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Biology of Human Tumors

Clinical Cancer Research

Sonic Hedgehog and Gli1 Expression Predict Outcome in Resected Pancreatic Adenocarcinoma

Raphaël Maréchal[1,2], Jean-Baptiste Bachet[3,4,5], Annabelle Calomme[1,2], Pieter Demetter[6], Jean Robert Delpero[7,8], Magali Svrcek[9], Jérôme Cros[10], Armelle Bardier-Dupas[3,11], Francesco Puleo[1,2], Geneviève Monges[12], Pascal Hammel[13], Christophe Louvet[3,14], François Paye[3,15], Philippe Bachelier[16], Yves Patrice Le Treut[17], Jean-Christophe Vaillant[3,18], Alain Sauvanet[19], Thierry André[20], Isabelle Salmon[6,21], Jacques Devière[1,2], Jean-François Emile[4,22], and Jean-Luc Van Laethem[1]

Abstract

Purpose: Aberrant activation of the hedgehog (Hh) pathway is implicated in pancreatic ductal adenocarcinoma (PDAC) tumorigenesis. We investigated the prognostic and predictive value of four Hh signaling proteins and of the tumor stromal density.

Experimental Design: Using tissue microarray and immunohistochemistry, the expression of Shh, Gli1, SMO, and PTCH1 was assessed in 567 patients from three independent cohorts who underwent surgical resection for PDAC. In 82 patients, the tumor stromal index (SI) was calculated, and its association with overall survival (OS) and disease-free survival (DFS) was investigated.

Results: Shh and Gli1 protein abundance were independent prognostic factors in resected PDACs; low expressors for those proteins experiencing a better OS and DFS. The combination of Shh and Gli1 levels was the most significant predictor for OS and defined 3 clinically relevant subgroups of patients with different prognosis (Gli1 and Shh low; HR set at 1 vs. 3.08 for Shh or Gli1 high vs. 5.69 for Shh and Gli1 high; $P < 0.001$). The two validating cohorts recapitulated the findings of the training cohort. After further stratification by lymph node status, the prognostic significance of combined Shh and Gli1 was maintained. The tumor SI was correlated with Shh levels and was significantly associated with OS ($P = 0.023$).

Conclusions: Shh and Gli1 are prognostic biomarkers for patients with resected PDAC. *Clin Cancer Res; 21(5); 1215–24. ©2014 AACR.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest solid malignancies with a 5-year survival rate of <5% (1). This highly invasive disease is characterized by an extensive desmoplastic reaction. The epithelial and stromal compartments interact to enhance the aggressive nature of this cancer by fostering tumor growth, angiogenesis, metastatic spread, and enhancing drug resistance (2). Recent studies in multiple pancreatic cancer models have implicated the hedgehog (Hh) signaling pathway in these tumor–stromal interactions (3–7).

Response to the Hh signaling proteins [Sonic hedgehog (Shh), Indian hedgehog (Ihh) and desert hedgehog (Dhh)] is regulated by the receptor Patched (PTCH), which represses the activity of the transducer protein smoothened (SMO). Hh protein binds PTCH1 and blocks its inhibition of SMO-inducing activation of the Gli family of transcription factors, including *Gli1* and *PTCH1*. Constitutive activation of the pathway by activating mutations in *SMO* or inactivating mutations in the tumor suppressor gene *PTCH* leads to cell proliferation and tumor formation (8–10). Such mutations have not been described in PDAC, but overexpression

Saint Antoine [1]Department of Gastroenterology and Gastrointestinal Cancer Unit, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium. [2]Laboratory of Experimental Gastroenterology, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium. [3]Medical University Pierre et Marie Curie, UFR Paris VI, Paris, France. [4]EA4340 "Epidémiologie et oncogènes des tumeurs digestives," Versailles Saint-Quentin-en-Yvelines University, Versailles, France. [5]Department of Hepato-Gastroenterology, Pitié Salpêtrière Hospital, APHP, Paris, France. [6]Department of Pathology, Erasme Hospital, Université Libre de Bruxelles, and DiaPath, Brussels, Belgium. [7]Department of Surgery, Institute Paoli Calmettes, Marseille, France. [8]Aix Marseille Université, Marseille, France. [9]Department of Pathology, Saint Antoine Hospital, APHP, Paris, France. [10]Department of Pathology, Beaujon Hospital, APHP, Clichy, France. [11]Department of Pathology, Pitié Salpêtrière Hospital, APHP, Paris, France. [12]Department of Pathology, Institute Paoli Calmettes, Marseille, France. [13]Department of Gastroenterology, Beaujon Hospital, APHP, Clichy, France. [14]Department of Oncology, Institut Mutualiste Montsouris, Paris, France. [15]Department of Surgery, Saint Antoine Hospital, APHP, Paris, France. [16]Department of Surgery, University Hospital of Hautepierre, Strasbourg, France. [17]Department of Digestive Surgery, Conception Hospital, Marseille, France. [18]Department of Digestive Surgery, Pitié Salpêtrière Hospital, Paris, France. [19]Department of Digestive Surgery, Beaujon Hospital, APHP, Clichy, France. [20]Department of Oncology, Saint-Antoine Hospital, APHP, Paris, France. [21]DIAPath – Center for Microscopy and Molecular Imaging (CMMI), Université Libre de Bruxelles, Gosselies, Belgium. [22]Department of Pathology, Ambroise Paré Hospital, APHP, Boulogne Billancourt, France.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Raphaël Maréchal, Erasme University Hospital, Department of Gastroenterology, Gastrointestinal Cancer Unit, Université Libre de Bruxelles (ULB), Route de Lennik 808, Brussels 1070, Belgium. Phone: 322-555-3715; Fax: 322-555-4697; E-mail: raphael.marechal@erasme.ulb.ac.be

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Translational Relevance

The hedgehog pathway has a critical role in the development of pancreatic cancer. The paracrine loop between cancer cells and stellate cells provides support for proliferation, spreading capacities, and treatment resistance of pancreatic cancer cells. In the current study, we found that Shh and Gli1 protein expression were independent prognosticators for the disease-free and the overall survival in resected pancreatic cancer. The combination of those biomarkers is more informative than the classical prognostic factors (stage, lymph node status). Importantly, two independent multicentric validations cohorts replicated those results. Our results suggest that Shh and Gli1 expression could be tested in a molecular-driven study as a "companion diagnostic" for anti-Shh– and anti-Gli1–targeted therapies.

of the Shh ligand has been reported to occur in 70% of primary PDAC and has been implicated in the development and the progression of pancreatic tumors (11, 12). A paracrine Hh signaling is reversed in PDAC with tumor cells secreting Shh that activate signaling within stromal cells to produce secondary factors, supporting angiogenesis and tumor cell proliferation and survival (3–7, 13–17). These data support a functionally important role for Hh signaling in PDAC tumorigenesis.

Surprisingly, Gli expression within the pancreatic tumor epithelium is maintained despite SMO deletion and does not correlate with Hh ligand levels (7), suggesting a more complex regulation of the pathway. KRAS and TGFβ, two signaling pathways prominently involved in PDAC tumorigenesis, regulate the SMO-independent expression of Gli1 target genes in mouse PDAC cells (7, 18). Furthermore, Gli1 expression facilitates the migration and invasion of PDAC cell lines and is required for KRAS-mediated cellular transformation (19, 20). Although aberrant Hh signaling has been found to affect pancreatic cancer behavior, the correlation between the hedgehog pathway components and patients outcome has not yet been evaluated.

The purpose of this study is to determine whether Gli1, Shh, PTCH1, and SMO expression within tumors have any relationship with the clinical outcome of the patients (prognostic value) and the benefit of adjuvant therapy (predictive value) in patients with PDAC who underwent a curative-intent surgery.

Patients and Methods

Patients and data study design

A cohort of 572 consecutive and unselected patients with a diagnosis of PDAC who underwent between 1996 and 2009 a curative-intent surgery, were obtained from five university hospitals with expertise in the management of PDAC. Exclusion criteria included preoperative chemotherapy and/or radiotherapy, macroscopically incomplete resection (R2), pancreatic tumor histology other than ductal adenocarcinoma. Patients who died of postoperative complications within 30 days following the surgery were also excluded because they were noninformative for this translational study. The study population comprised a consecutive series of 471 archived formalin-fixed, paraffin-embedded tumor (FFPE) specimens which was the combination of a training cohort of 237 patients accrued from 2 hospitals [E. (Erasme) and B. (Beaujon) and designated as the Brussels training cohort] and an independent validation cohort of 234 patients accrued from 3 hospitals [S.A. (Saint Antoine); L.P.S. (La Pitié Salpêtrière); A.P. (Ambroise Paré) and designated as the Paris validation cohort]. One additional validation cohort of 96 FFPE samples corresponding to 96 patients (designated as the Marseille validation cohort) who underwent a curative-intent surgery between 2008 and 2010 was obtained from a recently published prospective trial (21).

Data collection

For the Brussels and the Paris cohorts, each participating center prospectively maintains a PDAC database, including patient demographics, clinical, and pathologic variables. An aggregated database was created with standardized clinocopathologic variables. For the Marseille cohort, clinicopathologic factors and follow-up clinical information were collected as a part of the prospective trial.

Specimen characteristics and tissue microarray

Fixation of tumor specimens followed standard protocols, using either 10% nonbuffered or 10% buffered formalin for 12 hours, dehydrated in 70% EtOH, and paraffin embedded. FFPE PDACs were stored at room temperature. Tissue microarrays (TMA) were constructed from 0.6-mm tissue cores obtained from FFPE with each tumor represented by 3to 5 cores. Hematoxylin and eosin staining was performed on each TMA slide to confirm tumor presence.

Gli1, PTCH1, SMO, and Shh immunohistochemistry

Immunostaining was done using the following antibodies and concentrations: rabbit anti-SMO polyclonal antibody ab-72130 (Abcam; 1:400), rabbit anti-Gli1 polyclonal antibody sc-20687 (Santa Cruz Biotechnology; 1:20), rabbit anti-Shh polyclonal antibody sc-9024 (Santa Cruz Biotechnology; 1:100), goat anti-PTCH1 polyclonal antibody sc-6149 (Santa Cruz Biotechnology; 1:50). Basocellular carcinoma and gastric mucosa were used as positive control for Shh, SMO, Gli1, and PTCH1 antibodies. Antigen retrieval was performed by heating in a citrate buffer for Gli1 and EDTA buffer for Shh, SMO, and PTCH1. Amplification was performed with the Dako HRP system with 3,3′-diaminobenzidine revelation, and slides were counterstained with hematoxylin. All four immunohistochemical factors were tested in the training cohort and the Paris validation cohort, but only the two that were prognostic (Shh and Gli1) were tested in the Marseille validation cohort. Nonspecific rabbit or mouse IgG antibodies were used as negative control.

Immunohistochemistry scoring

TMA slide images were captured as high-resolution digital files. Evaluation of staining was done independently by one specialist pancreatic pathologist (P. Demetter) and 2 translational researchers experienced in pancreatic histopathology (R. Maréchal and A. Calomme) blinded to the clinicopathologic and outcome data. Any discrepancies were resolved by consensus after conferencing. The percentage of stained cells, cellular staining localization (nuclear for Gli1, cytoplasmic for Shh, membranous for SMO and PTCH1) and staining intensities (0–2) were assigned. A semiquantitative scale based on the distribution of staining in malignant cells was used: low staining was defined as staining intensity 0 in >95% tumor cells; high staining was defined as

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staining intensity 2 in >50% tumor cells; moderate staining was assigned to the remaining cases. Each immunohistochemical factors were categorized as low (staining intensity = 0 or 1) or high (staining intensity = 2) for statistical analyses.

Tumor stromal density

Sections were stained with a pricosirius red solution for the visualization of collagen deposits. A representative slide of the tumor was selected by a pathologist and the quantification was done on the whole slide for each sample. We determined the stromal index by adjusting the stromal area to the proportion of tumor cells area by using the Metamorph image-analysis system (Metamorph 7.0; Molecular Devices). Morphometric analysis was carried out by two independent examiners blinded to patient outcome and biomarkers assessment.

Ethics

The study protocol was approved by the institutional review board of each participating hospitals. Written informed consent was obtained from all patients included in the prospective cohort (second validation cohort).

Statistical analysis

Overall survival (OS) was measured as the time from the date of surgery to the time of last follow-up or death of any cause. Disease-free survival was defined as the time from surgery to the first disease-free failure event (local or distant disease relapse or death). The cutoff date for analysis was September 30, 2010. Follow-up was calculated using the reverse Kaplan–Meier method. The χ^2 test was used to compare proportions and t test or Mann–Whitney test was used to compare continuous variables. Median survival was estimated using the Kaplan–Meier method, and the difference was tested using the log-rank test. Clinicopathologic variables analyzed with a P value < 0.05 on log-rank test were entered into Cox proportional hazards multivariate analysis. The 4 biomarkers and known prognostic factors for resected PDAC: resection margins, nodal status, tumor differentiation, and T stage were included in multivariate models whatever their P value in univariate analysis. All multivariate Cox models were built according to the rule of 10 events per variable (22). The models assumptions were verified by graphical methods.

The predictive accuracy for multivariate models was examined by calculating the Harell concordance index (*C*-index). Results of Harrell *C* index range from 0.5 (no discrimination for predicting survival) to 1.0 (perfect discrimination).

All data analyses were carried out according to a prespecified analysis plan. The prognostic values of the immunohistochemical factors were evaluated in the Brussels cohort (n = 237). The prognostic model and the cutoff points evaluated in the training sample were then separately applied to two independent validation samples of 234 (Paris cohort) and 96 (Marseille cohort) patients.

For investigating the predictive effect of the immunohistochemical (IHC) factors, interactions between adjuvant therapy and the factors Shh, Gli1, SMO, and PTCH1 were examined. This was done for each of these factors, including the respective factor and separate treatment effects for both values (low and high) of the interesting factor, respectively. The interaction tests between each IHC factors and the administration of adjuvant therapy were performed using the Cox model to calculate the HRs with 95% confidence intervals (CI).

In the absence of previous data allowing anticipating an expected rate of high and low staining for each of the IHC factors, no formal sample size evaluation has been done.

All significant tests were two-sided and all used a 5% level of significance. Statistical analyses were performed using SPSS software (version 18.0; SPSS).

Results

Cohort characteristics

A total of 721 patients were assessed for the eligibility, and 567 were considered for this translational study. The majority of the patients have received an adjuvant therapy and most of them a gemcitabine-based regimen.

Brussels training cohort. The cohort of 237 patients consisted of 127 (53.6%) men and 110 women. The median age at diagnosis was 63 years. The median follow-up was 53.9 months. The median DFS and OS was 13.5 months and 26.8 months respectively, with a 5-year survival rate of 26.4%.

Paris validation cohort. The cohort of 234 patients consisted of 139 (59.4%) men and 95 women followed during a median time of 57.3 months. The median age at diagnosis was 64 years. The median DFS and OS was 14.7 months and 30.4 months, respectively, with a 5-year survival rate of 28.7%. Adjuvant therapy was more frequently administered (73.4%) than in the validation set (62%), P = 0.03.

Marseille validation cohort. Most patients who underwent surgery were men (56.3%). The median time of follow-up was shorter than in the 2 aforementioned cohorts with a median time of 24.9 months. The median DFS and OS was 22.1 months and 26.6 months, respectively. Characteristics of the three cohorts are listed in Table 1 and a detailed description of the patients flow was provided in the Data Supplement (Supplementary Fig. S1).

Clinicopathologic and immunohistologic factors

Each IHC factor was assessed in >95% of the tumors. A good interobserver agreement for dichotomized IHC factors was seen on the basis of the chosen cutoffs, including unweighted κ scores of 0.821 (95% CI, 0.751–0.892) for SMO, 0.80 (95% CI, 0.74–0.85) for PTCH1, 0.84 (95% CI, 0.799–0.88) for Shh, and 0.83 (95% CI, 0.77–0.89) for Gli1. Representative staining for IHC factors is shown in Supplementary Fig. S2.

No correlations were found between baseline clinicopathologic parameters and dichotomized IHC factors, with the exceptions of high Gli1 expression and R1 resection both in the Brussels training and the Paris validation cohorts (P = 0.016 and P = 0.010, respectively) and a significant association between high SMO expression and poorly differentiated tumor (P = 0.044); in the Paris validation cohort (Supplementary Table S1).

Shh and Gli1 abundance predict survival and DFS in resected pancreatic cancer

In the Brussels training cohort, high Shh and high Gli1 expressions were independent predictors of worse OS (Shh: HR = 3.47, P < 0.001 and Gli1: HR = 1.88; P = 0.001) and DFS (Shh: HR = 2.43, P < 0.001 and Gli1: HR = 1.98; P < 0.001), whereas no

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Table 1. Patients characteristics and univariate analyses of survival prediction

	Brussels cohort ($n = 237$)			Paris cohort ($n = 234$)			Marseille cohort ($n = 96$)		
Variable	***n* (%)**	**Median OS (months)**	**Log-rank *P* value**	***n* (%)**	**Median OS (months)**	**Log-rank *P* value**	***n* (%)**	**Median OS (months)**	**Log-rank *P* value**
Gender									
Female	120 (50.6)	33.2	0.116	95 (40.6)	33.3	0.656	41 (42.7)	31.7	0.388
Male	117 (49.4)	21.1		139 (59.4)	28.6		55 (56.3)	23.9	
Age, y									
Median		63			64			63	
Range		34–82			37–87			41–80	
Follow-up									
Median		53.9			66.3			24.9	
95% CI		44.9–62.8			52.9–79.7			24.3–25.4	
Outcome									
Median OS		26.8			30.4			26.6	
95% CI		19.6–34.1			24.5–36.4			23.2–30.0	
Median DFS		13.5			14.7			22.1	
95% CI		11.0–16.1			12.0–16.2			15.9–28.2	
Death	152 (64.0)			143 (61.1)			47 (49.0)		
Recurrence	158 (67.5)			159 (71.3)			51 (53.1)		
Resection margins									
R0	191 (80.6)	27.1	0.020	189 (80.8)	33.3	<0.001	64 (66.7)	NR	0.017
R1	46 (19.4)	17.7		45 (19.4)	19.4		32 (33.3)	17.5	
Tumor differentiation									
Well	97 (40.9)	33.2	0.014	104 (44.4)	30.5	0.865	31 (32.2)	36.3	0.371
Moderate	97 (40.9)	23.5		92 (39.3)	32.7		46 (49.0)	25.9	
Poor	39 (16.5)	12.2		28 (12.0)	22.9		19 (19.8)	24.4	
Unknown	4 (1.7)			10 (4.3)					
Tumor stage									
T1/T2	36 (15.2)	33.6	0.552	49 (20.9)	37.0	0.209	18 (18.8)	NR	0.153
T3	201 (84.8)	24.9		185 (79.1)	27.5		78 (81.2)	24.0	
Lymph nodes									
Negative, N0	63 (26.6)	36.2	0.001	57 (24.4)	57.9	0.005	25 (26.0)	NR	<0.001
Positive, N1	174 (73.4)	23.5		177 (75.6)	24.4		71 (74.0)	19.0	
Perineural invasion									
Negative	47 (19.8)	36.0	0.042	79 (33.8)	31.5	0.721	31 (32.3)	35.2	0.686
Positive	185 (78.1)	23.5		155 (66.2)	28.7		65 (67.7)	24.0	
Unknown	5 (2.1)								
Lymphovascular invasion									
Negative	81 (34.2)	34.1	0.376	99 (42.3)	33.3	0.245	50 (52.1)	24.1	0.737
Positive	156 (65.8)	24.4		135 (57.7)	26.7		46 (47.9)	22.3	
Adjuvant therapy									
Yes	174 (73.4)	26.9	0.173	145 (62.0)	33.3	0.253	NA		
No	63 (26.6)	24.1		79 (33.7)	25.6		NA		
Unknown				10 (4.3)			NA		
Non-gemcitabine–based	41 (23.6)	19.8	0.127	19 (13.1)	44.2	0.284	NA		
Gemcitabine-based	133 (76.4)	33.1		126 (86.9)	32.7		NA		
RT/RCT									
Yes	100 (57.5)	27.0	0.854	58 (40.0)	35.5	0.239	NA		
No	74 (42.5)	26.9		87 (60.0)	31.8		NA		
Shh expression									
Low	148 (62.4)	35.6	<0.001	133 (56.8)	43.3	<0.001	62 (64.6)	NR	0.001
High	85 (35.9)	16.4		101 (43.2)	20.5		34 (35.4)	13.8	
Unknown	4 (1.7)								
Gli1 expression									
Low	115 (48.5)	36.2	<0.001	107 (45.7)	38.0	<0.001	58 (60.4)	36.4	<0.001
High	122 (51.5)	18.2		126 (53.8)	20.7		38 (39.6)	11.6	
Unknown				1 (0.5)					
SMO expression									
Low	177 (74.7)	26.9	0.640	178 (76.1)	25.5	0.094			
High	45 (19.0)	26.8		56 (23.9)	38.0				
Unknown	15 (6.3)								
PTCH1 expression									
Low	165 (69.6)	26.4	0.823	159 (67.9)	32.0	0.313			
High	66 (27.7)	27.0		75 (32.1)	28.9				
Unknown	6 (2.5)								

Abbreviations: NA, not available; NR, not reached.

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Table 2. Multivariate Cox regression analyses of overall and disease-free survival

	OS		DFS	
Variable	**HR[a] (95% CI)**	***P***	**HR (95% CI)**	***P***
Brussels training cohort	(*n* = 222, 147 events)		(*n* = 222, 153 events)	
PTCH1 high	1.30 (0.89–1.89)	0.179	1.06 (0.68–1.52)	0.765
Gli1 high	1.88 (1.27–2.76)	0.001	1.98 (1.37–2.86)	<0.001
Shh high	3.47 (2.33–5.17)	<0.001	2.43 (1.66–3.55)	<0.001
SMO high	0.88 (0.55–1.42)	0.69	1.05 (0.66–1.66)	0.827
T3 tumor	1.55 (0.92–2.62)	0.098	1.89 (1.08–3.31)	0.025
Margin positive	1.61 (1.03–2.51)	0.035	1.41 (0.93–2.13)	0.107
Node positive	1.74 (1.11–2.74)	0.016	1.743 (1.12–2.71)	0.014
Poorly differentiated tumor	1.29 (0.92–1.63)	0.147	1.64 (1.03–2.63)	0.038
Perineural invasion	1.08 (0.67–1.73)	0.757	1.06 (0.74–1.52)	0.765
Adjuvant treatment	0.64 (0.43–0.91)	0.012	0.62 (0.41–0.82)	0.008
Harrell *C* statistic	0.722		0.711	
Paris validation cohort	(*n* = 224, 129 events)		(*n* = 224, 144 events)	
PTCH1 high	1.21 (0.83–1.75)	0.315	0.86 (0.61–1.20)	0.375
Gli1 high	1.76 (1.21–2.56)	0.003	1.73 (1.23–2.46)	0.002
Shh high	2.51 (1.70–3.71)	<0.001	2.62 (1.84–3.73)	<0.001
SMO high	1.16 (0.73–1.85)	0.518	1.28 (0.86–1.89)	0.217
T3 tumor	1.60 (1.01–2.55)	0.047	1.59 (1.10–2.30)	0.013
Margin positive	2.08 (1.35–3.22)	0.001	2.15 (1.43–3.23)	<0.001
Node positive	1.87 (1.15–3.02)	0.011	1.73 (1.12–2.66)	0.013
Poorly differentiated tumor	1.42 (0.95–2.13)	0.083	1.69 (1.10–2.30)	0.052
Adjuvant treatment	0.58 (0.40–0.85)	0.004	0.55 (0.38–0.79)	0.002
Harrell *C* statistic	0.693		0.706	
Marseille validation cohort	(*n* = 96, 47 events)		(*n* = 96, 51 events)	
Gli1 high	1.87 (1.06–3.46)	0.044	2.30 (1.28–4.15)	0.006
Shh high	5.55 2.92–10.54	<0.001	6.45 3.30–12.56	<0.001
T3 tumor	1.42 (0.57–3.57)	0.455	1.96 (0.68–5.57)	0.207
Margin positive	2.17 (1.17–4.02)	0.014	2.35 (0.93–5.96)	0.071
Node positive	3.22 (1.05–9.87)	0.401	1.89 (1.05–3.41)	0.034
Poorly differentiated tumor	1.05 (0.52–2.12)	0.890	1.43 (0.72–2.86)	0.309
Harrell *C* statistic	0.712		0.708	

[a]HR of 1 indicates no difference between the two groups of patients, whereas an HR > 1 indicates an increased risk of death/failure.

association was found between the levels of PTCH1 and SMO expression and patients' outcome (Table 2; Fig. 1). The median OS and DFS were worse for patients with high expression for Shh level (median OS: 16.4 vs. 35.6 months, $P < 0.001$; median DFS: 10.3 vs. 20.9 months, $P < 0.001$) and for those with a high expression of Gli1 (median OS, 18.2 vs. 36.2 months; $P < 0.001$; median DFS, 10.5 vs. 25.0 months; $P < 0.001$; Supplementary Table S2). Furthermore, the addition of these 2 biomarkers to the multivariate analysis for OS and DFS improved the discrimination ability of the model as the mean Harell *C*-index increased from 0.62 to 0.72 for the OS and from 0.62 to 0.71 for the DFS (Table 3 and Supplementary Table S3).

To independently validate the results of the training cohort, the two biomarkers were separately examined in two validating cohorts.

Paris validation cohort. High expression of Shh (median OS: 20.5 vs. 43.3 months, $P < 0.001$; median DFS: 10.1 vs. 20.0 months, $P < 0.001$) and high Gli1 (median OS: 20.7 vs. 38.0 months, $P < 0.001$; median DFS: 12.4 vs. 21.2 months, $P < 0.001$) were associated with a worse outcome (Fig. 1). In multivariate analysis, Shh and Gli1 remain independent and significant negative prognostic factors for OS (Table 2) and outcome prediction of the model was better with those two biomarkers (*C*-index from 0.63 to 0.69 for the OS, from 0.63 to 0.71 for the DFS; Table 3 and Supplementary Table S3). Similar results were observed in the Marseille validation cohort (Table 1, Table 2, and Supplementary Table S3; Fig. 1).

The combined Shh and Gli1 abundance stratify within lymph node status

For complementary identification of patient subgroups with the best and worst outcomes, we categorized patients according to their combined Shh and Gli1 levels. In the Brussels training set, patients with low Shh and Gli1 levels experienced the best outcomes (median OS = 45.1 months; HR set at 1.0). In contrast, patients with both Shh high and Gli1 high levels had the worst outcomes (median OS = 14.6 months; multivariate HR = 5.69, $P < 0.001$). Patients with Shh or Gli1 high level (median OS = 23.5 months; multivariate HR = 3.08, $P < 0.001$) constituted intermediate risk group. These results were confirmed in the two validation cohorts (Table 3; Supplementary Fig. S3).

Because low expression of Shh and Gli1 predict less aggressive tumor, we performed subgroup analysis on patients with PDAC stratified by N stage. The combined biomarkers discriminate differences in survival both for the subset of patients with node-negative and node-positive PDAC in both the training ($P < 0.001$) and the Paris validation cohort ($P < 0.001$; Fig. 2). These results remained significant in multivariate analysis (Supplementary Table S4). Shh and Gli1 low tumor had an excellent prognosis both in patients with lymph node metastasis (median OS in the training cohort: 50.9 months and 38.8 months in the validation set) and patients without metastasis (median OS in the training cohort not reached and 57.9 months in the Paris validation cohort). In contrast, patients with high expression for Shh and Gli1 had a worse outcome whatever the lymph node status

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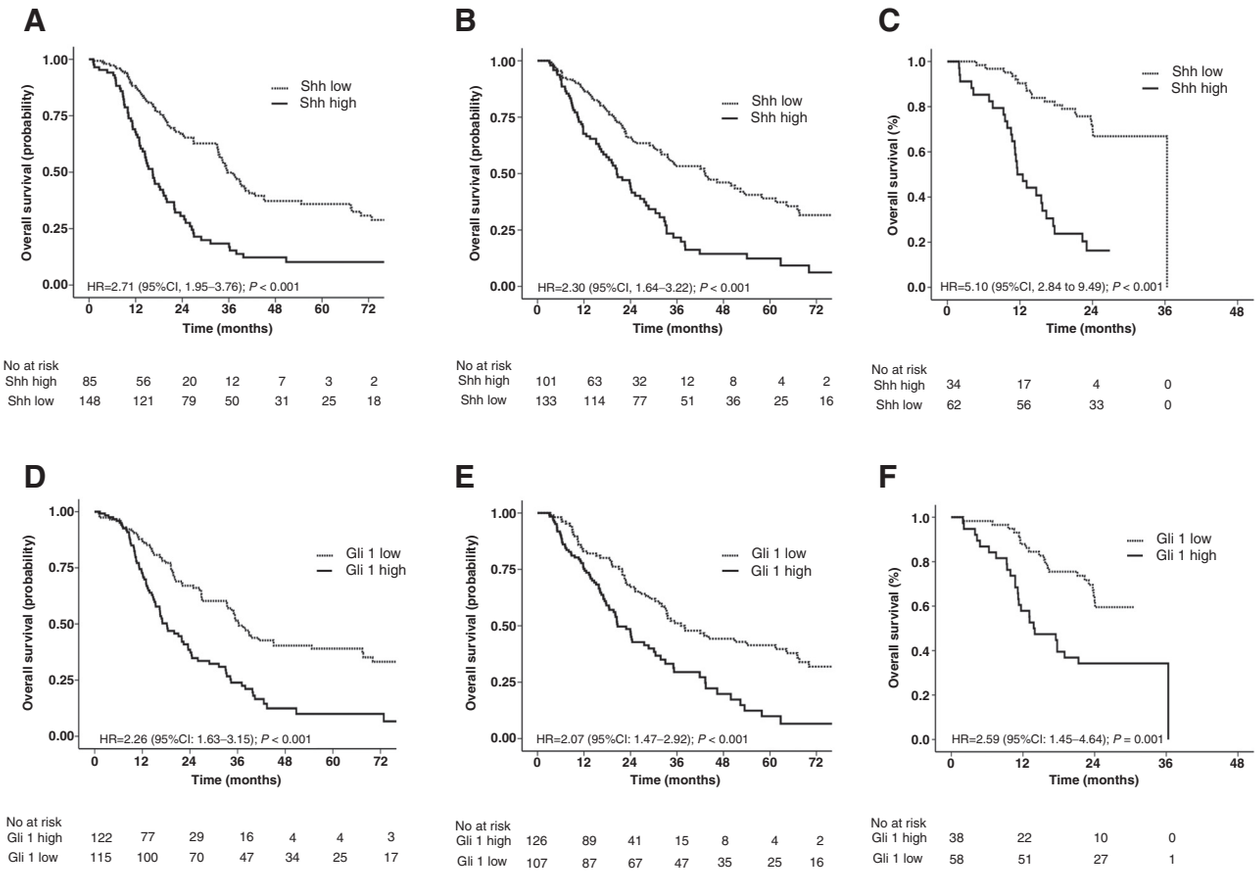

Figure 1.
Cumulative OS according to Shh (A–C) and Gli1 expression (D–F) in the Brussels training (A and D), Paris validation (B and E) and Marseille validation cohorts (C and F).

(Brussels training set; median OS in N0, 14.4 months and in N+, 14.8 months; Paris validation set, median OS in N0, 28.7 months and in N+, 17.0 months; Fig. 2; Supplementary Table S4). Such analysis was not performed in the Marseille validation cohort due to the small number of patients in each stratum.

Table 3. Multivariate Cox regression models of overall survival for the combination of Shh and Gli1

	OS	
Variable	**HR[a] (95% CI)**	***P***
Brussels training cohort (n = 232, 147 events)		
Gli1 and Shh low	1	<0.001
Gli1 or Shh high	3.08 (2.01–4.72)	
Gli1 and Shh high	5.69 (3.55–9.12)	
Harrell *C* statistic: 0.722		
Paris validation cohort (n = 224,129 events)		
Gli1 and Shh low	1	<0.001
Gli1 or Shh high	2.57 (1.62–4.08)	
Gli1 and Shh high	4.39 (2.56–7.53)	
Harrell *C* statistic: 0.705		
Marseille validation cohort (n = 96, 47 events)		
Gli1 and Shh low	1	<0.001
Gli1 or Shh high	4.95 (2.05–11.93)	
Gli1 and Shh high	11.34 (4.17–30.82)	
Harrell *C* statistic: 0.717		

NOTE: Each model was adjusted for the following covariates: tumor differentiation (not poor vs. poor), T stage (T1–T2 vs. T3), nodal status (N0 vs. N1), resection margin (R0 vs. R1), adjuvant therapy (no vs. yes).
[a]HR of 1 indicates no difference between the two groups of patients, whereas an HR>1 indicates an increased risk of death/failure.

Hh pathway components do not predict benefit of adjuvant therapy

We examined whether Gli1, PTCH1, SMO, and Shh abundance predict the benefit of adjuvant therapy. The interaction test was nonsignificant for all the four markers evaluated both in the training and the Paris validation sets (Supplementary Table S5). When stratifying the patients on the basis of the type of adjuvant therapy (chemotherapy versus radiotherapy, gemcitabine versus non-gemcitabine) the interaction tests remain insignificant.

Tumor stromal index correlates with Shh expression and predicts survival

Given that studies in animal models demonstrated that Hh ligands stimulate the desmoplastic reaction through a paracrine activation of pancreatic stellate cells, we examined the relationship between stroma abundance and (i) SMO expression (ii) Shh expression. To this end, whole consecutive tissue sections of 82 resected PDACs were analyzed. In 24% (20/82) and 46% (38/82), a high expression in pancreatic tumor cells was found for SMO and Shh, respectively. Stromal index (SI) was correlated with Shh protein abundance ($P < 0.001$; Fig. 3) while no correlation was

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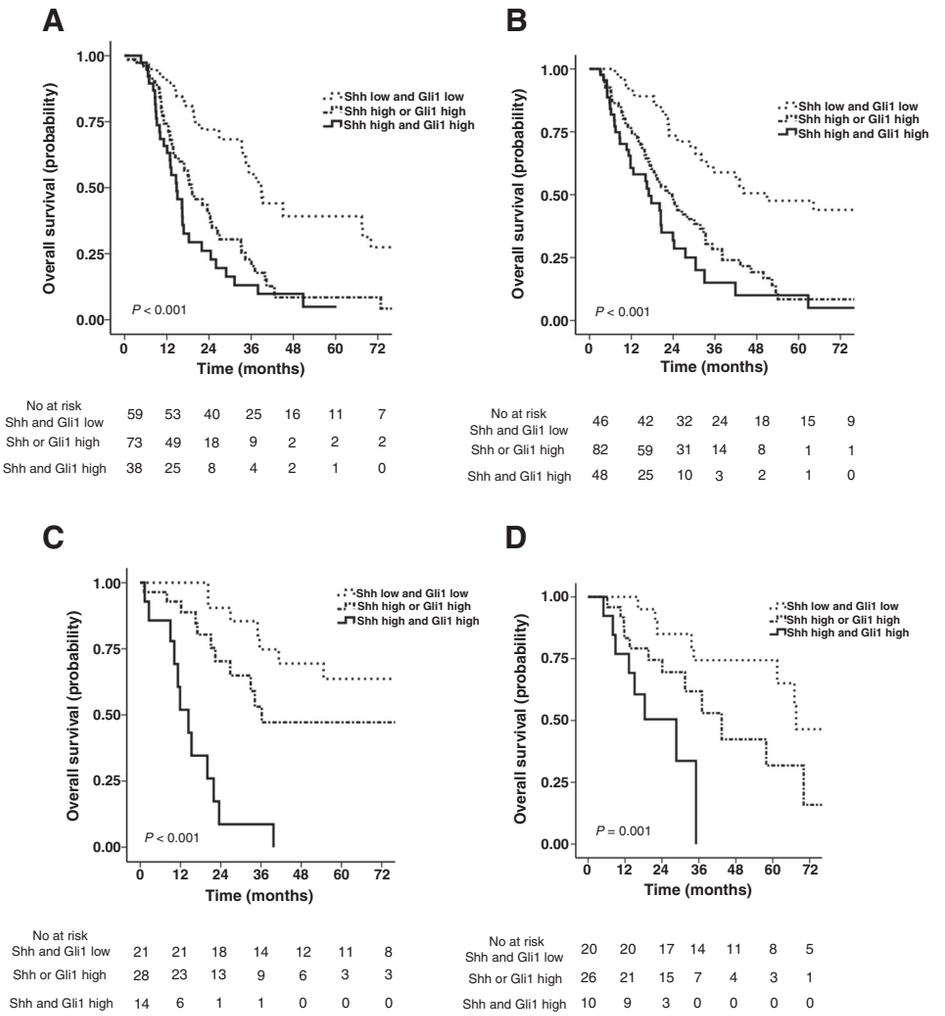

Figure 2.
Cumulative overall survival in (A and C) Brussels training and (B and D) Paris validation cohorts according to combined expression of Shh and Gli1 in lymph node–positive (A and B) and lymph node–negative tumors (C and D).

found between SI and SMO expression (data not shown). Furthermore, after dichotomizing the SI based on the median value, we found that patients with a high SI have a poorer outcome than those with a low SI (Fig. 3; Supplementary Table S6).

Discussion

Using immunohistochemistry assessment of abundance, we found that Shh and Gli1 provide significant and independent prognostic information in patients with PDACs who undergo a curative-intent surgery. Low level of expression of Gli1 or Shh by tumor cells was associated with longer DFS and OS times.

A dense stroma is characteristic of PDACs, and this dynamic compartment is critically involved in tumorigenesis (2). Hh signaling has been shown to play a functionally important role in the genesis of PDAC and in the aggressive phenotype of this cancer (3–7, 13–17). Canonical Hh signaling operates in a paracrine fashion (3–7, 17), contrasting with earlier work which focused on an autocrine role for Shh (12, 23).In an engineered mouse model of PDAC, mice treated with the gemcitabine and the SMO inhibitor saridegib exhibited a greater increase in survival relative to gemcitabine alone due to stroma depletion and a higher vascular density resulting in a better delivery of gemcitabine to tumor cells (24). A growing body of evidence suggests a cell-autonomous noncanonical Gli1 regulation through KRAS and TGFβ pathways in pancreatic cancer cells that distinct from the Hh ligand-dependant paracrine effect on the tumor stroma (7, 18). Gli1 expression is essential for PDAC cells' survival and facilitates migration and invasion of the cells by promoting the epithelial-to-mesenchymal transition process (18–20, 25).

In aggregate, the current study strengthened the consistency of these preclinical data: in multivariate analysis, Shh and Gli1 were independent negative prognostic biomarkers of DFS and OS. The combination of these 2 biomarkers was more informative than the separate evaluation of each one suggesting that an optimized prognostic algorithm should include both biomarkers, with Shh status weighted more heavily than Gli1 status. Importantly, the prognostic value of these two biomarkers was confirmed in two independent cohorts of patients with a similar discriminating magnitude effect between the different cohorts. In addition, these concordant results in the three cohorts provided evidence that these IHC biomarkers were generally applicable to patients with PDAC independent of differences in clinical practice. Furthermore, these two biomarkers are independent of the major clinical features known to influence prognosis and could serve as an adjunct to current staging systems. Importantly, no interaction

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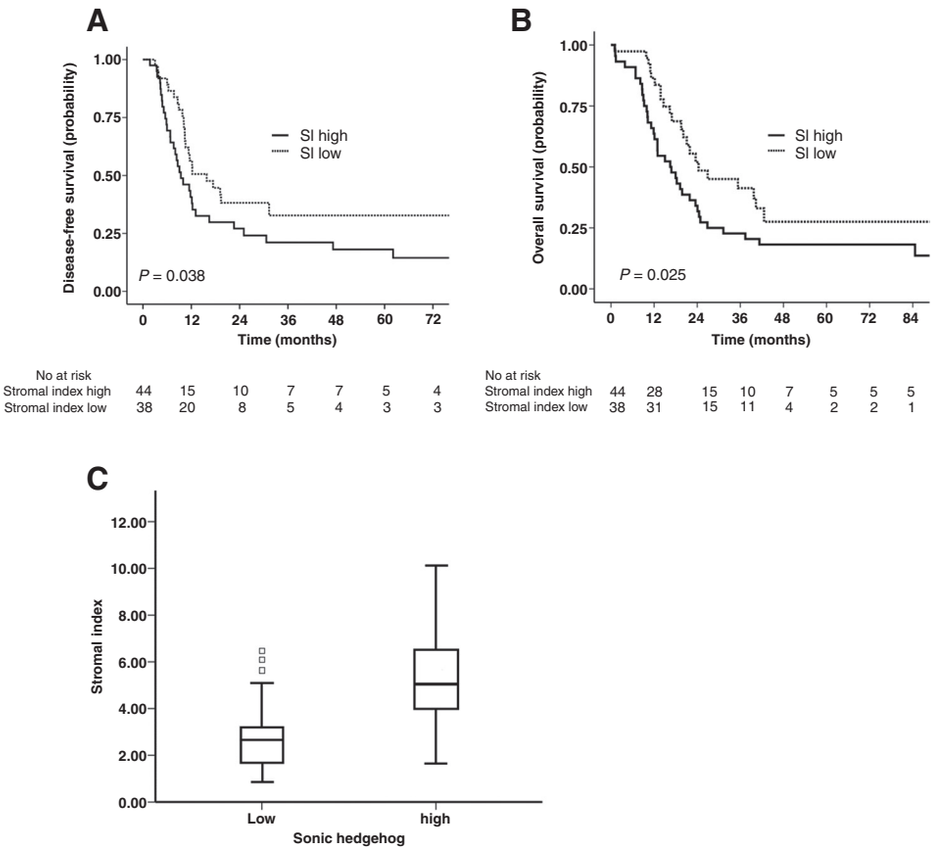

Figure 3.
A and B, Kaplan–Meier disease-free survival curves showing patients stratified by stromal index. C, association between the level of Shh into the tumor and the SI. Median value of fibrosis index (FI) for the whole population: 3.15 (range, 0.85–10.12). Mean FI value in the Shh-high subgroup: 2.59 ± 0.16 (SD) versus 5.23 ± 0.36 ($P = 0.002$) in the Shh-low subgroup.

was observed between Gli1, Shh, and adjuvant treatment activity making sure that this confounding factor did not affect our relationship between Gli1, Shh, and patients' outcome.

Although there is possibility of bias associated with retrospective studies, our study is strengthened by the large number of patients and the two independent replication cohorts. Furthermore, agreement coefficients almost perfect for Shh and Gli1 support the reliability of their semiquantitative scoring assessment and allow for accurate classification of the staining pattern. Whether these two robust and easy applicable biomarkers provide sufficient prognostic information requires prospective evaluation in randomized clinical trials.

The prognostic significance of Shh and Gli1 was further strengthened by their ability to stratify within a currently validated prognostic factor. Patients with positive lymph node disease with low levels of Shh and Gli1 experienced similar outcomes than patients with negative lymph node. The ability of these biomarkers to prognosticate within tumors with positive lymph node has a particularly important value, because this population represents 80% of the resected patients with PDAC.

Our study showed that the Hh pathway is not uniformly active or pathogenic in all cases of PDAC and those interindividual differences in the Hh pathway activation may contribute to the variable outcomes in PDAC. Patients with a low tumor expression for both Shh and Gli-1 experienced the best outcome. The prognostic value of Shh could be partially explained by its influence on the desmoplastic reaction (3–7, 13–17). SMO overexpression was frequently observed in pancreatic stellate cells and it has been shown that these cells are responsive to stimulation with Shh suggesting that the desmoplastic response mediated by the Hh signaling is depending on the ability of PDAC cells to produce Shh (4, 17). Our findings support this hypothesis: (i) Shh was positively correlated to the SI, (ii) the SI was significantly higher in Shh high than in Shh low tumors, and (iii) a high SI was a negative independent prognosticator for both the DFS and the OS. Overall, our results also support evidence that anti-Hh therapies targeting stroma depletion should be restricted to high Shh PDACs.

Despite the strong biologic data supporting Hh inhibition in PDAC, two recent studies failed to demonstrate any additional benefit of SMO inhibitors (saridegib and vismodegib) in unselected patients with metastatic PDAC compared with placebo (26, 27). Furthermore, in the KPC model of pancreatic cancer, deletion of Shh induced stroma depletion and resulted in an undifferentiated aggressive tumor phenotype suggesting a protective role of Shh and the desmoplastic reaction (28). On the contrary, our findings support a deleterious effect of Shh, and no association was found between the abundance of Shh and the tumor differentiation. The setting of the disease and the nature of the tissue could partially explain these discrepancies. In conclusion, high expression of Shh and Gli1 in resected PDACs is dramatically and independently associated with poorer prognosis. The observation that these two discriminating biomarkers maintained their prognostic value across three patient cohorts makes our findings

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compelling enough to subject Shh and Gli1 to further clinical evaluation.

Disclosure of Potential Conflicts of Interest

J.-B. Bachet is a consultant/advisory board member for and reports receiving commercial research support from Celegene. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

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Authors' Contributions

Conception and design: R. Maréchal, J.-B. Bachet, M. Svrcek, J. Cros, J.-F. Emile, J.-L. Van Laethem

Development of methodology: R. Maréchal, J.-B. Bachet, P. Demetter, F. Puleo, J.-L. Van Laethem

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Maréchal, J.-B. Bachet, J.R. Delpero, M. Svrcek, J. Cros, A. Bardier-Dupas, G. Monges, P. Hammel, C. Louvet, F. Paye, P. Bachellier, Y.P. Le Treut, J.-C. Vaillant, A. Sauvanet, T. André, I. Salmon, J. Devière, J.-F. Emile, J.-L. Van Laethem

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Maréchal, J.-B. Bachet, P. Hammel, J. Devière, J.-L. Van Laethem

Writing, review, and/or revision of the manuscript: R. Maréchal, J.-B. Bachet, P. Demetter, F. Puleo, P. Hammel, C. Louvet, P. Bachellier, T. André, J. Devière, J.-F. Emile, J.-L. Van Laethem

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Maréchal, J.-B. Bachet, P. Demetter, J.R. Delpero, J. Cros, J. Devière, J.-L. Van Laethem

Study supervision: R. Maréchal, J.-L. Van Laethem

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