Association of the combined parameters including the frequency of primary cilia, PD-L1, Smoothened protein, membranous β-catenin and cytoplasmic β-catenin expression with the outcome of patients with clear cell renal cell carcinoma

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Aims. The objective of this study was to investigate the association and combined prognostic significance of the PD-L1, Smoothened protein and β-catenin expressions in patients with clear cell renal cell carcinoma (ccRCC).
Methods. The PD-L1, Smoothened protein and β-catenin expression were evaluated in 104 ccRCC patients. All studied tumor samples were acquired from nephrectomy specimens of primary tumors and not from biopsies or metastases. An indirect immunohistochemistry using polyclonal rabbit anti-Smoothened antibody, monoclonal mouse anti-human β-catenin-1 antibody, immunohistochemical assay PD-L1 28-8 pharmDx using monoclonal rabbit anti-PD-L1 antibody and anti-VHL (C- terminal) rabbit antibody was used. Immunohistochemistry was scored semiquantitavely.
Results. Median overall survival (OS) was significantly better in patients with lower PD-L1 expression (≤5%), Smoothened protein (SMO) expression (<5%) or cytoplasmic β-catenin expression (≤75%) than in patients with higher expressions of these biomarkers (P<0.001, P=0.047, and P<0.001, respectively). Membranous β-catenin showed an opposite effect with its lower expression (≤75%) being associated with longer OS (P=0.020). There was significant association between PD-1 and PD-L1 expression (P=0.007) and significant association of tumor grade (WHO 2016) with membranous β-catenin (P<0.001), cytoplasmic β-catenin (P=0.005), pVHL (P=0.042), PD-L1 (P=0.049) and PD-1 (P=0.028) expression.
Conclusion. The present study provides the first data on the potential association and combined prognostic significance of frequency of primary cilia, PD-L1, Smoothened protein and β-catenin expression with the outcome in clear cell renal cell carcinoma.

Key words: clear cell renal carcinoma, primary cilia, programmed cell death protein ligand 1, smoothened protein, membranous β-catenin, cytoplasmic β-catenin

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INTRODUCTION

Sensory primary cilia are expressed by most mammalian cell types and exist as a singular cytosolic compartment, often projected into the extracellular environment[1-3]. There are several signal pathways active in primary cilia including Hedgehog and Wnt (ref.[4,5]). Primary cilium is considered to represent a functional homologue of the immune synapse due to morphological and functional similarities in architecture[6].

In our previous study on patients with clear cell renal cell carcinoma (ccRCC) we reported the negative prognostic significance of higher frequency of primary cilia, higher frequency of intratumoral CD8+ tumor infiltrating lymphocytes (TIL) expression and higher programmed cell death-1 (PD-1) cells expression[7]. In the present study we investigated the prognostic significance of several other biomarkers of the ccRCC microenvironment.

We hypothesized that negative prognostic significance of primary cilia observed in our previous study with ccRCC could be associated with the cancer reactivation of sonic hedgehog signalization[7,8]. Therefore, in the present study, we examined the expression of the protein Smoothened, which is a key component of the

Hedgehog signaling pathway. At the molecular level, it has been shown that the Hedgehog signaling drives the progression of cancers by regulating cancer cell proliferation, malignancy, metastasis, and the expansion of cancer stem cells[9].

The primary cilium and its basal body may function as a regulatory switch to control the canonical (β-catenin dependent) and non-canonical (β-catenin independent) Wnt signaling pathways[10]. Ciliogenesis itself is thought to induce a switch from canonical signaling towards non-canonical Wnt signaling[2]. It has been reported that the primary cilium is a negative regulator of canonical Wnt signaling by sequestering β-catenin away from the nucleus[11]. As canonical Wnt signaling is classically considered to promote proliferation while non-canonical Wnt signaling is thought to promote differentiation, this may represent a mechanism through which the primary cilium can regulate differentiation[2].

Given that the therapy with immune checkpoint inhibitors has been approved in renal cell carcinoma, it is important to know the immunologic status in the tumor microenvironment of ccRCC with the relevance to cancer progression[12,13]. One mechanism by which cancer cells limit T cell activation is via upregulating PD-L1 expression in cancer cells.

The von Hippel-Lindau (*VHL*) tumor suppressor gene encoding the VHL protein (pVHL) is inactivated frequently in sporadic ccRCCs (ref.[14]). However, the prognostic significance of *VHL* gene alteration has not been well defined[15]. ccRCC cell lines with or without reconstitution of wild-type *VHL*, show that pVHL contributes to primary cilia maintenance and stability[16,17].

The aim of this study was to investigate the potential association and combined prognostic significance of the Smoothened protein, β-catenin, PD-L1 and pVHL expression in patients with ccRCC.

METHODS

Patients

In a previous study we reported a negative prognostic significance of primary cilia, CD8+ tumor infiltrating lymphocytes (TIL) and PD-1 cells expression in 104 patients with ccRCC (ref.[7]). In the present investigation we used this prior cohort of patients (Table 1) with already determined frequency of primary cilia, CD8+ TIL and PD-1 cells expression and added the examination of the Smoothened protein, membranous β-catenin, cytoplasmic β-catenin, β-catenin in cell nucleus, PD-L1 and pVHL expression (Table 2). All patients were treated at the Thomayer Hospital, Prague, Czech Republic. All studied tumor samples were acquired from nephrectomy specimens of primary tumors and not from biopsies or metastases. The study was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital (Docket No. A-18-19). Patients' informed consents have been gained.

Immunohistochemistry

An indirect immunohistochemistry using polyclonal rabbit anti-Smoothened antibody (clone ab113438, Abcam, Cambridge, United Kingdom) (Fig. 1A), monoclonal mouse anti-human β-catenin-1 antibody (clone β-catenin-1, Dako, Glostrup, Denmark) (Fig. 1B, 1C), immunohistochemical assay PD-L1 28-8 pharmDx using monoclonal rabbit anti-PD-L1 antibody (clone 28-8, Dako, Glostrup, Denmark) (Fig. 1D) and anti-VHL (C- terminal) rabbit antibody (Sigma-Aldrich, St. Louis, Missouri, USA) (Fig. 1E) was used. Immunohistochemistry was scored semiquantitavely as shown in Table 2.

Immunofluorescence

Primary cilia of cells were demonstrated by immunofluorescence using anti-acetylated tubulin-alpha antibody

Table 1. Characteristics of the cohort.

Characteristics		n	%
Sex	Male	73	70.2
	Female	31	29.8
Age at diagnosis (years)	median (range)	64 (37–82)	
	mean (SD)	62 (10)	
Stage at diagnosis	I	28	26.9
	II	15	14.4
	III	32	30,8
	IV	29	27.9
Vascular invasion	Yes	37	35.6
	No	67	64.4
Tumor grade (WHO 2016)	1	27	26.0
	2	44	42.3
	3	16	15.4
	4	17	16.3
Patient status	Alive	61	58.7
	Died	43	41.3

Table 2. Characteristics of the tumors.

Characteristics		n	%
Without primary cilia[1]	Yes	27	28.1
	No	69	71.9
Frequency of primary cilia[1]	median (range)	0.003 (0.000–0.188)	
	mean (SD)	0.009 (0.022)	
SMO[2]	<5%	27	27.0
	5–25%	42	42.0
	26–50%	16	16.0
	51–75%	12	12.0
	>75%	3	3.0
Membranous β-catenin[3]	<5%	2	2.0
	5–25%	3	3.0
	26–50%	7	7.0
	51–75%	32	32.0
	>75%	56	56.0
Cytoplasmic β-catenin[4]	<5%	0	0.0
	5–25%	12	12.0
	26–50%	34	34.0
	51–75%	36	36.0
	>75%	18	18.0
PD-L1[5]	0%	60	60.0
	1–5%	24	24.0
	6–25%	10	10.0
	>25%	6	6.0
pVHL[6]	<5%	0	0.0
	5–25%	0	0.0
	26–50%	0	0.0
	51–75%	7	7.0
	>75%	93	93.0

[1]No information about primary cilia in 8 patients.
[2]No information about SMO in 4 patients.
[3]No information about membranous β-catenin in 4 patients.
[4]No information about cytoplasmic β-catenin in 4 patients.
[5]No information about PD-L1 in 4 patients.
[6]No information about pVHL in 4 patients.

and the nuclei of the cells were visualised using DAPI labeling (Fig. 1F). The percentage of primary cilia on cells was counted as a primary cilia to cell nuclei ratio as described previously[7].

Statistical analysis

Standard descriptive statistics were used to characterize the sample data set. Categorical variables were described by absolute and relative frequencies; continuous variables were described by median values and mean with standard deviation. Comparison of the categorical parameters was performed using the Fisher exact test. Overall survival (OS) was estimated using the 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. Patients who had not died were censored at the date of last update. Comparison of OS between subgroups of patients was carried out by means of log-rank test. Univariate and multivariate Cox proportional hazards models were used to evaluate the effect of potential prognostic factors on the survival measures. Point estimates of hazard ratio (HR) are shown with 95% confidence intervals. Statistical significance of hazard ratios was assessed by means of the Wald test. As a level of statistical significance α=0.05 was used.

RESULTS

The expression of PD-L1, Smoothened protein, membranous β-catenin, cytoplasmic β-catenin and pVHL, including descriptive statistics is summarized in Table 2. The expression of β-catenin in cell nucleus was negative in all patients. Median overall OS according to PD-L1 expression was significantly shorter in patients with higher expression (>5%) than in patients with lower expression (P<0.001, Fig. 2A). Median OS according to SMO expression was significantly shorter in patients with higher expression (≥5%) than in patients with lower expression (P=0.047, Fig. 2B). Median OS according to membranous β-catenin expression was significantly longer in patients with higher expression (>75%) than in patients with lower expression (≤75%) (P=0.020, Fig. 2C). Median OS according to cytoplasmic β-catenin expression was significantly shorter in patients with higher expression (>75%) than

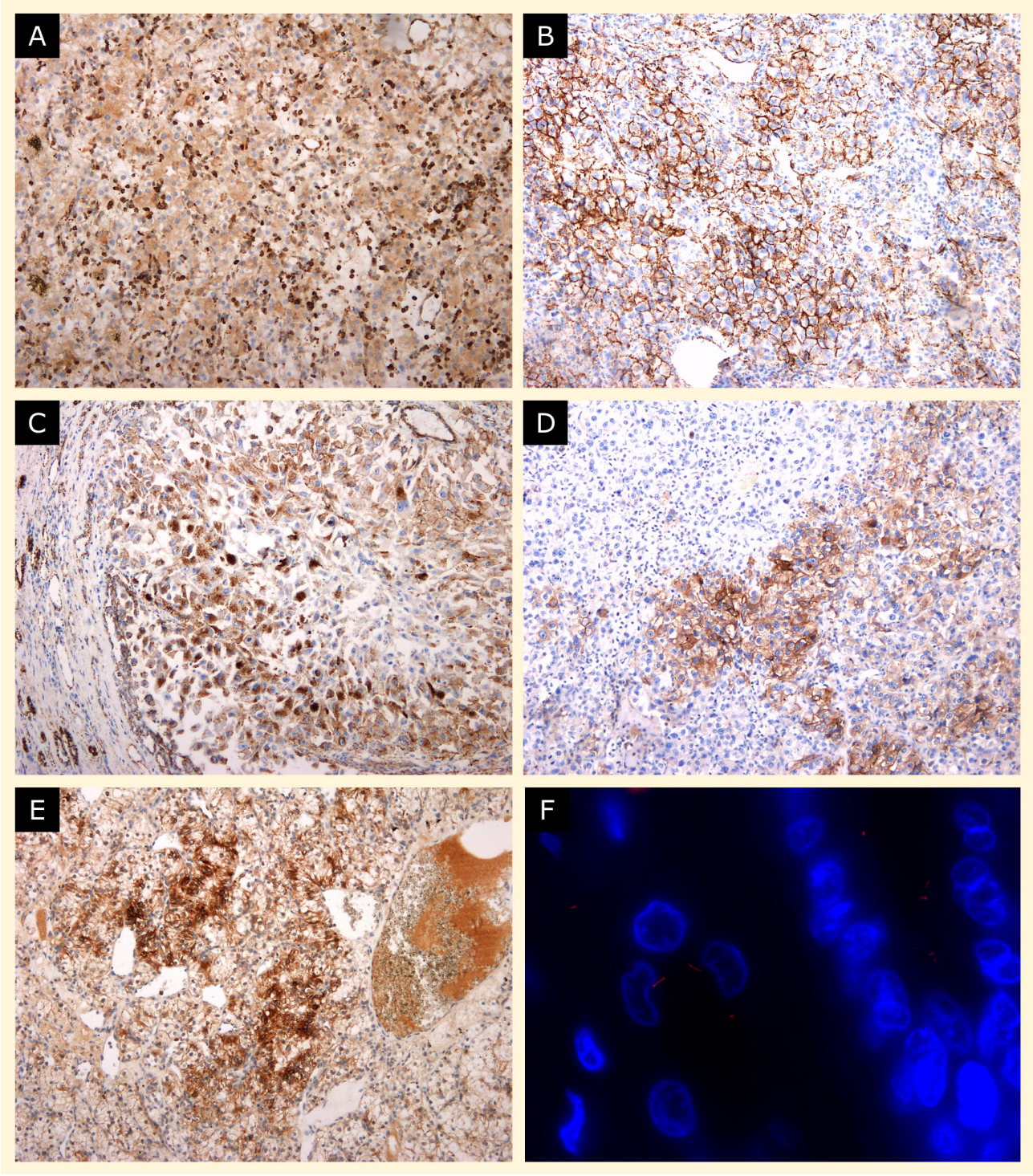

Fig. 1. A. Cytoplasmatic smoothened protein expression in ccRCC with strong positivity in inflammatory cells. Immunohistochemical staining with polyclonal rabbit anti-Smoothened antibody (clone ab113438, Abcam, Cambridge, United Kingdom) was used. Magnification 100x; **B.** Strong complete membranous β-catenin expression in all cells of ccRCC. Immunohistochemical staining with monoclonal mouse anti-human β-catenin-1 antibody (clone β-catenin-1, Dako, Glostrup, Denmark) was used. Magnification 100x; **C.** Intense granular cytoplasmic β-catenin expression in ccRCC, almost complete loss of membranous positivity. Immunohistochemical staining with monoclonal mouse anti-human β-catenin-1 antibody (clone β-catenin-1, Dako, Glostrup, Denmark) was used. Magnification 100x; **D.** Typical membranous PD-L1 expression is detected in third cells of ccRCC. Immunohistochemical assay PD-L1 28-8 pharmDx using monoclonal rabbit anti-PD-L1 antibody (clone 28-8, Dako, Glostrup, Denmark) was used. Magnification 100x; **E.** Focally strong cytoplasmatic Von Hippel-Lindau (VHL) protein expression with partial and weak expression in membrane in ccRCC. Immunohistochemical staining with anti-VHL (C-terminal) rabbit antibody (Sigma-Aldrich, St. Louis, Missouri, USA) was used. Magnification 100x; **F.** Primary cilia of ccRCC cells labeled using anti-acetylated tubulin-alpha antibody and cell nuclei labeled using DAPI. Magnification 100x. Scale bar 10 μm.

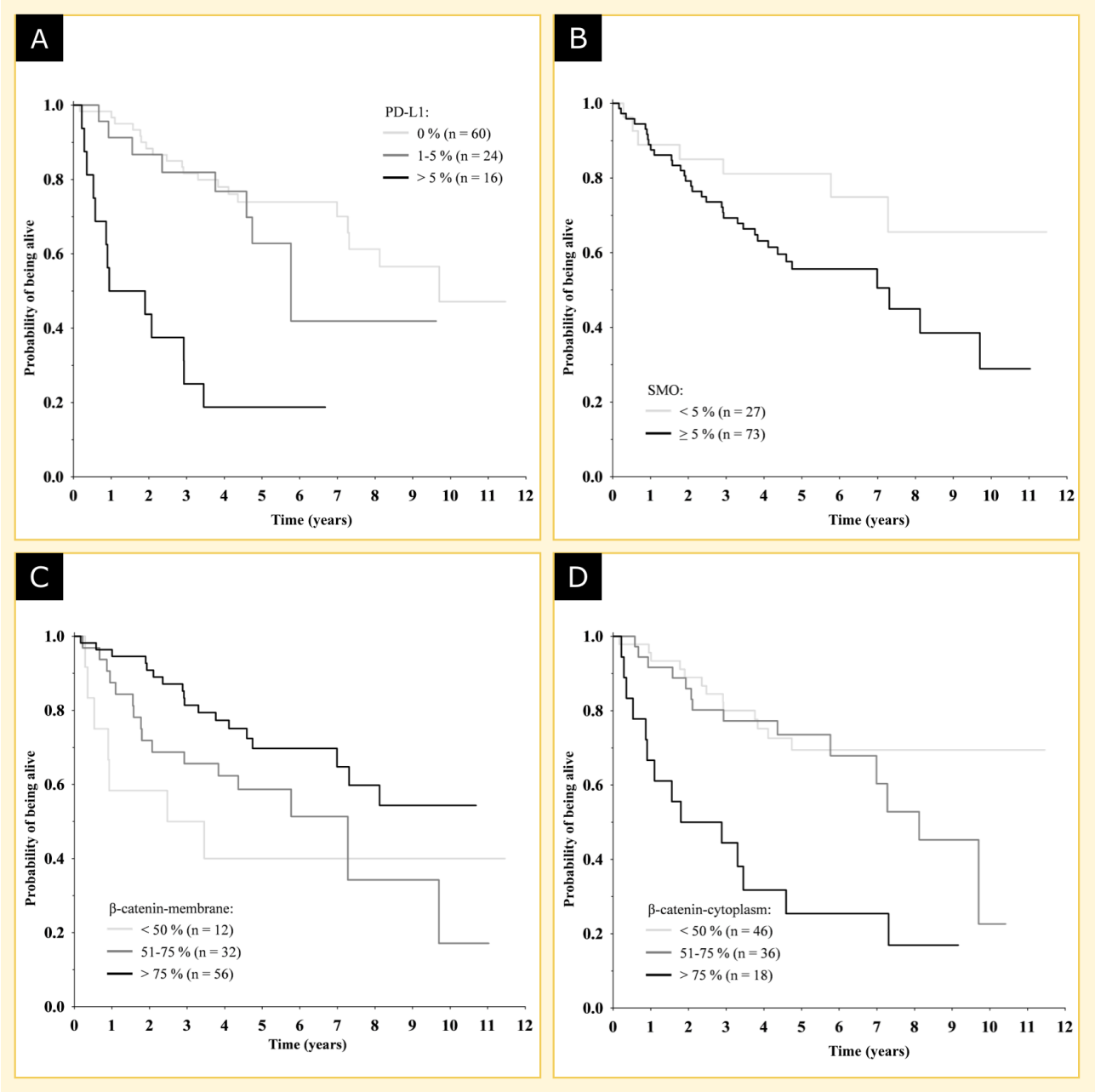

Fig. 2. Overall survival. **A.** Overall survival from diagnosis according to PD-L1 (P<0.001); **B.** Overall survival from diagnosis according to SMO (P=0.047); **C.** Overall survival from diagnosis according to membranous β-catenin (P=0.020); **D.** Overall survival from diagnosis according to cytoplasmic β-catenin (P<0.001).

in patients with lower expression (≤75%) (*P*<0.001, Fig. 2D). There was a trend of longer OS according expression was insignificantly longer in patients with higher pVHL expression (>75%) compared to patients with lower expression (51–75%) (*P*=0.093).

Table 3 shows a significant association between PD-1 and PD-L1 expression (*P*=0.007) and Table 4 shows a significant correlation between PD-1 and intratumoral CD8+ TIL (*P*<0.001) expression. There was significant association of tumor grade (WHO 2016) with membranous β-catenin (*P*<0.001), cytoplasmic β-catenin (*P*=0.005), pVHL (*P*=0.042), PD-L1 (*P*=0.049) and PD-1 (*P*=0.028) and no association of tumor grade with SMO (*P*=0.085) (Table 5).

The univariate Cox proportional hazard models of individual risk factors and combined risk factors are summarized in Table 6 and Table 7. Multivariate testing showed that vascular invasion, primary cilia count, membranous β-catenin, cytoplasmic β-catenin and PD-L1 expression were associated with prognosis (Table 8).

DISCUSSION

The assessment of biomarkers is a crucial component of patient management in medical oncology[18]. Biomarkers are even more important in patients treated with targeted therapy.

Table 3. Relationship between PD-1 and PD-L1.

Characteristics, n (%)		PD-L1 ≤5% (n=84)	PD-L1 >5% (n=16)	*P*-value[1]
PD-1, n (%)	<5%	47 (56.0)	4 (25.0)	
	5–25%	27 (32.1)	5 (31.3)	0.007
	>50%	10 (11.9)	7 (43.8)	

[1]Fisher exact test.

Table 4. Relationship between PD-1 and intratumoral CD8+ TIL.

Characteristics, n (%)		Intratumoral CD8+ TIL <25% (n=63)	Intratumoral CD8+ TIL >25% (n=40)	*P*-value[1]
PD-1, n (%)	<5%	44 (69.8)	7 (17.5)	<0.001
	5–25%	19 (30.2)	15 (37.5)	
	>50%	0 (0.0)	18 (45.0)	

[1]Fisher exact test.

Table 5. Association of tumor grade (WHO 2016) with β-catenin, pVHL, SMO, PD-L1 and PD-1.

Characteristics, n (%)		Tumor grade 1	2	3	4	*P*-value[1]
Membranous β-catenin, n (%)	<5%	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	<0.001
	5–25%	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	
	26–50%	3 (42.9)	0 (0.0)	1 (14.3)	3 (42.9)	
	51–75%	10 (31.3)	8 (25.0)	4 (12.5)	10 (31.3)	
	>75%	13 (23.2)	31 (55.4)	9 (16.1)	3 (5.4)	
Cytoplasmic β-catenin, n (%)	<5%	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.005
	5–25%	7 (58.3)	4 (33.3)	0 (0.0)	1 (8.3)	
	26–50%	9 (26.5)	19 (55.9)	3 (8.8)	3 (8.8)	
	51–75%	10 (27.8)	11 (30.6)	7 (19.4)	8 (22.2)	
	>75%	0 (0.0)	8 (44.4)	6 (33.3)	4 (22.2)	
pVHL, n (%)	<5%	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.042
	5–25%	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
	26–50%	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
	51–75%	1 (14.3)	2 (28.6)	0 (0.0)	4 (57.1)	
	>75%	25 (26.9)	40 (43.0)	16 (17.2)	12 (12.9)	
SMO, n (%)	<5%	12 (44.4)	6 (22.2)	4 (14.8)	5 (18.5)	0.085
	5–25%	9 (21.4)	20 (47.6)	9 (21.4)	4 (9.5)	
	26–50%	4 (25.0)	6 (37.5)	3 (18.8)	3 (18.8)	
	51–75%	1 (8.3)	7 (58.3)	0 (0.0)	4 (33.3)	
	>75%	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	
PD-L1, n (%)	0%	17 (28.3)	29 (48.3)	8 (13.3)	6 (10.0)	0.049
	1–5%	8 (33.3)	9 (37.5)	4 (16.7)	3 (12.5)	
	6–25%	1 (10.0)	3 (30.0)	3 (30.0)	3 (30.0)	
	>25%	0 (0.0)	1 (16.7)	1 (16.7)	4 (66.7)	
PD-1, n (%)	<5%	19 (36.5)	21 (40.4)	8 (15.4)	4 (7.7)	0.028
	5–25%	6 (17.6)	14 (41.2)	8 (23.5)	6 (17.6)	
	26–50%	2 (15.4)	7 (53.8)	0 (0.0)	4 (30.8)	
	>50%	0 (0.0)	2 (40.0)	0 (0.0)	3 (60.0)	

[1]Fisher exact test.

To the best of our knowledge, the present study provides the first information about correlation and prognostic significance of the frequency of primary cilia, Smoothened protein cytoplasmic β-catenin and PD-L1 expression in ccRCC. The limitations of this study are the number of 104 patients, its retrospective nature and the known limitations of immunohistochemical methods.

The cilium has been suggested to function as the Hedgehog transduction hub[10]. In the present study a negative prognostic significance of Smoothened protein expression and the frequency of primary cilia combined with the Smoothened protein expression were observed. The Hedgehog pathway cross talks with the Wnt/β-catenin signal transduction.

Table 6. Survival analysis – univariate Cox proportional hazard model.

Characteristic	Category	n	HR (95% CI)	*P*-value[1]
Frequency of cilia	≤ 0.002	44	1.000	-
	> 0.002	52	5.268 (2.296–12.085)	< 0.001
SMO	< 5 %	27	1.000	-
	5–25 %	42	2.586 (1.098–6.088)	0.030
	26–50 %	16	1.398 (0.443–4.416)	0.568
	> 50 %	15	2.307 (0.801–6.642)	0.121
Membranous β-catenin	< 50 %	12	1.000	-
	51–75 %	32	0.668 (0.274–1.628)	0.375
	> 75 %	56	0.361 (0.150–0.869)	0.023
Cytoplasmic β-catenin	< 50 %	46	1.000	-
	51–75 %	36	1.369 (0.642–2.918)	0.416
	> 75 %	18	4.339 (2.016–9.337)	< 0.001
PD-L1	≤ 5 %	84	1.000	-
	> 5 %	16	5.780 (2.895–11.538)	< 0.001
pVHL	≤ 75 %	7	1.000	-
	> 75 %	93	0.419 (0.147–1.194)	0.103

[1]Wald test.

Table 7. Survival analysis: combined risk factors – univariate Cox proportional hazard model.

Characteristic	n	HR (95% CI)	*P*-value[1]
Frequency of cilia and SMO			
Frequency of cilia >0.002 and SMO >5 %	40	4.578 (2.234–9.380)	<0.001
Frequency of cilia and membranous β-catenin			
Frequency of cilia >0.002 and membranous β-catenin >75%	30	1.165 (0.588–2.307)	0.661
Frequency of cilia ≤0.002 and membranous β-catenin >75%	22	0.209 (0.064–0.689)	0.010
Frequency of cilia and cytoplasmic β-catenin			
Frequency of cilia >0.002 and cytoplasmic β-catenin >75%	10	7.015 (3.236–15.204)	<0.001
Frequency of cilia and PD-L1			
Frequency of cilia >0.002 and PD-L1 ≥5%	8	8.410 (3.625–19.509)	<0.001

[1]Wald test.

B-catenin is rarely mutated in cancer, but mutations of its main protein partners can confer enhanced stability to β-catenin, causing aberrant accumulation[19,20]. In accordance with the literature, we found a negative prognostic significance of the cytoplasmic β-catenin in ccRCC (ref.[21]).

The lack of nuclear β-catenin positivity in the present study is interesting. We do not think this is an artifact. B-catenin is a membrane marker of cell adhesion[19]. Translocation of beta-catenin to the nucleus is a sign of an active Wnt pathway that drives to the epithelial-mesenchymal transition[22].

New information in the present study is the prognostic significance of cytoplasmic β-catenin combined with frequency of primary cilia and favorable prognostic significance of the membranous β-catenin in ccRCC. In the present study we found favorable prognostic significance of membranous β-catenin, negative prognostic significance of cytoplasmic β-catenin and statistically significant association between the frequency of primary cilia and Smoothened protein. We did not observe inverse relationship between intrinsic β-catenin signaling and intratumoral T cell infiltration, which was found in melanoma[23].

Medical therapy of metastatic RCC (mRCC) is currently based on targeted therapy, including immunotherapy and the findings of the present study could suggest some additional targets for future drug development. Current targets in mRCC include the vascular endothelial growth factor receptor (VEGF) and mammalian target of rapamycin (mTOR) pathways and immune checkpoint interactions. Until recently, active agents were administered in mRCC sequentially in a paradigm that has evolved more or less spontaneously in the clinical practice[24]. The paradigm of sequential therapy is currently being replaced by the combination therapy as the primary strategy. While trials combining anti-VEGF agents with mTOR inhibitors have been negative[25], the combinations based on immune checkpoint inhibitors have resulted in improved outcomes[26-28].

Increased tumor cell PD-L1 was associated with shorter survival in patients with metastatic RCC in the multicentric randomized controlled COMPARZ trial[29]. This findings are in agreement with the negative prognostic significance of PD-L1 observed in the present study that reflects the situation before the advent of immune checkpoint inhibitors.

Table 8. Survival analysis – multivariate Cox-proportional hazard model.

Characteristic	Category	n	HR (95% CI)	*P*-value[1]
Sex	females	28	1.000	-
	males	64	1.593 (0.624–4.066)	0.330
Age at diagnosis	<60 yrs	31	1.000	-
	60–69 yrs	41	2.059 (0.844–5.027)	0.113
	≥70 yrs	20	1.698 (0.448–6.432)	0.436
Tumor grade (WHO 2016)	1	26	1.000	-
	2	38	1.032 (0.290–3.677)	0.961
	3	14	1.601 (0.394–6.497)	0.510
	4	14	0.611 (0.127–2.924)	0.537
Vascular invasion	No	59	1.000	-
	Yes	33	4.509 (1.962–10.361)	<0.001
Frequency of cilia	<0.002	42	1.000	-
	>0.002	50	16.487 (4.603–59.054)	<0.001
Membranous β-catenin	≤75%	40	1.000	-
	>50%	52	0.300 (0.122–0.739)	0.009
Cytoplasmic β-catenin	≤75%	78	1.000	-
	>50%	14	4.519 (1.591–12.833)	0.005
SMO	<5%	24	1.000	-
	≥5%	68	2.582 (0.857–7.782)	0.092
Intratumoral CD8+ TIL	<25%	57	1.000	-
	>25%	35	1.194 (0.448–3.183)	0.723
PD-L1	≤5%	77	1.000	-
	>5%	15	15.875 (3.792–66.467)	<0.001
PD-1	<25%	76	1.000	-
	>25%	16	0.354 (0.090–1.383)	0.135

[1]Wald test.

CONCLUSION

In conclusion, the present pilot study demonstrates a statistically significant association between the frequency of primary cilia and Smoothened protein, the frequency of primary cilia and cytoplasmic β-catenin and the frequency of primary cilia and PD-L1 in patients with ccRCC. The data obtained may become the basis and justification for further functional studies and trials of targeted therapies and, moreover, these markers could be potentially used as a biomarkers of disease prognosis.

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