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Negative prognostic significance of primary cilia and cytoplasmic β-catenin expression in non-small cell lung cancer

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The objective of this study was to investigate the prognostic significance of the frequency of primary cilia (PC) and β-catenin expression in 218 patients (pts) with non-small cell lung cancer (NSCLC), including 125 pts with adenocarcinoma and 93 pts with squamous cell carcinoma. In the whole group of 218 pts with NSCLC, overall survival (OS) was significantly inferior among pts with present PC than without PC (p=0.024) and with higher cytoplasmic β-catenin expression (25–75%) than with lower cytoplasmic β-catenin expression (<25%) (p=0.008). In the univariate Cox proportional hazard model, the hazard ratio was 1.653 in pts with present PC (p=0.026) and 1.851 in pts with higher cytoplasmic β-catenin (25–75%) (p=0.009). Multivariate testing of the whole group of 218 pts with NSCLC showed that the presence of PC was associated with a worse prognosis (p=0.018). In the subgroup of 125 pts with adenocarcinoma, OS was significantly improved in pts with higher membranous β-catenin expression (≥50%) than in pts with lower expression (<50%) (p=0.0300) and OS was significantly inferior in pts with higher cytoplasmic β-catenin expression (25–75%) than in pts with lower expression (<25%) (p=0.0004). Multivariate testing of the subgroup of pts with adenocarcinoma showed that cytoplasmic β-catenin (p<0.001) and pleural invasion (p=0.017) were associated with worse prognosis. The present results indicate a negative prognostic significance of PC and cytoplasmic β-catenin expression in NSCLC and a negative prognostic significance of cytoplasmic β-catenin expression in adenocarcinoma.

Key words: non-small cell lung cancer; primary cilia; membranous β-catenin expression; cytoplasmic β-catenin expression; CD8+ tumor-infiltrating lymphocytes

Despite significant advances in systemic treatment, lung cancer holds a leading position in the incidence and mortality of cancer [1]. Histopathologically, lung carcinoma is principally categorized into small-cell lung carcinoma (SCLC) or non–small cell lung carcinoma (NSCLC). The former is a type of highly aggressive neuroendocrine neoplasm of the lung, and the latter mainly comprises two main groups: adenocarcinoma (ACA) and squamous cell carcinoma (SQCC) [2, 3].

Cilia are traditionally classified as motile or primary. Motile cilia are restricted to specific well-differentiated epithelial cells, including the airway, brain ventricles, and oviduct epithelia [4]. Primary cilia (PC) are nonmotile, solitary, antenna-like structures that mediate signaling sensation and transduction in many different tissues and cells [4, 5]. PC are regarded as the regulatory hub of cell functions due to their indispensable roles in several signaling pathways, such as Wnt, Hedgehog, and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways [6, 7].

β-catenin is a pivotal component of the Wnt signaling pathway [8] that comprises 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 [9]. The canonical aspect of the Wnt signaling pathway is mediated by β-catenin, which, upon activation, translocates to the nucleus [10]. The Wnt/β-catenin signaling accelerates lung tumorigenesis *in vivo* [11]. The Wnt/β-catenin pathway cross-talks with the Hedgehog signal

transduction. The primary cilium is proposed to function as the Hedgehog transduction [12]. Abnormal activation of the Hedgehog signaling is involved in cellular proliferation and differentiation, invasion and metastasis, apoptosis, and angiogenesis, leading to tumorigenesis, including medulloblastoma and basal cell carcinoma [13]. Smoothened (Smo) is a transmembrane protein that is a key component of the Hedgehog signaling pathway. The temporary localization of Smo to the PC activates the Hedgehog signaling pathway [14]. There is an increasing prevalence of aberrant activation of Hedgehog signaling in lung cancer patients [15].

Cancer cells communicate with multiple nontumor cell types in the tumor microenvironment, such as lymphocytes. Lymphocytes do not morphologically have PC but form immunological synapses, referred to as "frustrated cilia", to destroy tumorigenic cells [5]. The immune synapse is a temporary interface between an antigen-presenting or cancer cell and the effector lymphocyte [16]. One mechanism by which cancer cells limit the formation of the immune synapse is via upregulation of programmed death-1 ligand (PD-L1) and subsequent ligation to programmed death protein-1 receptor (PD-1) on CD8+ tumor-infiltrating lymphocytes (TIL) [17]. The introduction of immune checkpoint inhibitors, which target PD-1 or its ligand PD-L1 treatment, led to improvement in overall survival in NSCLC [18, 19].

PC are microtubule-based organelles containing an anti-acetylated tubulin-α positive axoneme that requires disassembly before cells can enter mitosis [20, 21]. Histone deacetylase 6 (HDAC6) primary deacetylating targets are nonhistone substrates, particularly anti-acetylated α-tubulin, which is a major component of the cell cytoskeleton [20, 22, 23]. HDAC6 plays a key role in mediating the disassembly of PC during the G0 or G1 cell cycle phases [20, 21].

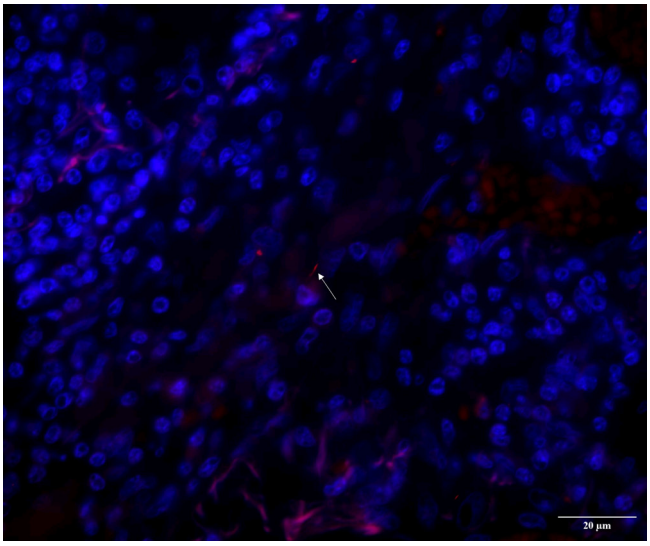

Figure 1. Primary cilia of cells of lung adenocarcinoma immunofluorescently labeled using anti-acetylated tubulin-α antibody and cell nuclei labeled using DAPI. The images were taken on an Axio Observer 7 fluorescence microscope ZEISS. Magnification 63×.

Recently, the close association between PC and cancer growth has emerged as an intriguing topic drawing great attention [5, 6, 24]. However, few studies have systematically investigated the role of PC in different types of cancer [5].

Although PC comprise a number of oncogenic molecules (including Smo, KRAS, EGFR, and PDGFR), their precise role in cancer remains unclear. Indeed, dependent on the nature of the oncogenic driver mutation, PC can have opposing roles in tumorigenesis even in the same tumor type [25], for example, in medulloblastoma [26]. Hence, uncertainty remains over the precise role of PC in cancer, which is probably context-dependent [25].

The objective of this study was to investigate the prognostic significance of the frequency of PC, β-catenin expression, CD8+ TIL, and other biomarkers that could be related to the presence of PC, β-catenin expression, and CD8+ TIL density, including the expression of PD-1, Smo, and HDAC6 in patients with NSCLC.

Patients and methods

Patients. In the present investigation, we retrospectively evaluated tumor tissue blocks of 218 NSCLC patients (pts), including 125 pts (67 females and 58 males) with ACA, median age 66 (range 26–77) years, and 93 pts (28 females and 65 males) with SQCC, median age 69 (range 48–88) years (Table 1). The study was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer University Hospital at its meeting on 10.4.2019 (Docket No. G-19-13). All patients were treated at the Thomayer University Hospital, Prague, Czech Republic. All the tumor samples used in this study were specimens obtained from lung resections of primary tumors rather than from metastases or biopsies.

Immunofluorescence. Primary cilia of cells were demonstrated by immunofluorescence using an anti-acetylated tubulin-α antibody, and the nuclei of the cells were visualized using DAPI labeling. The percentage of primary cilia on cells was counted as a primary cilium to the cell nuclei ratio as described previously [27] (Figure 1).

Immunohistochemistry. An indirect immunohistochemistry method using monoclonal mouse anti-human β-catenin-1 antibody (Agilent #M3539, RRID:AB_2086135) (Figure 2A), mouse monoclonal primary antibody against CD8+ (Agilent #M7103, RRID:AB_2075537) (Figure 2B), mouse monoclonal primary antibody against PD-1 (Cell Marque #315M-94, RRID:AB_1160822) (Figure 2C), polyclonal rabbit anti-Smo antibody (Abcam #ab113438, RRID:AB_10890999) (Figure 2D), and monoclonal primary antibody against HDAC6 (Santa Cruz Biotechnology #sc-28386, RRID:AB_627708) (Figure 2E) was used. Immunohistochemistry was scored semiquantitatively, as shown in Table 1.

Statistics. Recorded pts' characteristics and parameters of their diagnosis were evaluated using descriptive statis-

tics. Categorical variables were described 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 Chi-Square test. For the comparison of continuous variables, the Mann-Whitney test was used. Overall survival (OS) was assessed using Kaplan-Meier analysis for estimating survival probabilities and the Cox proportional hazards model for identifying prognostic factors. OS was defined as the interval from the date of diagnosis to the date of death from any cause or the date of last recorded contact with the patient. The Cox model was used for variables that met its assumptions, initially for each variable separately. Variables with a p-value below 0.1 were subsequently included in a multivariate model using stepwise selection, with a significance level set at 0.05 for variable inclusion and exclusion. Hazard ratios (HR) were supplemented with 95% confidence intervals (CI), and the

Table 1. Patients and tumor characteristics by the histological type.

Parameter		Adenocarcinoma (N=125)	Squamous cell carcinoma (N=93)	p-value*
Gender	Female	67 (53.6%)	28 (30.1%)	<0.001
	Male	58 (46.4%)	65 (69.9%)	
Age at diagnosis (years)	Mean	65.1	62.9	<0.001
	SD	8.57	6.33	
	Median	66.00	64.00	
	Range	26.00–77.00	40.00–75.00	
Primary tumor site	Upper	72 (57.6%)	37 (40.2%)	<0.001
	Lower	39 (31.2%)	35 (38.0%)	
	Middle	9 (7.2%)	20 (21.7%)	
	2 lobes	5 (4.0%)		
	Not determined		1	
Clinical stage	I–II	81 (65.9%)	62 (66.7%)	0.900
	III–IV	42 (34.1%)	31 (33.3%)	
	Not determined	2		
Histological grade	1–2	40 (34.2%)	53 (58.2%)	<0.001
	3–4	77 (65.8%)	38 (41.8%)	
	Not determined	8	2	
Vascular invasion	No	23 (20.4%)	34 (36.6%)	0.010
	Yes	90 (79.6%)	59 (63.4%)	
	Not determined	12		
Pleural invasion	0–1	105 (88.2%)	88 (96.7%)	0.026
	2–3	14 (11.8%)	3 (3.3%)	
	Not determined	6	2	
EGFR mutation	No	94 (79.0%)		
	Yes	25 (21.0%)		
	Not determined	6	93	
Primary cilia	No	57 (64.8%)	28 (31.5%)	<0.001
	Yes	31 (35.2%)	61 (68.5%)	
	Not determined	37	4	
ß-catenin membranous	< 50%	5 (5.1%)	11 (13.8%)	0.045
	≥ 50%	93 (94.9%)	69 (86.3%)	
	Not determined	27	13	
β-catenin cytoplasmatic	<25%	85 (86.7%)	43 (53.8%)	<0.001
	25–75%	13 (13.3%)	37 (46.3%)	
	Not determined	27	13	
β-catenin nuclear	< 5%	88 (97.8%)	77 (96.3%)	0.556
	5–25%	2 (2.2%)	3 (3.8%)	
	Not determined	35	13	
Score CD8+ TIL	<25%	69 (70.4%)	52 (65.0%)	0.437
	25–50%	29 (29.6%)	27 (33.8%)	
	Not determined	27	13	

Table 1. ***Continued*** **...**

Parameter		Adenocarcinoma (N=125)	Squamous cell carcinoma (N=93)	p-value*
PD-1	<25%	64 (71.9%)	45 (55.6%)	0.026
	25–75%	25 (28.1%)	36 (44.4%)	
	Not determined	36	12	
SMO cytoplasmatic	<50%	50 (53.2%)	18 (19.8%)	<0.001
	≥50%	44 (46.8%)	73 (80.2%)	
	Not determined	31	2	
SMO cell nucleus	<5%	58 (63.0%)	64 (70.3%)	0.296
	5–25%	34 (37.0%)	27 (29.7%)	
	Not determined	33	2	
HDAC6	<5%		76 (81.7%)	
	5–50%		17 (18.3%)	
	Not determined	125		
Recurrence	No	58 (55.2%)	71 (78.0%)	<0.001
	Yes	47 (44.8%)	20 (22.0%)	
	Not determined	20	2	

*p-value of Chi-square test for categorical parameters or Mann-Whitney test for continuous parameters

Table 2. Results of univariate Cox PH models for overall survival of 218 patients.

Parameter	Group	Reference group	Hazard ratio	p-value
Gender	Male	Female	1.151	0.461
Clinical stage	III–IV	I–II	2.311	<0.001*
Histological type	SCC	AC	1.069	0.742
Histological grade	3–4	1–2	1.349	0.137
Vascular invasion	Yes	No	1.282	0.302
Pleural invasion	2–3	0–1	1.698	0.065
Primary tumor site	Upper	2 lobes	1.437	0.546
	Lower	2 lobes	1.598	0.442
	Middle	2 lobes	1.307	0.678
EGFR mutation	Yes	No	1.18	0.558
Score CD8+TIL	<25%	≥25%	1.398	0.171
PD-1	<25%	25–75%	1.598	0.058
HDAC6	5–50%	<5%	1.217	0.661
SMO cystoplasmatic	<50%	≥50%	1.237	0.313
SMO cell nucleus	<5%	5–25%	1.165	0.524
β-catenin membranous	<50%	≥50%	1.529	0.209
β-catenin cytoplasmic	25–75%	<25%	1.851	0.009*
β-catenin cell nucleus	< 5%	5–25%	3.71	0.197
Primary cilia	Yes	No	1.653	0.026*

Note: *statistical significance identifier

Table 3. Results of multivariate selective Cox PH model for overall survival in 218 patients.

Parameter	Group	Reference group	Hazard ratio	95% confidence interval	p-value
Clinical stage	III–IV	I–II	2.144	1.354-3.395	0.001
Primary cilia	Yes	No	1.757	1.100-2.806	0.018

Note: Cox PH model with stepwise selection with entry and drop significance level 0.05; initially, all parameters with significance less than 0.1 in the univariate model were included

statistical significance of the HRs was assessed using the Wald test. Kaplan-Meier survival curves were plotted with a log-rank test evaluating differences between categories. Statistical significance was determined at a level of $\alpha=0.05$.

Results

Descriptive statistics of the investigated biomarkers in all groups of 218 pts with NCSLC are summarized in Table 1. At the time of the last follow-up update of the whole group of 218 pts with NCSLC, 113 pts were alive and 105 pts had died. For each histologic group separately, the recorded characteristics of the pts were evaluated using the methods of descriptive statistics.

In the whole group of 218 pts with NSCLC, OS was significantly shorter in pts with present PC than in pts without PC (p=0.0244; Figure 3) and in pts with higher cytoplasmic β-catenin expression (25–75%) than with lower cytoplasmic β-catenin expression (<25%; p=0.0082; Figure 4). In the univariate Cox proportional hazard models HR (95% CI) was 1.653 in pts with present PC (p=0.026) and 1.851 in pts with higher cytoplasmic β-catenin (25–75%; p=0.009; Table 2). Multivariate testing of the whole group of pts with NSCLC showed that the presence of PC was associated with a worse prognosis (Table 3).

The pts were divided according to histological subtype into the ACA subgroup (125 pts) and the SQCC subgroup (93 pts).

In the subgroup of 125 pts with ACA, OS was significantly longer in pts with higher membranous β-catenin expression (≥50%) than in pts with lower expression (<50%; p=0.0300; Figure 5), and OS was significantly shorter in pts with higher cytoplasmic β-catenin expression (25–75%) than in pts with

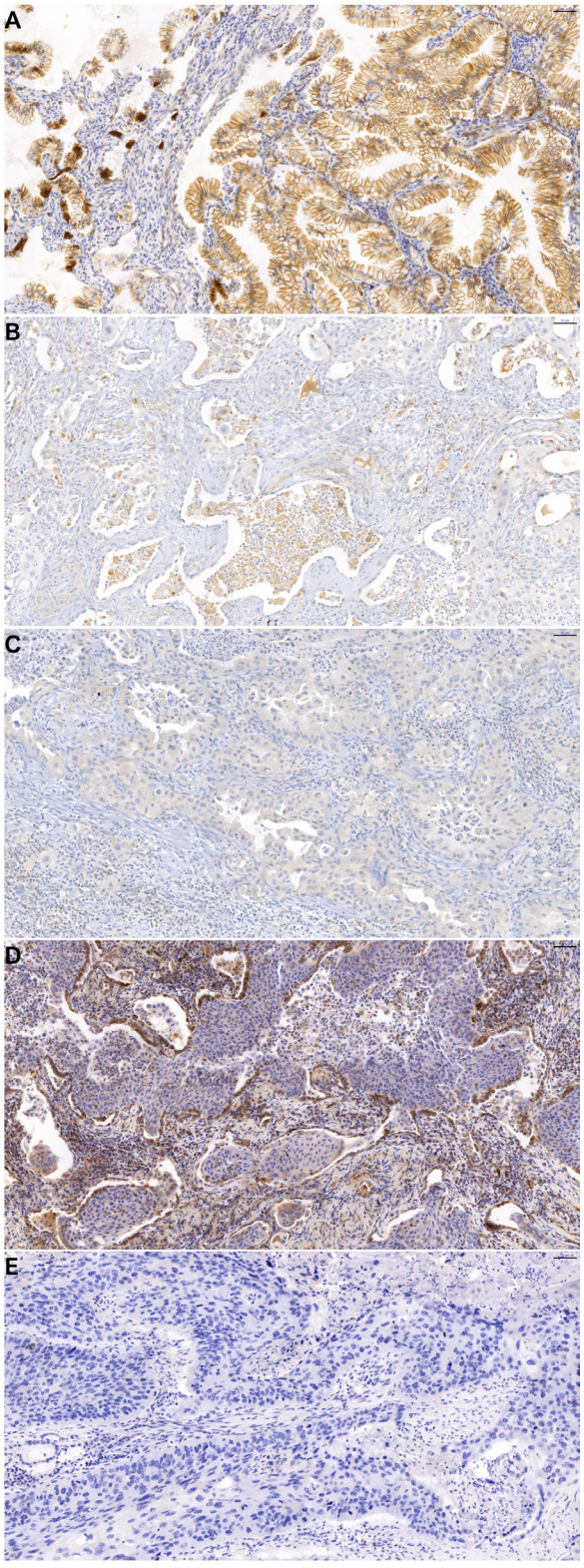

Figure 2. A) β-catenin expression in lung adenocarcinoma. B) CD8+ expression of cytotoxic T lymphocytes using human antibody in lung squamous cell carcinoma. C) PD-1 expression in lung adenocarcinoma. D) SMO expression in lung squamous cell carcinoma. E) HDAC6 expression in lung squamous cell carcinoma. Magnification 20×.

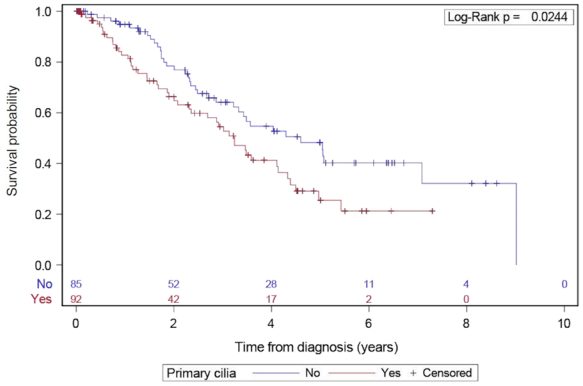

Figure 3. Kaplan-Meier survival plot of primary cilia presence groups in 218 patients.

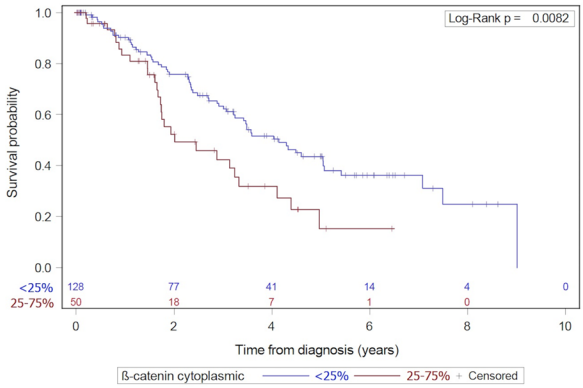

Figure 4. Kaplan-Meier survival plot of cytoplasmic β-catenin groups in 218 patients.

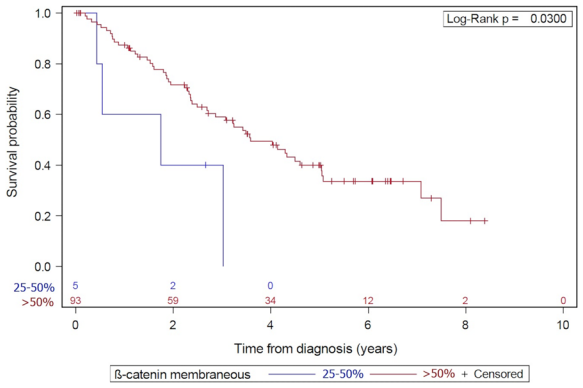

Figure 5. Kaplan-Meier survival plots of membranous β-catenin for adenocarcinoma.

lower expression (<25%; p=0.0004; Figure 6). The univariate Cox proportional hazard models HR (95% CI) was 2.990 in pts with membranous β-catenin expression (25–50%; p=0.039), 3.159 in pts with cytoplasmic β-catenin expression (25–75%; p<0.001), 1.684 in pts with histological grade 3–4 (p=0.057) and 2.031 in pts with pleural invasion 2–3 (p=0.026; Table 4). Multivariate testing of the subgroup of

Table 4. Results of Cox PH models with one variable for adenocarcinoma.

Parameter	Group	Reference group	Hazard ratio	p-value
Clinical stage	III–IV	I–II	2.130	0.002*
Histological grade	3–4	1–2	1.684	0.057*
Vascular invasion	Yes	No	1.292	0.422
Pleural invasion	2–3	0–1	2.031	0.026*
Primary tumor site	Upper	2lobes	1.387	0.587
	Lower	2lobes	1.287	0.684
	Middle	2lobes	1.781	0.419
EGFR mutation	Yes	No	1.180	0.558
Score CD8+	<25%	≥25%	1.053	0.863
PD-1	<25%	25–75%	1.533	0.193
SMO cystoplasmatic	25–50%	>50%	1.456	0.178
β-catenin membranous	25–50%	>50%	2.990	0.039*
β-catenin cytoplasmic	25–75%	<25%	3.159	< 0.001*
β-catenin cell nucleus	<5%	5–25%	1.992	0.499
Primary cilia	Yes	No	1.585	0.120

Note: *parameter included in multivariate analysis

Table 5. Results of multivariate selective Cox PH model for adenocarcinoma.

Parameter	Group	Reference group	Hazard ratio	Confidence interval	p-value
Pleural invasion	2–3	0–1	2.768	1.201–6.381	0.017
β-catenin cytoplasmic	25–75%	<25%	4.411	2.092–9.3	<0.001

Notes: Cox PH model with stepwise selection with entry and drop significance level 0.05; initially, all parameters with significance <0.1 in the univariate model were included

Table 6. Results of Cox PH models with one variable for squamous cell carcinoma.

Parameter	Group	Reference group	Hazard ratio	p-value
Gender	Male	Female	1.814	0.124
Age at the time of diagnosis			1.003	0.908
Clinical stage	III–IV	I–II	2.636	0.004*
Histological grade	3–4	1–2	1.072	0.837
Vascular invasion	Yes	No	1.308	0.472
Pleural invasion	2–3	0–1	0.923	0.913
Primary tumor site	Upper	Middle	1.306	0.561
	Lower	Middle	1.938	0.128
Score CD8+	<25%	≥25%	2.359	0.046*
PD-1	<25%	25–75%	1.592	0.224
HDAC6	5–25%	<5%	1.217	0.661
SMO cystoplasmatic	25–50%	>50%	0.999	0.998
SMO cell nucleus	<5%	5–25%	0.678	0.354
β-catenin membranous	5–25%	>25%	1.209	0.679
β-catenin cytoplasmic	25–75%	<25%	1.489	0.280
Primary cilia	Yes	No	1.930	0.124

Note: *parameter included in multivariate analysis

pts with ACA showed that cytoplasmic β-catenin and pleural invasion were associated with worse prognosis (Table 5).

In the subgroup of 93 pts with SQCC, OS was significantly shorter in patients with lower CD8+ TIL score (<25%) than in pts with higher (≥25%) CD8+ TIL score (p=0.0401; Figure 7). In the univariate Cox proportional hazard models, HR (95% CI) with SQCC was 2.359 in pts with lower CD8+ TIL score (<25%) than in pts with higher CD8+ TIL score (≥25%; p=0.046; Table 6). Multivariate testing of the subgroup of pts with SQCC showed that only the clinical stage was associated with the prognosis.

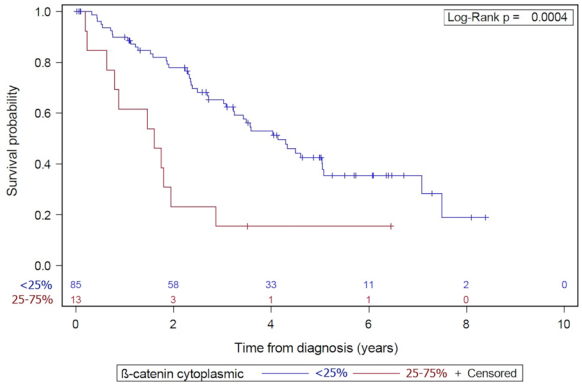

Figure 6. Kaplan-Meier survival plots of cytoplasmic β-catenin for adenocarcinoma.

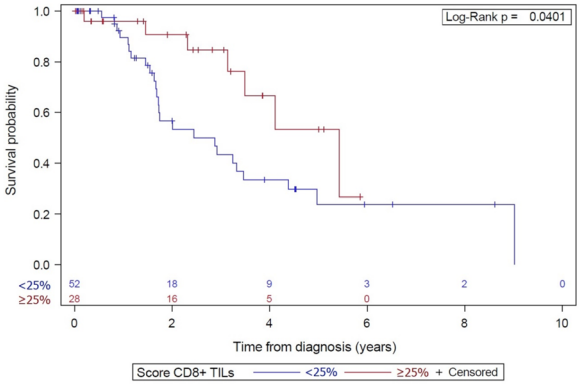

Figure 7. Kaplan-Meier survival plots of score CD8+ TIL for squamous cell carcinoma.

Discussion

As far as we know, the present study is the first to demonstrate the prognostic significance of the presence of PC in NSCLC.

Surgical resection is a principal curative treatment modality of NSCLC. In the present study, all tumor samples were obtained from lung resection specimens of primary tumors, which produced both a sufficient amount of histological material for research purposes and, at the same time, enough histological material for the investigation of predictive biomarkers for target therapy.

PC have already been demonstrated sporadically in normal lung cells by electron microscopy [4] and in a low

percentage in lung ACAs and SQCC cells by immunohistochemical analysis using an antibody against ARL13B, a marker of PC [3]. In our study, we used a different methodology of immunofluorescence using an anti-acetylated tubulin-α antibody. In our study, we also evaluated the prognostic significance of the presence of PC.

The negative prognostic significance of the presence of PC in NSCLC is consistent with the results of our previous study in pts with clear cell renal cell carcinoma [28].

The negative prognostic significance of cytoplasmic β-catenin expression in NSCLC is in agreement with the meta-analysis of 24 studies published between 2000 and 2016, including 2,807 pts with NSCLC. According to this meta-analysis, reduced membranous β-catenin, positive expression of cytoplasmic or nuclear β-catenin were all correlated with a poor prognosis, although this meta-analysis did not identify a significant association between abnormal β-catenin expression and clinical outcome of NSCLC pts [29]. In the present study, we found a negative prognostic significance of cytoplasmic β-catenin expression in NSCLC and a negative prognostic significance of cytoplasmic β-catenin expression in ACA lung cancer. The negative prognostic significance of cytoplasmic β-catenin expression in NSCLC is also consistent with the results of our previous study conducted using the same methodology in pancreatic cancer in which we reported the negative prognostic significance of cytoplasmic β-catenin expression and favorable prognostic significance of membranous β-catenin expression [30].

Beta-catenin has been shown to interact with EGFR [31]. The activation of β-catenin can induce resistance to EGFR tyrosine kinase inhibitors [32]. In NSCLC, there is a positive correlation between activated EGFR mutation and nuclear accumulation of β-catenin [31, 33], but in the present study, we did not observe the interaction between β-catenin and EGFR mutation status.

The favorable prognostic significance of high CD8+ TIL density found in this study in SQCC has already been repeatedly demonstrated across the spectrum of different primary tumors [34, 35], including NSCLC [36, 37]. In the literature, a more significant favorable prognostic impact of CD8+ TIL density on NSCLC survival is reported in the histological type of lung SQCC than in lung ACA [37]. In this study, the favorable effect on survival was statistically significant only in the histological type of lung SQCC, in the histological type of lung ACA, the effect of CD8+ TIL density on survival was not statistically significant, which is probably due to the limitation of the group size of 125 pts.

The prognostic significance of other evaluated NSCLC biomarkers that could be related to the presence of PC and β-catenin expression, including the expression of PD-1, Smo, and HDAC6, could not be demonstrated.

This study highlights the importance of PC in predicting the prognosis of NSCLC; however, more studies in the enlarged group of pts should be evaluated. Among the study limitations are the relatively small patient cohort, the retrospective design as well as the limitations associated with the use of immunohistochemical and immunofluorescent methods. The patient cohort was heterogeneous with regard to the stage and location of the primary tumor, and in pts with ACA, with regard to the subsequent therapy.

In conclusion, the data of the present study indicate a negative prognostic significance of PC and cytoplasmic β-catenin expression in NSCLC, a negative prognostic significance of cytoplasmic β-catenin expression in ACA lung cancer, and a positive prognostic significance of CD8+ TILs in SQCC. The present study results suggest a potential role for PC as a biomarker in NSCLC.

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