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ORIGINAL ARTICLE

Negative prognostic significance of primary cilia, CD8+ tumor infiltrating lymphocytes and PD1+ cells expression in clear cell renal cancer

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Summary

Purpose: *The aim of this study was to investigate the potential association and combined prognostic significance of the frequency of primary cilia (PC), programmed cell death protein-1 receptor (PD1) and CD8+ tumor infiltrating lymphocytes (TIL) in patients with clear cell renal cancer (ccRCC).*

Methods: *The frequency of PC, PD1 expression and the frequency of intratumoral CD8+ TIL were evaluated in 104 ccRCC patients.*

Results: *The median frequency of PC was 0.003. The expression of PD1+ cells were <5% in 52 patients, 5-25% in 34 patients and 26-50% in 13 patients and >50% in 5 patients. Intratumoral CD8+ TIL were evaluable in all patients: negative in 1 patient, <25% in 63, 26-50% in 29 and >50% in 11 patients. Overall survival (OS) according to the frequency of PC was significantly shorter in patients with higher frequency (≥0.002) than in patients with lower frequency (<0.002) (p<0.001). Median OS was significantly shorter in patients with higher (25%) CD8+ TIL and higher (>25%) PD1+ expression than in patients with lower (<25%) expression (4.6 vs. 97. years, p=0.006 and 2.9 vs. 8.9 years, p=0.006, respectively).*

Conclusions: *The present study provides the first data on the potential association and combined prognostic significance of frequency of PC, PD1+ cells and CD8+ TIL in patients with clear cell renal cancer.*

Key words: *clear cell renal cancer, primary cilia, CD8+ tumor infiltrating lymphocytes, programmed cell death protein 1*

Introduction

Primary cilia (PC) are specialized sensory microtubule-based organelles that project from the surface of almost all human cells except hematopoietic cells [1,2]. PC are considered to represent a functional homologue of the immune synapse due to morphological and functional similarities in architecture [3-5]. The immune synapse is a temporary interface between an antigen-presenting or cancer cell and the effector lymphocyte [6]. One mechanism by which cancer cells limit the

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formation of immune synapse is via upregulation of programmed death-1 ligand (PD-L1) and subsequent ligation to programmed death protein-1 receptor (PD1) on CD8+ tumor infiltrating lymphocytes (TIL) [7,8]. Both microtubule structures, i.e. primary cilia and immune synapses between cytotoxic CD8+ TIL and antigen-presenting or cancer cells, are regularly found in varying amounts in the microenvironment of solid tumors.

Recently we reported the positive prognostic significance of primary cilia in tumor microenvironment in intestinal cancer [9]. Favorable prognostic and predictive significance of high density of CD8+ TIL has been repeatedly demonstrated across a spectrum of different primary tumors [10, 11] with the exception of renal cell carcinoma (RCC), where the high density of CD8+ TIL is a contrastingly negative prognostic factor [12,13]. Similarly as in our previous studies, we have also been interested in the relationship between PC and immune synapses in tumor microenvironment [4,5]. Simultaneous immunohistochemical demonstration of CD8+ TIL and PD-1 expression in tumor is a close approximation of the imaging of immune synapse [4].

Clear cell RCC (ccRCC) is the most common type of kidney cancer. Immunotherapy and targeted therapy have improved the outcome for metastatic ccRCC patients [14,15].

The aim of the present pilot study was to examine the association and correlate the prognostic significance of the frequency of PC, PD1+ cells expression and CD8+ TIL expression in the same group of patients with ccRCC.

Methods

Patients

The presence of PC in cells was retrospectively evaluated in primary tumor tissue blocks of 104 ccRCCpatients (Table 1). All patients were treated at the Thomayer Hospital, Prague, Czech Republic. All studied tumor samples were acquired from nephrectomy specimens. The study was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital.

Immunofluorescence

Direct immunofluorescence was used to demonstrate the presence of PC using anti-acetylated tubulin-alpha antibody (Sigma-Aldrich, USA), followed by a Cy3 conjugated secondary antibody (Sigma-Aldrich, USA). Cell nuclei were stained with DAPI (Sigma-Aldrich, USA). PC analysis was done in a fluorescent microscope Nikon Eclipse Ti (Nikon, Tokyo, Japan). The percentage of primary cilia on cells was counted as a ratio of PC to cell nuclei (Figure 1A) [9,16].

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)	
Clinical 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		
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
Without PC [1]		
Yes	27	28.1
No	69	71.9
Frequency of PC [1]		
Median (range)	0.003 (0.000-0.188)	
Mean (SD)	0.009 (0.022)	
Intratumoral CD8+ TIL		
Negative	1	1.0
<25 %	63	60.6
25–50 %	29	27.9
> 50 %	11	10.6
PD1+ cells		
<5 %	52	50.0
5–25 %	34	32.7
25–50 %	13	12.5
51–75 %	4	3.8
>75 %	1	1.0

[1] No information on primary cilia in 8 patients. PC: Primary cilia; TIL: Tumor infiltrating lymphocytes; PD1: Programmed death protein receptor

Immunohistochemistry

An indirect immunohistochemistry using mouse monoclonal primary antibodies against CD8 (Figure 1B) and PD1 (Figure 1C) was used. Each slide was assessed for quantity of intratumoral CD8+ TIL and for PD1+ cells by an experienced pathologist, not informed of the patient treatment results. The immunohistochemical evaluation of CD8+ TIL and PD1+ cells expression was scored semi-quantitatively. Presence of intratumoral CD8+ TIL expression compared with the total amount of nucleated cells was quantified as follows: 0 - negative CD8+ TIL, 1 - <25% CD8+ TIL, 2 - 25≤50% CD8+ TIL and 3 - >50% CD8+ TIL. The extent of PD1+ cells staining was evaluated as follows: 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%), as described previously [4].

Statistics

Absolute and relative frequencies were used to describe the categorical variables, and continuous variables were described by median values, range and mean

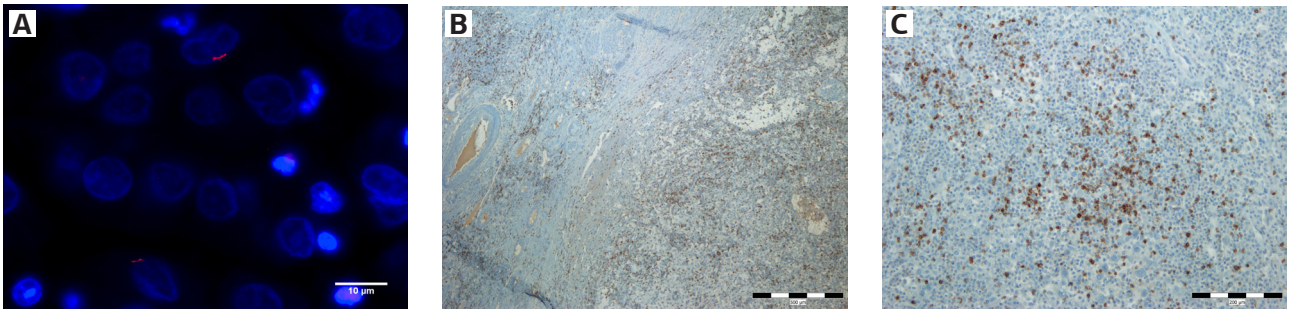

Figure 1. A: Primary cilia of ccRCC cells labeled using anti-acetylated tubulin-alpha antibody and cell nuclei labeled using DAPI. Magnification 100x. Scale bar 10 µm. **B:** CD8+ expression in cytotoxic T lymphocytes detected using human antibody in ccRCC intratumoral areas. Indirect immunohistochemistry using mouse monoclonal primary anti-CD8 antibodies (M7103, Dako, Glostrup, Denmark). Magnification 40x. Scale bar 500 µm. **C:** Immunodetection of PD1+ expression in ccRCC. Indirect immunohistochemistry using mouse monoclonal primary anti-PD1+ antibodies (NAT105, Cell Marque, Darmstadt, Germany) was used. Magnification 100x. Scale bar 200 µm.

Table 2. Prediction of mortality by frequency of primary cilia (diagnostic test)

	Valid N	*Cut-off* [1]	*AUC*	*P value*	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Accuracy*
All patients	96	0.002	0.727	< 0.001	0.821	0.649	0.615	0.841	0.719

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

Table 3. Overall survival from diagnosis according to frequency of primary cilia

Variables	*Frequency of PC*		*p value*[1]
	< 0.002 (n= 44)	*> 0.002 (n= 52)*	
Median OS (95% CI)	Not reached	4.6 year (2.5-6.7)	< 0.001
3-year OS (%; 95% CI)	85.9 (75.4-96.4)	61.5 (48.3-74.8)	
5-year OS (%; 95% CI)	85.9 (75.4-96.4)	46.1 (32.0-60.2)	

[1] Log-rank test. PC: Primary cilia; OS: Overall survival

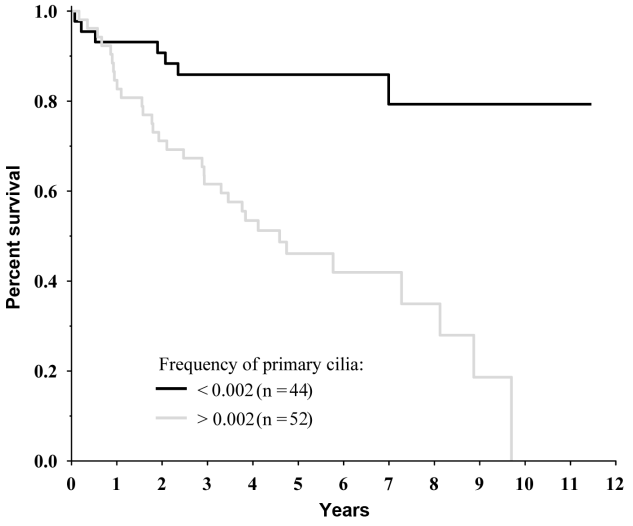

Figure 2. Overall survival from diagnosis according to frequency of primary cilia (p<0.001).

Table 4. Overall survival with univariate Cox proportional hazard model

Characteristics	*Category*	*n*	*HR (95% CI)*	*p value* [1]
Frequency of PC	(continuous - 0.01 change)	96	1.075 (0.989-1.169)	0.090
	≤ 0.002	44	1.000	-
	> 0.002	52	5.268 (2.296-12.085)	< 0.001
Intratumoral CD8+ TIL	< 25 %	63	1.000	-
	25-50 %	29	1.964 (0.990-3.898)	0.054
	> 50 %	11	3.199 (1.396-7.330)	0.006
PD1+ cells	< 5 %	52	1.000	-
	5-25 %	34	0.876 (0.418-1.835)	0.725
	> 25 %	18	2.366 (1.154-4.852)	0.019

[1] Wald test. PC: Primary cilia; TIL: Tumor infiltrating lymphocytes; PD1: Programmed death protein receptor; HR: Hazard ratio

Table 5. Overall survival from diagnosis according to intratumoral CD8+ TIL

Variables	*Intratumoral CD8+ TIL*		*p value* [1]
	< 25 % (n=63)	*> 25 % (n=40)*	
Median OS (95% CI)	9.7 years (6.1-13.3)	4.6 years (2.1-7.1)	0.006
3-year OS (%; 95% CI)	80.4 (70.4-90.3)	62.3 (47.2-77.4)	
5-year OS (%; 95% CI)	76.5 (65.7-87.3)	45.3 (29.3-61.4)	

[1] Log-rank test. TIL: Tumor infiltrating lymphocytes; OS: Overall survival

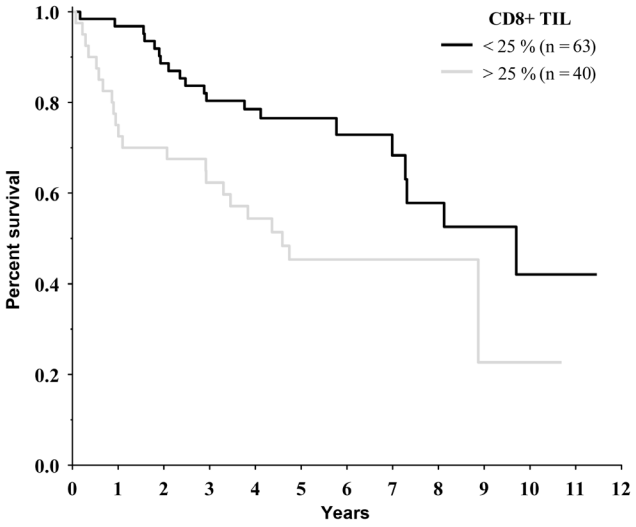

Figure 3. Overall survival from diagnosis according to intratumoral CD8+ TIL (p=0.006).

with standard deviation. Categorical parameters were compared using the Fisher exact test. For continuous variables, Mann-Whitney or Kruskal-Wallis test were used. A ROC analysis was calculated to estimate the optimal cut-off value for 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 assessed using Kaplan Meier method and all point estimates came with 95% confidence intervals (95% CI). OS was defined as the time from diagnosis to death from any cause. Patients still alive were censored at the date of last update. Comparison of OS between subgroups of patients were performed using the Log-rank test. Univariate and multivariate Cox proportional hazards models were used to evaluate the effect of potential prognostic factors on survival. P value<0.05 was considered as statistically significant.

Results

Immunofluorescence and immunohistochemistry

PC were detected in 69 (71.9%) patients. The median frequency of the PC was 0.003 (range0 - 0.188). The expression of PD1+ cells was <5% in 52 patients, 5-25% in 34 patients and 26-50% in 13 patients and >50% in 5 patients. Intratumoral CD8+ TIL were evaluable in all patients: negative in one patient, <25% in 63, 26-50% in 29 and >50% in 11 patients, respectively. Descriptive statistics are summarized in Table 1.

Impact on overall survival

Results of the diagnostic test of the prediction of mortality by frequency of PC is shown in

Table 6. Overall survival from diagnosis according to PD1+ cells

Variables	*PD1+ cells*		*p value* [1]
	< 25 % (n= 86)	*> 25 % (n= 18)*	
Median OS (95% CI)	8.9 years (6.6-11.1)	2.9 years (1.8-4.0)	0.006
3-year OS (%; 95% CI)	77.4 (68.4-86.3)	50.0 (26.9-73.1)	
5-year OS (%; 95% CI)	70.4 (60.4-80.5)	33.3 (11.6-55.1)	

[1] Log-rank test. PD1: Programmed death protein receptor; OS: Overall survival

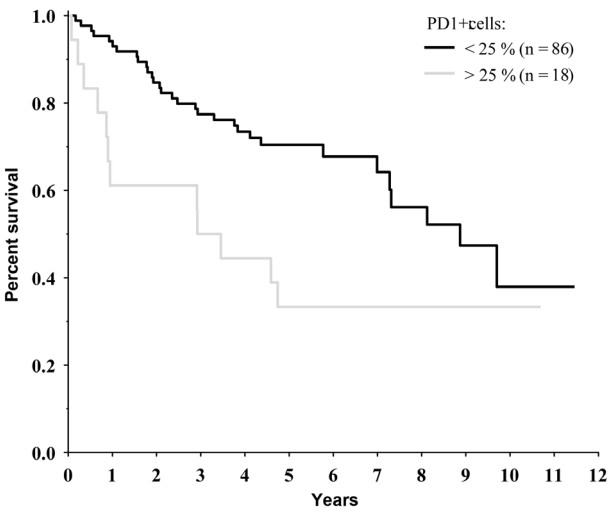

Figure 4. Overall survival from diagnosis according to PD1+ cells (p=0.006).

Table 7. Overall survival with univariate Cox proportional hazard model

Characteristics	*PD1+ cells*		*p value* [1]
	< 25 % (n= 86) n (%)	*> 25 % (n= 18) n (%)*	
Sex			0.400
Male	62 (72.1)	11 (61.1)	
Female	24 (27.9)	7 (38.9)	
Age at diagnosis, years			0.385
< 60	32 (37.2)	4 (22.2)	
60-69	39 (45.3)	9 (50.0)	
≥ 70	15 (17.4)	5 (27.8)	
Clinical stage at diagnosis			0.032
I	27 (31.4)	1 (5.6)	
II	14 (16.3)	1 (5.6)	
III	24 (27.9)	8 (44.4)	
IV	21 (24.4)	8 (44.4)	
Vascular invasion			0.183
Yes	28 (32.6)	9 (50.0)	
No	58 (67.4)	9 (50.0)	
Tumor grade			0.006
1	25 (29.1)	2 (11.1)	
2	35 (40.7)	9 (50.0)	
3	16 (18.6)	0 (0.0)	
4	10 (11.6)	7 (38.9)	
Frequency of PC2			0.374
median (range)	0.002 (0.000–0.077)	0.005 (0.000–0.188)	
CD8+ TIL, n (%)			< 0.001
Negative	1 (1.2)	0 (0.0)	
< 25 %	63 (73.3)	0 (0.0)	
25–50 %	20 (23.3)	9 (50.0)	
> 50 %	2 (2.3)	9 (50.0)	

[1] Fisher exact test or Kruskal-Wallis test. 2 No information about cilia in 7 patients in "<25 %" subgroup and 1 in ">25 %" subgroup PC: Primary cilia; TIL: Tumor infiltrating lymphocytes; PD1: Programmed death protein receptor; HR: Hazard ratio

Table 2. The median OS was significantly shorter in patients with higher (≥0.002) frequency of PC than in patients with lower (<0.002) frequency of PC (log rank test; p<0.001) (Table 3 and Figure 2). This difference was significant also in the Cox proportional hazard model (p<0.001) (Table 4). The median OS was significantly shorter in patients with >25% intratumoral CD8+ TIL expression compared to patients with <25% expression [4.6 (95% CI: 2.1–7.1) vs. 9.7 (95% CI: 6.1–13.3) years, p=0.006, respectively] (Table 5 and Figure 3). The difference was also significant in the univariate Cox proportional hazard model (p=0.006, Table 4). The median OS was significantly shorter in patients with higher (>25%) PD1+ cell counts (median 2.9, 95% CI 1.8-4.0 years) compared to patients with lower (<25%) expression (median 8.9, 95% 6.6-11.1 years; p=0.006; Table 6 and Figure 4). This difference was also significant in the univariate Cox proportional hazard model (p=0.019; Table 4). Significant association with PD1+ cells was observed with the stage at diagnosis, tumor grade and intratumoral CD8+ TIL (Table 7). Table 8 shows prognostic significance of the combination of frequency of PC, expression of CD8+ TIL (p=0.002) and combination of frequency of PC and PD1+ cells expression (p<0.001). Multivariate testing (Table 9) confirmed that cilia counts but not infiltration with CD8+ cells or PD-1+ cells were independently associated with prognosis.

Discussion

To the best of our knowledge, the present study provides the first information about an association between the frequency of PC, PD1+ cells and intratumoral CD8+ TIL counts and prognosis in ccRCC.

Table 8. Survival analysis: combined risk factors (univariate Cox proportional hazard model)

Variables	*n*	*HR (95% CI)*	*p value* [1]
Frequency of PC and CD8+ TIL			
Frequency of PC > 0.002 and CD8+ TIL > 25 %	68	6.726 (2.045–22.125)	0.002
Frequency of PC and PD1+ cells			
Frequency of PC > 0.002 and PD1+ cells > 25 %	57	6.156 (2.398–15.801)	< 0.001

[1] Wald test. PC: Primary cilia; TIL: Tumor infiltrating lymphocytes; PD1: Programmed death protein receptor; HR: Hazard ratio

Table 9. Overall survival with univariate Cox proportional hazard model

Variables	*Categories*	*n*	*HR (95% CI)*	*p value* [1]
Sex	Females	29	1.000	-
	Males	66	1.486 (0.683–3.233)	0.318
Age at diagnosis, years	< 60	32	1.000	-
	60–69	43	0.623 (0.277–1.399)	0.252
	≥ 70	20	0.352 (0.113–1.098)	0.072
Tumor grade	1	26	1.000	-
	2	40	1.509 (0.471–4.832)	0.488
	3	14	7.355 (1.944–27.823)	0.003
	4	15	5.324 (1.551–18.273)	0.008
Vascular invasion	No	60	1.000	-
	Yes	35	2.805 (1.348–5.839)	0.006
Frequency of primary cilia	< 0.002	44	1.000	-
	> 0.002	51	8.399 (3.166–22.282)	< 0.001
Intratumoral CD8+ TIL	< 25 %	58	1.000	-
	> 25 %	37	1.010 (0.383–2.666)	0.983
PD1+ cells	< 25 %	78	1.000	-
	> 25 %	17	2.739 (0.964–7.783)	0.059

[1] Wald test. TIL: Tumor infiltrating lymphocytes

In a Swiss study investigating 20 ccRCC patients, the median frequency of PC of 7.8 (0 to 22%) was higher than in the present study [17]. Methodology of present study was also used in previous studies [4,9], including a study of gastrointestinal stromal tumors [16] in which we observed similar frequency of PC by immunofluorescence as other authors in an ultrastructure study using transmission electron microscopy [18].

The hedgehog signaling pathway is a central developmental pathway uniquely associated with the function of PC [19]. It is critical for the embryonic and postnatal organ and tissue development, including the kidney. The sonic hedgehog signaling pathway has also been shown to be dysregulated in pancreatic and colorectal carcinomas and melanoma. It was shown, that the sonic hedgehog signaling pathway is reactivated in human RCC and holds an important role in tumor growth [20]. We hypothesize that the negative prognostic significance of PC observed in the present study could be associated with the reactivation of sonic hedgehog signaling, however this needs further investigation.

In agreement with the literature, we observed that more abundant infiltration of tumor tissue by CD8+ TIL was associated with shorter OS [12,13]. This suggests a possibility that cellular immune response is more pronounced in tumor of higher grade and more aggressive biological behavior,

likely due to increased antigenicity of tumor cells [12]. In the present study on ccRCC, both host factors, immune response (CD8+ TIL and PD1+ cells expression) and vascular reaction (vascular invasion) were significant.

Interestingly, in our previous study of intestinal carcinomas, in which a higher CD8 + TIL density and higher PD1+ cells expression were associated with longer OS, higher frequency of PC was also significantly associated with longer OS [4]. This contrasts with ccRCC, where higher CD8 + TIL density, higher PD1+ cells expression as well as higher frequency of PC are significantly associated with shorter OS. For both groups of tumors, the prognostic significance of PC correlates with the prognostic significance of CD8 + TIL density and PD1+ cells counts.

These findings are of importance in light of the recent advances that introduced immunotherapy as a major approach in metastatic RCC [14]. Although biomarkers play an essential role in the management of many tumors [21], the utilization of biomarkers in RCC is still very limited. In particular, there is an unmet medical need for biomarkers predicting the response to immunotherapy in metastatic RCC. Future studies should determine whether the expression of PC, CD8 + TIL and PD1+ cell counts or more complex indices combining these biomarkers could serve to predict response to immunotherapy in this setting.

In conclusion, the present pilot study provides initial information on the negative prognostic significance of higher frequency of PC, higher frequency of CD8+ TIL expression and higher PD1+ cells expression. A model combining the frequency of PC and CD8+ TIL expression ($p=0.002$) and the frequency of PC and PD1+ cells expression ($p<0.001$) have negative prognostic significance in ccRCC. The present study results suggest a potential role for PC as a biomarker in ccRCC.

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Conflict of interests

The authors declare no conflict of interests.

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