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Favorable prognostic significance of membranous β-catenin expression and negative prognostic significance of cytoplasmic β-catenin expression in pancreatic cancer

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The aim of this study was to investigate the prognostic significance of membranous β-catenin and cytoplasmic β-catenin expression in pancreatic cancer patients (pts). One hundred pts with histologically verified exocrine pancreatic ductal adenocarcinoma were retrospectively studied. The membranous β-catenin, cytoplasmic β-catenin, and cell nucleus β-catenin expression were immunohistochemically evaluated. The expression of membranous β-catenin was <5% in none of the pts, 5–25% in one patient, 26–50% in 2 pts, 51–75% in 14 pts, and >75% in 81 pts. The expression of cytoplasmic β-catenin was <5% in 34 pts, 5–25% in 42 pts, 26–50% in 18 pts, 51–75% in 3 pts, and >75% in one patient. The expression of β-catenin in the cell nucleus was negative in all pts. At the time of the last follow-up, 21 pts were alive and 79 pts had died. Median OS was 1.3 (0.4–2.3) years in pts with membranous β-catenin expression ≤75% and 1.7 (1.3–2.1) years in pts with membranous β-catenin expression >75% (p=0.045). Median OS was (1.3–2.0) 1.6 years in pts with cytoplasmic β-catenin expression ≤25% and 0.9 (0.5–1.2) years in pts with cytoplasmic β-catenin expression >25% (p=0.040). In the univariate Cox proportional hazard models HR (95% CI) was 0.556 (0.311–0.995) in pts with membranous β-catenin expression >75% (p=0.048) and 2.200 (1.216–3.980) in pts with cytoplasmic β-catenin expression >25% (p=0.009). The present results indicate a favorable prognostic significance of membranous β-catenin expression in pancreatic cancer.

Key words: β-catenin cytoplasmic; β-catenin membranous; CD8+ tumor infiltrating lymphocytes; pancreatic ductal adenocarcinoma; primary cilia; programmed cell death protein 1

Pancreatic ductal adenocarcinoma (PDAC) is a cancer associated with a poor prognosis. Currently, the only potentially curative treatment is radical surgical resection with microscopically negative margins (R0). Due to an asymptomatic course in most patients with early-stage disease, late diagnosis is a rule, and radical resection is possible in only 20–30% of patients. PDAC is a relatively chemo- and radioresistant tumor with annual mortality close to the annual incidence rates. Recently, much progress has been achieved in the understanding of the mechanisms of anti-tumor immunity, however, immunotherapy has not yet been shown to improve treatment outcomes in PDAC. This apparent lack of efficacy of immunotherapy may be due to a low mutation burden, with a subsequent paucity of T-cell infiltration and the development of an immune-suppressive tumor microenvironment [1, 2].

β-catenin is a pivotal component of the Wnt signaling pathway [3] that includes several signaling branches, the most important of which is the Wnt/β-catenin pathway (also called the canonical pathway), which is determined by the β-catenin activity [4]. The canonical aspect of the Wnt signaling pathway is mediated by β-catenin, which upon

activation translocates to the nucleus [5]. In more than half of all neoplastic disorders, including colorectal carcinoma, breast cancer, liver carcinoma, melanoma, and leukemia, β-catenin accumulates within the cytoplasm or nucleus [6–10]. In normal pancreatic tissue, β-catenin expression is predominantly localized in the membrane of ductal cells. In pancreatic cancer, downregulation of membrane expression and increased cytoplasmic expression are observed [11–15]. Although β-catenin is rarely mutated in cancer, mutations of its main protein partners can confer enhanced stability to β-catenin, causing an aberrant accumulation [16, 17]. β-catenin can promote the progression of tumors by suppressing T-cell responses [18].

The primary cilium is a sensory microtubule-based organelle, which protrudes during the quiescent phase of the cell cycle from the surface of most human cells with the exception of hematopoietic cells [19]. Several signaling pathways are active in primary cilia, including the Wnt [20, 21] and Hedgehog [22, 23] signaling pathways. The formation of primary cilia itself can induce a switch from canonical to non-canonical Wnt signaling [24]. The Wnt/β-catenin signaling pathway cross-talks with the Hedgehog signal transduction. One of the key proteins of the Hedgehog signaling pathway is the transmembrane protein Smoothened (Smo). The temporary localization of Smo to the primary cilia activates the Hedgehog signaling pathway [25].

Table 1. Baseline characteristics[1].

Variable		n	%
Gender	Male	53	53.0
	Female	47	47.0
Age at diagnosis (years)	median (range)	66 (36-82)	
	mean (SD)	65 (9)	
Tumor localization	Head of the pancreas	79	79.0
	Body of the pancreas	10	10.0
	Tail of the pancreas	11	11.0
Stage at diagnosis	I	26	26.0
	II	56	56.0
	III	13	13.0
	IV	5	5.0
Vascular invasion	Yes	84	84.8
	No	15	15.2
Perineural invasion	Yes	74	88.1
	No	10	11.9
Resection	R0	77	77
	R1	23	23
Tumor grade (WHO 2016)	G1	1	1.2
	G2	35	42.2
	G3	45	54.2
	G4	2	2.4
Patient status	Alive	21	21.0
	Died	79	79.0

Note: [1]No information about vascular invasion in one patient, perineural invasion in 16 patients and grade in 17 patients

The primary cilium is considered to represent a functional homolog of the immune synapse due to morphological and functional similarities in architecture [26, 27].

The aim of this study was to investigate the prognostic significance of membranous β-catenin and cytoplasmic β-catenin expression in PDAC patients. The prognostic significance of several other biomarkers of PDAC microenvironment in potential association with β-catenin expression, including the frequency of primary cilia, Smo expression, CD8+ tumor-infiltrating lymphocytes (TIL) expression, and PD-1 expression were also investigated.

Patients and methods

Study design. The Ethics Committee with multi-center competence of the Institute for Clinical and Experimental Medicine (IKEM) and Thomayer Hospital (TN) discussed and approved submitted documents for the study at its meeting on 8. 2. 2017 (reference number: 2200/16+218/17 (G-16-12-02). One hundred PDAC patients, 53 males and 47 females, median age of 66 (range 36–82) years, were retrospectively evaluated. All patients had histologically verified PDAC; grade 1 in one patient, grade 2 in 35 patients, grade 3 in 45 patients, grade 4 in 2 patients, and unknown grade in 17 patients. The anatomical localization included the head of the pancreas in 79 patients, the body of the pancreas in 10 patients, and the tail of the pancreas in 11 patients. Resection with microscopic residual tumor (R1) was performed in 23 patients while in the 77 patients, radical resection with microscopically negative margins (R0) was possible (Table 1). All studied tumor samples were acquired from pancreatectomy specimens of primary tumors and not from biopsies or metastases.

Immunohistochemistry. An indirect immunohistochemistry method using monoclonal mouse anti-human β-catenin-1 antibody (clone β-catenin-1, Dako, Glostrup, Denmark) (Figure 1A), polyclonal rabbit anti-Smoothened antibody (clone ab113438, Abcam, Cambridge, United Kingdom) (Figure 1B), mouse monoclonal primary antibody against CD8 (M7103, Dako, Glostrup, Denmark; Figure 1C) and mouse monoclonal primary antibody against PD-1 (NAT105, Cell Marque, Darmstadt, Germany; Figure 1D) was used. All slides were assessed by an experienced pathologist not aware of the treatment and outcome of the patients. Immunohistochemistry was scored semiquantitatively as shown in Table 2.

Immunofluorescence. Primary cilia of cells were demonstrated by immunofluorescence using anti-acetylated tubulin-alpha antibody and the nuclei of the cells were visualized using DAPI labeling. The percentage of primary cilia on cells was counted as primary cilia to the cell nuclei ratio as described previously [28] (Figure 1E).

Statistics. Standard descriptive statistics were used to characterize the sample data set. Categorical variables were defined by absolute and relative frequencies; continuous

variables were described by median values (including range) and mean values (including standard deviation). A comparison of the categorical parameters was performed using the Fisher Exact test. In the case of continuous variables, the Mann-Whitney or Kruskal-Wallis test was used. ROC analysis was calculated to estimate the optimal cut-off value for the frequency of cilia. The optimal cut-off was selected according to the criterion of maximizing the product of sensitivity and specificity. Overall survival (OS) was estimated using Kaplan Meier method and all point estimates were accompanied by 95% confidence intervals (95% CI). OS was defined as the time from diagnosis to death from any cause. Surviving patients were censored at the date of the last update. Comparison of OS between subgroups of patients was carried out by log-rank test. Univariable Cox proportional hazards models were used to evaluate the effect of potential prognostic factors on the survival measures. Point estimates of hazard ratio (HR) were calculated with 95% confidence intervals. The statistical significance of hazard ratios was assessed by the mean of the Wald test. As a level of statistical signifikance, α=0.05 was used.

Results

Descriptive statistics of the investigated biomarkers are summarized in Table 2. The expression of membranous β-catenin was <5% in none patients, 5–25% in one patient, 26–50% in 2 patients, 51–75% in 14 patients, and >75% in 81 patients. The expression of cytoplasmic β-catenin was <5% in 34 patients, 5–25% in 42 patients, 26–50% in 18 patients, 51–75% in 3 patients, and >75% in one patient. The expression of β-catenin in the cell nucleus was negative in all patients.

At the time of the last follow-up update, 21 patients were alive and 79 patients had died. Median overall survival (OS) was 1.6 years (95% CI 1.3–1.8), 1-year OS rate was 64.7% (95% CI 55.2–74.3), 2-year OS was 36.3% (95% CI 26.2–46.4), and 3-year OS was 19.9% (95% CI 11.3–28.6).

Median OS was 1.3 (95% CI 0.4–2.3) years in patients with membranous β-catenin expression ≤75% and 1.7 (95% CI 1.3–2.1) years in patients with membranous β-catenin expression >75% (p=0.045; Table 3 and Figure 2). There was no correlation between tumor grade and membranous cytoplasmic β-catenin expression (Table 4). Median OS was 1.6 (95% CI 1.3–2.0) years in patients with cytoplasmic β-catenin expression ≤25% and 0.9 (95% CI 0.5–1.2) years in patients with cytoplasmic β-catenin expression >25% (p=0.040; Table 5 and Figure 2). There was a significant association between higher membranous β-catenin and tumor grade with cytoplasmic β-catenin (Table 6). The frequency of primary cilia did not have a statistically significant effect on OS (Table 7 and Figure 3). Table 8 shows the OS according to other evaluated biomarkers, including frequency of primary cilia, Smo expression, CD8+ TIL expression, and PD-1 expression. In the univariable Cox proportional hazard models, HR

Table 2 Prognostic factors[1]

Variable		n	%
Membranous β-catenin	<5%	0	0.0
	5–25%	1	1.0
	26–50%	2	2.0
	51–75%	14	14.3
	>75%	81	82.7
Cytoplasmic β-catenin	<5%	34	34.7
	5–25%	42	42.9
	26–50%	18	18.4
	51–75%	3	3.1
	>75%	1	1.0
Without cilia	Yes	42	54.5
	No	35	45.5
Frequency of cilia	median (range)	0.001 (0.000-0.004)	
	mean (SD)	0.001 (0.001)	
Smo	<5%	0	0.0
	5–25%	3	3.0
	26–50%	6	6.1
	51–75%	11	11.1
	>75%	79	79.8
CD8+ TIL	Negative	0	0.0
	<25%	85	85.0
	25–50%	15	15.0
	> 50%	0	0.0
PD-1	<5%	68	68.0
	5–25%	30	30.0
	26–50%	2	2.0
	51–75%	0	0.0
	>75%	0	0.0

Note: [1]No information about membranous β-catenin in 2 patients, cytoplasmic β-catenin in 2 patients, cilia in 23 patients and Smo in one patient

Table 3. Overall survival from diagnosis according to membranous β-catenin expression

	Membranous β-catenin		p-value[1]
	≤75% (n=17)	>75% (n=81)	
Median OS (95% CI)	1.3 years (0.4–2.3)	1.7 years (1.3–2.1)	0.045
1-year OS (%; 95% CI)	57.4 (33.3–81.4)	67.9 (57.4–78.3)	
2-year OS (%; 95% CI)	15.3 (0.1–34.3)	41.5 (30.0–53.0)	
3-year OS (%; 95% CI)	7.6 (0.1–21.9)	22.8 (12.8–32.9)	

Note: [1]Log-rank test

Table 4. Association of tumor grade and cytoplasmic β-catenin expression with membranous β-catenin expression

Characteristics		Membranous β-catenin		p-value[1]
		≤75% (n=17)	>75% (n=81)	
Cytoplasmic β-catenin	<5%	0 (0.0)	34 (42.0)	<0.001
	5–25%	5 (29.4)	37 (45.7)	
	>25%	12 (70.6)	10 (12.3)	
Tumor grade (WHO 2016)	G1	0 (0.0)	1 (1.5)	0.262
	G2	4 (26.7)	31 (47.0)	
	G3	10 (66.7)	33 (50.0)	
	G4	1 (6.7)	1 (1.5)	

Note: [1]Fisher exact test

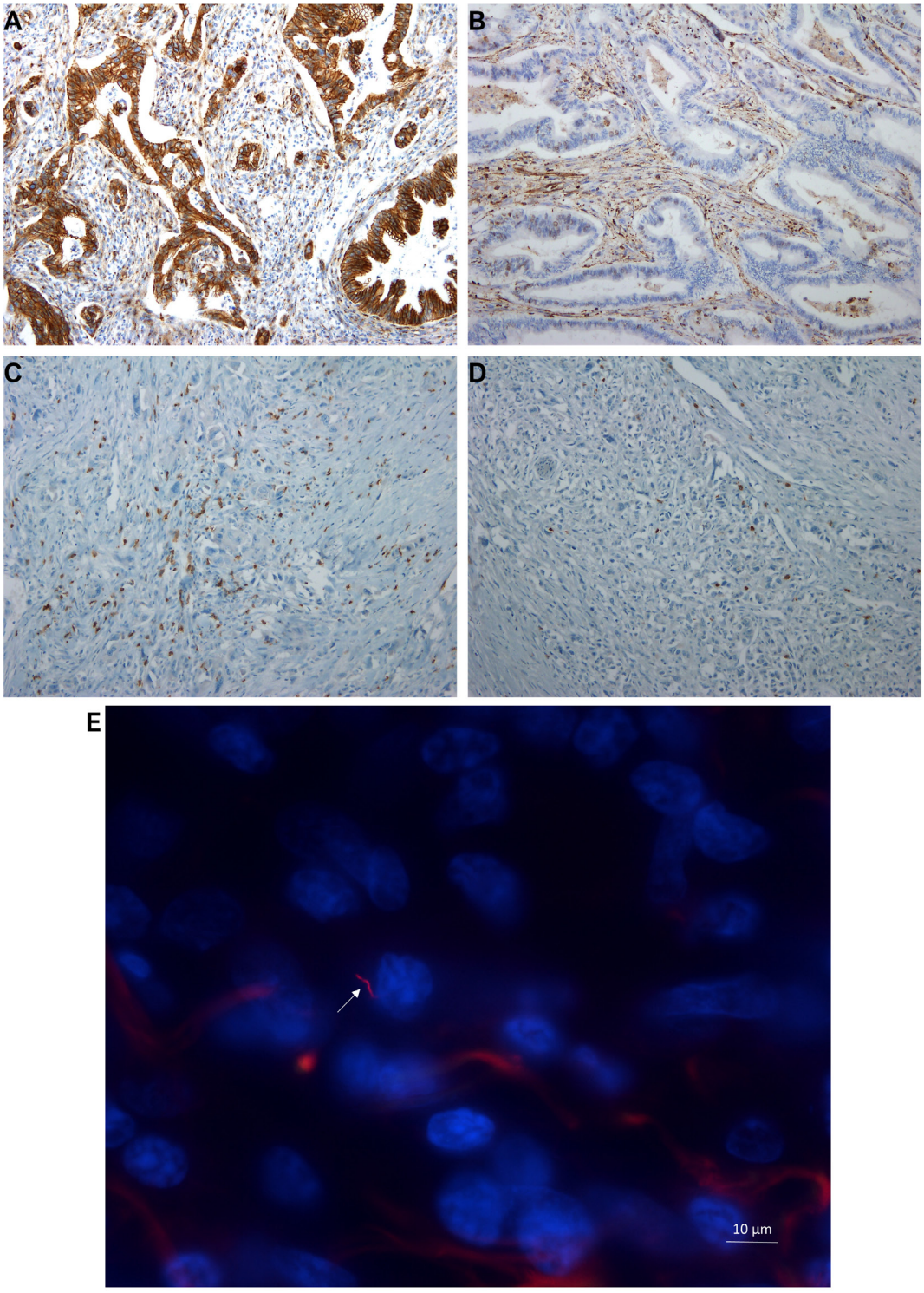

Figure 1. A) β-catenin expression. Immunohistochemical staining with monoclonal mouse anti-human β-catenin-1 antibody (clone β-catenin-1, Dako, Glostrup, Denmark) was used. Magnification 100×. B) Smoothened protein expression. Immunohistochemical staining with polyclonal rabbit anti-smoothened antibody (clone ab113438, Abcam, Cambridge, United Kingdom) was used. Magnification 100×. C) CD8+ expression of cytotoxic T lymphocytes using human antibody in stromal areas of pancreatic adenocarcinoma. An indirect immunohistochemistry using mouse monoclonal primary antibodies against CD8 (M7103, Dako, Glostrup, Denmark) was used. Magnification 100×. D) PD-1 expression using mouse monoclonal primary antibody in pancreatic adenocarcinoma. An indirect immunohistochemistry using mouse monoclonal primary antibodies against PD-1 (NAT105, Cell Marque, Darmstadt, Germany) was used. Magnification 100×. E) Primary cilia of cells of pancreatic adenocarcinoma immunofluorescently labeled using anti-acetylated tubulin-alpha antibody and cell nuclei labeled using DAPI. Magnification 100×.

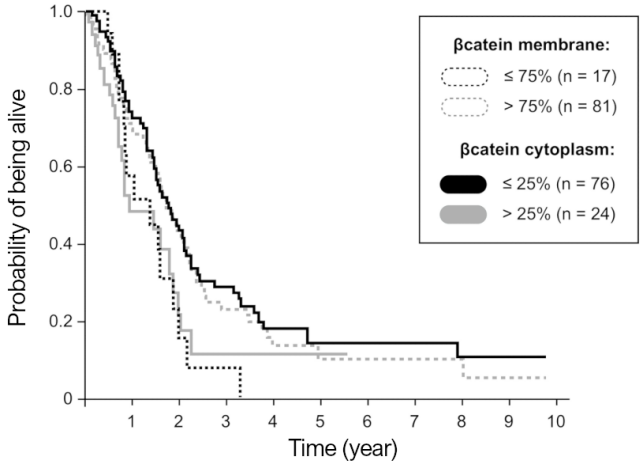

Figure 2. Overall survival from diagnosis according to membranous β-catenin expression and overall survival from diagnosis according to cytoplasmic β-catenin expression.

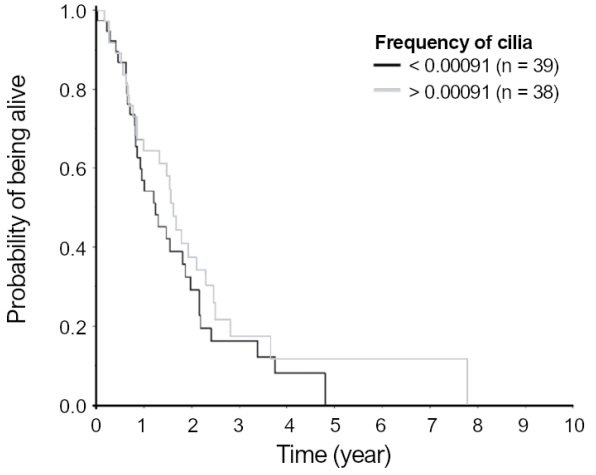

Figure 3. Overall survival from diagnosis according to the frequency of cilia.

(95% CI) was 0.556 (0.311–0.995) in patients with membranous β-catenin expression >75% (p=0.048) and HR (95% CI) 2.200 (1.216–3.980) in patients with cytoplasmic β-catenin expression >25% (p=0.009; Table 9). Multivariate testing showed that cytoplasmic β-catenin expression and tumor grade were associated with prognosis (Table 10).

Table 5. Overall survival from diagnosis according to cytoplasmic β-catenin expression.

	Cytoplasmic β-catenin		p-value[1]
	≤25% (n=76)	>25% (n=24)	
Median OS (95% CI)	1.6 years (1.3–2.0)	0.9 years (0.5–1.2)	0.040
1-year OS (%; 95% CI)	69.7 (59.1–80.3)	49.4 (29.1–69.6)	
2-year OS (%; 95% CI)	40.5 (28.7–52.3)	22.8 (4.2–41.4)	
3-year OS (%; 95% CI)	24.1 (13.5–34.6)	5.7 (0.1–16.4)	

Note: [1]Log-rank test

Table 6. Association of tumor grade and membranous β-catenin expression with cytoplasmic β-catenin expression.

Characteristics		Cytoplasmic β-catenin		p-value[1]
		≤25% (n=76)	>25% (n=24)	
Membranous β-catenin	≤75%	5 (6.6)	12 (54.5)	<0.001
	>75%	71 (93.4)	10 (45.5)	
Tumor grade (WHO 2016)	G1	1 (1.6)	0 (0.0)	0.004
	G2	32 (51.6)	3 (14.3)	
	G3	27 (43.5)	18 (85.7)	
	G4	2 (3.2)	0 (0.0)	

Note: [1]Fisher exact test

Discussion

Present data indicate a favorable prognostic significance of membranous β-catenin expression and a negative prognostic significance of cytoplasmic β-catenin expression in pancreatic cancer. Due to the negative expression of β-catenin in the cell nucleus, there are only two possibilities for the localization of β-catenin (membranous and cytoplasmic). Essentially, they must be correlated if they are not distributed in the same proportion in the membrane cytoplasm. Because in the case of a two-location system, the positive prognosis linked to one location means a negative prognosis of the second location.

Survival rates of PDAC patients are disappointing. The Wnt/β-catenin signaling pathway is involved in pancreatic carcinogenesis and resistance to current therapies [2]. In the treatment of PDAC, targeting the Wnt/β-catenin signaling pathway is an actively pursued experimental strategy. Several novel inhibitors for the Wnt/β-catenin signaling pathway that have been developed are presently tested in clinical trials [2, 29]. Since Wnt signaling is shared by both cancer cells and normal body tissues, controlling the side effects associated with targeting the Wnt pathway may be difficult [2].

In the normal pancreas, β-catenin expression is predominantly localized in the membrane of ductal cells. In PDAC, the downregulation of membrane expression and increased cytoplasmic expression were noted [11–15]. The results of the present study are consistent with the above literature. In this study, a statistically significant inverse correlation between membranous and cytoplasmic β-catenin expression was observed. The expression of β-catenin in the cell nucleus was negative in all patients of the present cohort.

The correlation of reduced membranous β-catenin expression with the loss of tumor differentiation in PDAC has been reported in the literature [11]. In the present study statistically significant association with tumor grade on cytoplasmic β-catenin expression, but not with membranous β-catenin expression was observed.

Furthermore, the prognostic significance of other evaluated biomarkers of pancreatic cancer that could be related to β-catenin expression could not be demonstrated.

Table 7. Prediction of mortality by frequency of cilia-diagnostic test.

	Valid N	Cut-off[1]	AUC	p-value	Sensitivity	Specificity	PPV	NPV	Accuracy
All patients	77	0.00091	0.553	0.507	0.467	0.412	0.737	0.179	0.455

Note: [1]Smaller value than cut-off corresponds to events (death)
Abbreviations: AUC-Area under curve; PPV-Positive predictive value; NPV-Negative predictive value

Table 8. Overall survival from diagnosis.

Overall survival from diagnosis according to the frequency of cilia			
	Frequency of cilia		**p-value[1]**
	<0.00091 (n=39)	**>0.00091 (n=38)**	
Median OS (95% CI)	1.2 years (0.7–1.8)	1.6 years (1.4–1.9)	0.363
1-year OS (%; 95% CI)	57.0 (41.0–73.0)	64.4 (48.9–80.0)	
2-year OS (%; 95% CI)	29.1 (13.7–44.6)	37.5 (20.6–54.4)	
3-year OS (%; 95% CI)	16.2 (3.4–29.0)	17.3 (2.8–31.9)	
Overall survival from diagnosis according to Smo expression			
	Smo		**p-value[1]**
	≤75% (n=20)	**>75% (n=79)**	
Median OS (95% CI)	1.6 years (0.0–3.5)	1.6 years (1.4–1.8)	0.549
1-year OS (%; 95% CI)	55.0 (33.2–76.8)	68.2 (57.7–78.7)	
2-year OS (%; 95% CI)	44.0 (21.9–66.1)	34.6 (23.2–46.0)	
3-year OS (%; 95% CI)	22.0 (3.2–40.8)	19.7 (9.8–29.5)	
Overall survival from diagnosis according to CD8+ TIL expression			
	CD8+ intratumoral		**p-value[1]**
	<25% (n=85)	**25–50% (n=15)**	
Median OS (95% CI)	1.6 years (1.2–1.9)	1.7 years (1.1–2.2)	0.388
1-year OS (%; 95% CI)	63.5 (53.1–74.0)	71.8 (48.3–95.3)	
2-year OS (%; 95% CI)	38.5 (27.5–49.6)	23.9 (0.5–47.4)	
3-year OS (%; 95% CI)	22.1 (12.3–31.8)	8.0 (0.1–22.9)	
Overall survival from diagnosis according to PD-1 expression			
	PD–1		**p-value[1]**
	<5% (n=68)	**>5% (n=32)**	
Median OS (95% CI)	1.5 years (1.2–1.7)	1.7 years (1.4–1.9)	0.421
1-year OS (%; 95% CI)	63.3 (51.5–75.0)	67.8 (51.4–84.2)	
2-year OS (%; 95% CI)	41.1 (28.7–53.4)	24.3 (7.7–40.8)	
3-year OS (%; 95% CI)	24.9 (13.7–36.1)	8.1 (0.1–18.8)	

Note: [1]Log-rank test

Table 9. Survival analysis-univariable Cox proportional hazard model.

Character-istic	Category	n	HR (95% CI)	p-value[1]
Membranous β-catenin	≤75%	17	1.000	–
	>75%	81	0.556 (0.311–0.995)	0.048
Cytoplasmic β-catenin	<5%	34	1.000	–
	5–25%	42	1.649 (0.978–2.780)	0.060
	>25%	24	2.200 (1.216–3.980)	0.009
Frequency of cilia	(continuous-0.01 change)	77	0.103 (0.004–2.449)	0.160
Frequency of cilia	<0.00091	39	1.000	–
	>0.00091	38	0.788 (0.471–1.318)	0.364
Smo	≤75%	20	1.000	–
	>75%	79	1.186 (0.677–2.078)	0.550
CD8+ TIL	>25%	85	1.000	–
	25-50%	15	1.301 (0.714–2.370)	0.390
PD-1	<5%	68	1.000	–
	>5%	32	1.220 (0.750–1.985)	0.422
Smo	≤75%	20	1.000	–
	>75%	79	1.186 (0.677–2.078)	0.550

Note: [1]Wald test

Table 10. Survival analysis-multivariable Cox-proportional hazard model.

Characteristic	Category	n	HR (95% CI)	p-value[1]
Membranous β-catenin	≤75%	17	1.000	–
	>75%	81	0.874 (0.402–1.899)	0.734
Cytoplasmic β-catenin	<5%	34	1.000	–
	5–25%	42	1.608 (0.931–2.777)	0.089
	>25%	22	2.664 (1.208–5.875)	0.015
Tumor grade	1	26	1.000	–
(WHO 2016)	2	54	1.031 (0.583–1.825)	0.915
	3	13	1.744 (0.760–3.999)	0.189
	4	5	5.709 (1.907–17.091)	0.002
Sex	female	46	1.000	–
	male	52	1.130 (0.686–1.862)	0.631
Age at diagnosis (years)	<60	26	1.000	–
	60–69	36	1.187 (0.643–2.190)	0.584
	≥70	36	1.386 (0.742–2.590)	0.306

Note: [1]Wald test

Loss of primary cilia in the early stage of PDAC has been reported in the literature [30, 31]. One study noted that the presence of primary cilia correlates with a poor prognosis in PDAC [32]. In the present study patients with a higher frequency of primary cilia (>0.00091) had longer median OS than patients with a lower frequency of primary cilia (<0.00091) with a shorter median OS, but this difference was not statistically significant.

Aberrant expression of Smo has been described in many cancers [25]. Increased Smo protein expression has been reported in the literature in most PDAC cases [25, 33]. In the present study, we observed higher (>75%) Smo expression in 79 (79.8%) patients, but no prognostic significance of the Smoothened protein expression was evident.

Several studies reported positive prognostic significance of CD8+ TIL expression [34–37] and PD-1 expression [34] in PDAC. In this study, patients with higher (25–50%) CD8+ TIL expression had a longer median OS survival than patients with lower (<25%) CD8+ TIL expression, but the difference was not statistically significant. Similarly, this cohort of patients with higher PD-1 expression (>5%) had a longer median OS than patients with lower PD-1 expression (<5%), but again, this difference was not statistically significant.

The limitations of this study include the relatively small patient cohort, the retrospective design as well as the limitations of immunohistochemical and immunofluorescent methods. The patient cohort was heterogeneous with regard to the stage, location of the primary tumor within the pancreas as well as the therapy.

In conclusion, the data of the present study indicate favorable prognostic significance of membranous β-catenin expression and negative prognostic significance of cytoplasmic β-catenin expression in pancreatic cancer and, thus, relatively cheap and easier immunohistochemical investigation could be used as a useful prognostic marker in PDAC. Moreover, the correlative data obtained can become the basis and justification for functional studies.

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