemotherapy (≥3 cycles)	0.59 (0.39–0.87)	.0081
	S100A2 expression (high)	1.81 (1.20–2.71)	.0045
G. Resected PC (n = 296)	Tumor size (≥20 mm)	1.59 (1.11–2.29)	.0122
(final model)			
	Lymph node metastases (positive)	1.50 (1.11–2.03)	.0080
	Margin involvement (positive)	1.68 (1.25–2.25)	.0005
	Adjuvant chemotherapy (≥3 cycles)	0.58 (0.39–0.87)	.0079
	S100A2 expression (high)	1.87 (1.25–2.81)	.0024
H. Resected PC (n = 296)	Tumor size (≥20 mm)	1.51 (1.04–2.18)	.0297
	Tumor location (body/tail)	1.33 (0.93–1.92)	.1225
	Lymph node metastases (positive)	1.45 (1.07–1.96)	.0161
	Margin involvement (positive)	1.69 (1.26–2.27)	.0005
	Perineural invasion (positive)	1.34 (0.95–1.88)	.0910
	Vascular invasion (positive)	1.17 (0.88–1.55)	.2888
	Adjuvant chemotherapy (≥3 cycles)	0.69 (0.50–0.95)	.0233
	S100A2 expression (mod/high)	1.46 (1.06–2.02)	.0211
I. Resected PC (n = 296)	Tumor size (≥20 mm)	1.52 (1.05–2.20)	.0254
	Tumor location (body/tail)	1.33 (0.92–1.91)	.1248
	Lymph node metastases (positive)	1.45 (1.07–1.96)	.0163
	Margin involvement (positive)	1.67 (1.24–2.24)	.0006
	Perineural invasion (positive)	1.37 (0.98–1.92)	.0692
	Adjuvant chemotherapy (≥3 cycles)	0.69 (0.50–0.96)	.0250
	S100A2 expression (mod/high)	1.46 (1.06–2.02)	.0209

Supplementary Table 2. (Continued)

	Variable	Hazard ratio (95% CI)	*P* value
J. Resected PC (n = 296) (final model)	Tumor size (≥20 mm)	1.54 (1.06–2.23)	.0221
	Lymph node metastases (positive)	1.45 (1.07–1.95)	.0170
	Margin involvement (positive)	1.68 (1.25–2.25)	.0005
	Perineural invasion (positive)	1.40 (1.00–1.96)	.0476
	Adjuvant chemotherapy (≥3 cycles)	0.71 (0.52–0.98)	.0382
	S100A2 expression (mod/high)	1.46 (1.09–2.07)	.0124

NOTE. Multivariate Cox proportional hazard analysis showing final model for all patients in the validation cohort (heading A). For patients who underwent operative resections, all clinicopathologic variables of interest and of significance were inserted in the initial model (heading B). The model was examined, and variables were eliminated using stepwise removal of redundant variables (headings B and C) to produce the final model (heading C). The same procedure was performed for clinicopathologic and biomarker variables using different cut points of S100A2 expression (high, headings D to G; and mod/high, headings H to J). These produced 2 final models (headings G and J) showing S100A2 expression to be an independent prognostic factors at either cut point.



Supplementary Figure 5. KM survival curves using mod/high as cutoff.

Supplementary Table 3. Immunohistochemistry Methodology

Gene	Antigen retrieval	Primary antibody and incubation	Dilution	Cut point for positive staining
HOXB2	10% EDTA MPH, 30 min	Goat/Poly (P-20, Santa Cruz Biotechnology, Santa Cruz, CA), 30 min	1:200	Intensity ≥2 in >20% nuclear
LMO4	S 1699 WB, 30 min	Rat/Mono (20F8), 30 min	1:200	Intensity ≥1 in >50% nuclear
S100A2	S 2367 PC, 5 min	Mouse/Mono (DAK-S100A2/1; Dako Corporation, Carpenteria, CA), 60 min	1:50	Intensity ≥3 in >30% cytoplasmic
S100A6	10% EDTA WB, 30 min	Rabbit/Poly (clone A5115; Dako Corporation, Carpenteria, CA), 30 min	1:2000	Intensity ≥1 in >10% cytoplasmic
S100P	Protein K solution, 7 min	Mouse/Mono (clone 16; BD Transduction Labs, San Jose, CA), 30 min	1:150	Intensity ≥1 in >20% cytoplasmic
Cyclin E1	10% EDTA MPH, 30 min	Mouse/Mono (clone 13A3; Novocastra, Newcastle Upon Tyne, UK), 90 min	1:50	>10% Nuclear
Cyclin D1	S1699 WB, 30 min	Mouse/Mono (clone DCS-6; Novocastra, Newcastle Upon Tyne, UK), 30 min	1:80	>5% Nuclear
p16^{INK4A}	S1699 WB, 20 min	Mouse/Mono (clone ZJ11; Neomarker, Fremont, CA), 30 min	1:40	>0% Nuclear
p21$^{WAF1/CIP1}$	S1699 WB, 20 min	Mouse/Mono (clone 70; BD Transduction Labs, San Jose, CA), 30 min	1:80	>10% Nuclear
p27^{Kip1}	10% EDTA MPH, 30 min	Mouse/Mono (clone 57; BD Transduction Labs, San Jose, CA), 90 min	1:200	>5% Nuclear
p53	S1699 WB, 30 min	Mouse/Mono (clone DO-7; Dako Corporation, Carpenteria, CA), 30 min	1:500	>10% Nuclear
EGFR	Proteinase K solution, 7 min	Mouse/Mono (M3563; Dako Corporation, Carpenteria, CA), 30 min	1:50	>5% Cell membrane
Smad4	S1600 WB, 20 min	Mouse/Mono (clone B-8; Santa Cruz Biotechnology, Santa Cruz, CA), 30 min	1:150	>5% Cytoplasmic
sFRP4	S 2367 WB, 30 min	Sheep/Poly (Minotopes), 30 min	1:250	>20% Cell membrane
β-catenin	0.01 mol/L citrate buffer WB, 20 min	Mouse/Mono (clone 14; BD Transduction Labs, San Jose, CA), 30 min	1:200	>5% Cytoplasmic or nuclear
CRBP1	S1699 PC, 5 min	Rabbit/Poly (FL-135; Santa Cruz Biotechnology, Santa Cruz, CA), 60 min	1:50	Intensity ≥1 in >50% cytoplasmic

MPH, microwave pressure heating; PC, pressure cooker; WB, water bath.