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Independent association of PD-L1 expression with non-inactivated *VHL* clear cell renal cell carcinoma - a finding with therapeutic potential

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Novelty and impact statements: In our retrospective series of 98 consecutive clear cell renal cell carcinoma (ccRCC), we identified an independent association between PD-L1 immunostaining and non-inactivated *VHL* ccRCC. In particular wild-type *VHL* ccRCC all expressed PD-L1. Wild-type *VHL* ccRCC are a sub-group that are associated with a worse prognosis. These patients with PD-L1 expression may benefit from targeted immunotherapy.

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ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is an aggressive tumor that is characterized in most cases by inactivation of the tumor suppressor gene *VHL*. The VHL/HIF/VEGF pathway thus plays a major role in angiogenesis and is currently targeted by anti-angiogenic therapy. The emergence of resistance is leading to the use of targeted immunotherapy against immune checkpoint PD1/PDL1 that restores antitumor immune response. The correlation between *VHL* status and PD-L1 expression has been little investigated.

In this study, we retrospectively reviewed 98 consecutive cases of ccRCC and correlated PD-L1 expression by immunohistochemistry (IHC) with clinical data (up to 10-year follow-up), pathological criteria, VEGF, PAR-3, CAIX and PD-1 expressions by IHC and complete *VHL* status (deletion, mutation and promoter hypermethylation).

PD-L1 expression was observed in 69 ccRCC (70.4%) and the corresponding patients had a worse prognosis, with a median specific survival of 52 months ($p=0.03$). PD-L1 expression was significantly associated with poor prognostic factors such as a higher ISUP nucleolar grade ($p=0.01$), metastases at diagnosis ($p=0.01$), a sarcomatoid component ($p=0.04$), overexpression of VEGF ($p<0.01$), and cytoplasmic PAR-3 expression ($p=0.01$). PD-L1 expression was also associated with dense PD-1 expression ($p=0.007$) and with ccRCC with 0 or 1 alteration(s) (non-inactivated *VHL* tumors) ($p<0.01$) that remained significant after multivariate analysis ($p=0.004$ and $p=0.024$, respectively). Interestingly, all wild-type *VHL* tumors (no *VHL* gene alteration, 11.2%) expressed PD-L1.

In this study, we found PD-L1 expression to be associated with non-inactivated *VHL* tumors and in particular wild-type *VHL* ccRCC which may benefit from therapies inhibiting PD-L1/PD-1.

INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) accounts for approximately 3% of adult cancers and is a very aggressive tumor with a 50% risk of metastases at initial diagnosis or follow-up.[1]

The *VHL (von Hippel-Lindau)* gene, located on chromosome 3p25, is a major tumor suppressor gene involved in ccRCC oncogenesis.[2] Alterations of this gene occur in the majority of ccRCC through mutations or deletions and/or hypermethylation of its promoter [3]. *VHL* gene inactivation necessarily involves biallelic alterations in tumor cells, as both first and second "hits" must occur to be inactivated. However, a minority of ccRCC exhibit no or a single allele alteration on the *VHL* gene.[4]

The protein, pVHL, is a component of an E3 ubiquitin-ligase which targets the transcription factor: hypoxia-inducible factor (HIF).[3] The consequence of pVHL impairment is a stabilization of HIF resulting in overexpression of HIF. This leads to the transcription of genes regulated by HIF, such as vascular endothelial growth factor (*VEGF*) or carbonic anhydrase IX (*CAIX*). Consequently, *VHL* is one of the main triggers of the angiogenesis process in ccRCC.[5] Based on an understanding of these mechanisms, therapies targeting angiogenesis emerged in the last decade, significantly improving the prognosis of metastatic ccRCC.[6] However, the response is short-lived due to the development of resistance to these therapeutic agents.[7]

A new approach is based on targeted immunotherapy using checkpoint inhibitors since ccRCC is considered an immunogenic tumor with high numbers of mononuclear immune cells such as tumor-infiltrating lymphocytes (TIL). Recent studies showed that 15-56.5% of ccRCC have aberrant programmed death-ligand 1 (PD-L1/B7-H1/CD274) expression in the primary tumor.[8-10] PD-L1 is a transmembrane protein which binds to its co-stimulatory receptor, PD-1 (B7-1), expressed by activated TILs, as a means to down-regulate antitumor immune responses by promoting TIL apoptosis and thus favor tumor progression. Recent clinical trials demonstrated that blocking the interaction between PD-1 and PD-L1 with monoclonal antibodies may result in anti-tumor activity as demonstrated in ccRCC.[11]

The aim of this study was to correlate PD-L1 expression with pathological criteria, expression of VEGF, PAR-3 (partitioning defective 3), CAIX, and PD-1, and complete *VHL* status in a population of patients with ccRCC and a long-term clinical follow-up of 10 years.

MATERIALS AND METHODS

Patients

Consecutive patients operated for sporadic ccRCC in the Department of Urology at Rennes Hospital between 2002 and 2005 with a clinical follow-up of up to 10 years were retrospectively included in the study. All patients underwent radical nephrectomy without presurgical medication. The study protocol was approved by the local advisory board and informed consent was received from all patients prior to inclusion in the study.

Tissue sample management

Tumor samples were derived from biological sample processing by the Rennes Hospital Biological Resources Center (BB-0033-00056, http://www.crbsante-rennes.com/). The research protocol was conducted under French legal guidelines and met the requirements of the local institutional ethics committee. All consecutive ccRCC and paired renal cortex samples were analyzed. Immediately after macroscopic examination, small samples were collected from surgical specimens, frozen in liquid nitrogen and stored at -80°C until DNA extraction. Genomic DNA was extracted from 25 to 35 mg of frozen tissue sections using a QIAamp DNA minikit (Qiagen, Courtaboeuf, France). DNA quantity and quality were assessed by optical density (OD 260/280) measurement and 0.8% agarose gel electrophoresis using standard protocols.

Pathological analysis

After fresh tissue sampling surgical specimens were formalin-fixed. Paraffin sections were stained with hematoxylin and eosin-safran for light microscopy. All slides were reviewed by a dedicated uropathologist (NRL). The macroscopic and histological parameters analyzed were: tumor size, multifocality, nucleolar grade under the International Society of Urological Pathology (ISUP) grading system[12], sarcomatoid pattern, tumor necrosis, granular component, lymphocyte infiltrate and microvessel invasion. Tumor stage was graded using the latest International Union Against Cancer Classification (2009).[13]

Immunohistochemistry

For each ccRCC case, a representative slide of the tumor with the highest nucleolar ISUP grade and the corresponding paraffin block were selected. The immunohistochemical analysis was double blinded and evaluated by two independent pathologists (SFKJ and NRL). Discordant cases were reevaluated collegially. Expression of VEGF (anti-VEGF antibody, sc-152, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CAIX (anti-CAIX antibody, ab15086, dilution 1:1500; Abcam, Cambridge, UK), PAR-3 (anti-PAR-3, HPA0300443, dilution 1:50; Sigma-Aldrich, St. Louis, USA) and PD-L1 (anti-PD-L1 antibody, clone 130021, dilution 1:200; R&D Systems, Minneapolis, USA) was assessed by immunohistochemistry as previously described.[4, 14, 15] The cut-off for overexpression was set at 85% of tumor cells for CAIX and 30% for VEGF.[4, 14] Only cytoplasmic PAR-3 expression was considered positive as previously assessed.[15] PD-L1 was considered positive when any membranous or cytoplasmic tumor cell staining was observed.[16] Regarding TILs, expression of CD3 (anti-CD3 antibody, clone SP7, dilution 1:100; Thermo Scientific, Waltham, MA, USA) and CD20 (anti-CD20 antibody, clone L26, dilution 1:25; Dako, Glostrup, Denmark) was assessed. The inflammatory extent was coded as 1 (sparse lymphocytes in the tumor) or 2 (marked dense lymphocytes or lymphoid nodules). Expression of PD-1 (anti-PD-1 antibody, clone NAT105, dilution 1:50; Abcam, Cambridge, UK) in TILs was scored as absent, rare, moderate or marked dense. Absence or rare density was considered negative whereas moderate or marked density was considered positive for statistical analysis.

***VHL* gene analysis**

We determined the complete *VHL* status for each tumor by analyzing *VHL* gene mutation, deletion and promoter methylation. Four primers pairs were designed (Primer3 software, Whitehead Research Institute, Cambridge, MA), to amplify two overlapping fragments for exon 1 (1A and 1B) and one fragment for each of exons 2 and 3 (Eurogentech, Belgium), covering part of the VHL 5'UTR, the entire coding sequence and exon-intron junctions (VHL Genbank accession AF010238). The 4 primers are presented in Table I (Supplementary tables). We amplified 50–150 ng of tumor DNA and of renal cortex DNA in parallel, using AmpliTaq Gold (Applera, Courtaboeuf, France) and the following PCR conditions: 95°C 9 min and 95°C 1 min, annealing T° 45 sec, 72°C 45 sec, 35 cycles, MgCl2 1.5 mM, dNTP 200

lM. DMSO [5% (v/v), Eurobio] was added to amplify exons 1A and 1B. *VHL* mutations were detected with denaturing high-performance liquid chromatography (DHPLC) sequencing. DHPLC screening was carried out on a WAVE Nucleic Acid. Fragment Analysis system (Transgenomic, Glasgow, UK) with a DNAsep column. Forward and reverse automatic sequencing was performed using BigDye Terminator v1.1 Cycling Sequencing kit on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Courtaboeuf, France). All mutations were confirmed in a second round of PCR and via sequencing reactions. *VHL* gene deletions and promoter methylation were detected by multiplex ligation–dependent probe amplification (MLPA) analysis using the SALSA MLPA P016B *VHL* probe kit and SALSA MS-MLPA kit, respectively, as previously described.[4] As *VHL* functions as a tumor suppressor gene, impairments necessarily involve biallelic alterations in tumor cells as two hits are needed for inactivation to occur.

Statistical analysis

χ^2, Fisher's exact and Mann-Whitney tests were performed to compare qualitative and quantitative parameters between the groups with and without PD-L1 expression, respectively. A logistic regression multivariate analysis was then performed using all significant variables from the univariate analysis ($p<0.05$). Cancer specific survival (CSS) according to PD-L1 expression was calculated from nephrectomy to death from cancer and Kaplan-Meier curves were compared by log-rank test. All p-values were 2-sided, and p-values less than 0.05 were considered statistically significant. All statistical analyses (AP) were performed using Stata 14.1 (StataCorp, College Station, TX, USA) software.

RESULTS

Patients and pathological parameters

The study retrospectively included 98 patients according to participant flow diagram (Figure I, supplementary figures). The median age at diagnosis was 64 years (40-84). Sixty-nine patients (70.4%) had an ECOG performance status of 0. In 11 cases (11.2%), nodal invasion was present at diagnosis and in 23 cases (23.5%) patients had synchronous metastases. The mean tumor size was 7.3 cm +/- 3.4 cm with tumors ranging from 1.5 to 18 cm. The

population characteristics and pathological parameters are summarized in Table 1. Table II (supplementary tables) details characteristics of metastatic patients.

Genetic and epigenetic *VHL* gene alteration

All patients tested negative for germline mutations. A *VHL* gene mutation was found in 68 cases (69.4%) and detailed in Table III (supplementary tables). Mutations occurred in exons 1, 2 and 3 in 28 (28.6%), 26 (26.5%) and 14 cases (14.3%), respectively. Stop, frameshift, missense, and splice site mutations were found in 10 (10.2%), 33 (33.7%), 19 (19.4%) and 6 (6.1%) cases, respectively. *VHL* loss of heterozygosity and promoter methylation occurred in 71 (72.4%) and 13 cases (13.3%), respectively. At least one or more *VHL* alterations were found in 87 cases (88.8%). Tumors with two alterations of the *VHL* gene (n=65 cases, 66.3%) were deemed inactivated for that gene (*inVHL*). Those with no or only one alteration (n=33, 33.7%) were grouped as non-inactivated *VHL* tumors (*niVHL*). These included tumors with no *VHL* gene alteration (n=11, 11.2%) that were classed as wild type (*wtVHL*).

Morphological and molecular phenotype of tumors with PD-L1 expression

PD-L1 expression was observed in 69 tumors (70.4%) with different patterns of expression (Figure 1 and Figure II, supplementary figures). These tumors were compared to tumors with no PD-L1 expression. Morphological and immunostainings for each markers are shown in Figures II to VI, supplementary figures. The results and statistical analysis are provided in Table 2. Tumors with PD-L1 expression were significantly associated with a higher tumor stage (p=0.035) and metastasis at diagnosis (p=0.010). Besides, these tumors had a higher ISUP nucleolar grade (p=0.009), sarcomatoid component (p=0.036), overexpression of VEGF (p=0.006), cytoplasmic expression of PAR-3 (p=0.010) and dense TIL PD-1 expression (p=0.007), as shown in Figure 2. PD-L1 expression was associated with *niVHL* ccRCC (p=0.007). Within the *niVHL* ccRCC group all *wtVHL* subgroup tumors expressed PD-L1 (p=0.030). The multivariate analysis (logistic regression) included all the significant factors except the sarcomatoid component which was already included in the ISUP nucleolar grade. Moderate or dense expression of PD-1 and the *niVHL* gene was significantly associated with PD-L1 expression in the multivariate analysis (p=0.004 and p=0.024, respectively).

Correlation between PD-L1 expression and survival

The progression of patients in the PD-L1 expression subgroup differed in comparison to patients with no PD-L1 expression in their tumors. The survival curves of the two subgroups are shown in Figure 3. Patients with PD-L1 expression had a worse prognosis with a median specific survival of 52 months from nephrectomy compared to patients with no PD-L1 expression for whom the median specific survival time was not reached (p=0.030).

DISCUSSION

In our study, we correlated PD-L1 expression by IHC with pathological criteria, expression of VEGF, PAR-3, CAIX, and PD-1, and complete *VHL* status in a large series of 98 patients with ccRCC and a long-term clinical follow-up of up to 10 years.

As previously reported for ccRCC, in our series PD-L1 expression was associated with poor prognostic factors such as tumor stage, ISUP nucleolar grade, sarcomatoid component, dense PD-1 expression and VEGF expression.[8, 10, 17-19] PD-L1 was also associated with cytoplasmic PAR-3 protein expression which was previously identified as a poor prognostic factor in ccRCC.[15] Furthermore, as expected, patients with PD-L1 expression in ccRCC samples had a worse clinical outcome.[8]

As commonly reported, PD-L1 expression was associated with sarcomatoid components.[19, 20] For the first time, we found an association between PD-L1 expression and cytoplasmic PAR-3 expression. PAR-3 is a crucial component of partitioning-defective complex proteins that controls cell polarity and contributes to cell migration and cancer cell epithelial-to-mesenchymal transition (EMT).[21] PD-L1 expression associated with both sarcomatoid components and PAR-3 expression suggested potential EMT involvement in ccRCC. Recently, PD-L1 expression was reported to induce EMT in renal cell carcinoma through activation of SREBP-1c, a key transcription factor for lipogenesis genes.[22]

Two general mechanisms for the regulation of PD-L1 by tumor cells have emerged, namely innate immune resistance and adaptive immune resistance.[23] For some tumors, it has been shown that PD-L1 expression is driven by constitutive oncogenic signaling pathways in tumor cells such as PI3K-AKT and MAPK pathways and is termed innate or constitutive immune resistance.[24, 25] A second alternative mechanism for PD-L1 regulation in tumor cells reflects

their adaptation to endogenous tumor-specific immune responses, a process termed adaptive immune resistance. In this model, tumor cells use the natural physiology of PD-1 ligand induction to produce IFNs, notably IFNγ.[26] In our series this mechanism is illustrated by dense PD-1 expression independently associated with PD-L1 expression.

For the first time, we correlated PD-L1 expression by IHC with *VHL* complete status including *VHL* gene locus deletion, *VHL* mutation and promoter hypermethylation. In our study, PD-L1 expression was independently associated with a *niVHL* status defined by 0 or 1 alterations of the *VHL* gene. As a tumor suppressor gene, *VHL* requires at least two events, one on each allele, to be inactivated as it is not a haploinsufficient tumor-suppressor gene.[2] PD-L1 expression and *niVHL* ccRCC were also associated with poor prognostic factors such as VEGF expression.[4, 19] Furthermore, Beuselinck *et al.* reported PD-L1 expression and fewer *VHL* gene mutations in ccrcc4 tumors. This molecularly defined subtype of metastatic primary ccRCC was associated with poor prognosis under anti-angiogenic therapy.[27] These data may indicate the occurrence of ccRCC with both PD-L1 expression and *niVHL*, as found in our series.

Recently, Messai *et al.* demonstrated that PD-L1 expression positively correlated with *VHL* inactivation through HIF-2α.[28] The difference in their conclusions may be due to population variations. In their study, 32 patients were retrospectively included: 21 patients with sporadic ccRCC and 11 with *VHL*-tumor associated ccRCC. Only one patient (3.1%) had no *VHL* gene alteration at all. In our series, sporadic ccRCC was an inclusion criterion and *VHL*-tumor associated ccRCC cases were excluded. Among the 98 patients included, 11 patients (11.2%) had a *wtVHL* tumor with no *VHL* gene alteration.

In our study, *wtVHL* ccRCC were particularly associated with PD-L1 expression. These results support the theory of alternative oncogenic pathways in ccRCC leading to PD-L1 overexpression despite HIF degradation due to the presence of an activated VHL protein (*niVHL*). Tumors with no inactivation of *VHL* could use alternative pathways independent of *VHL* mechanisms such as the MAP kinase and PI3K-AKT-mTOR pathways involved in ccRCC oncogenesis.[29-31] These alternative pathways have already been reported to induce PD-L1 expression in constitutive immune responses in other cancers.[23, 32]

CONCLUSION

For the first time, we found PD-L1 expression by IHC to be associated with *niVHL* tumors, and in particular with *wtVHL* ccRCC. These tumors may benefit from therapies inhibiting PD-L1/PD-1.

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Tables

Table 1. Summary of the clinical and histopathological characteristics of 98 patients with sporadic ccRCC.

Variables	Number of patients (%)
Sex	
M	60 (61.2%)
F	38 (38.8%)
ECOG	
0	64 (65.3%)
1	34 (34.7%)
Age (years)	64 (40-84)
Tumour size (cm)	7.3 (1.5-18)
Nucleolar grade	
1	2 (2%)
2	33 (33.7%)
3	32 (32.7%)
4	31 (31.6%)
Tumour stage (pT)	
1	37 (37.8%)
2	18 (18.4%)
3	39 (39.8%)
4	4 (4.1%)
Lymph node status (pN)	
0	88 (89.8%)
1-2	10 (10.2%)
Metastasis status (pM)	
0	70 (71.4%)
1	28 (28.6%)
Recurrence	
Local recurrence	6 (6.1%)
Metastases	20 (20.4%)

Table 2. Summary of histopathological and immunohistochemical characteristics and *VHL* status of tumors according to PD-L1 expression.

Variables	No expression of PD-L1 (n=29)	Expression of PD-L1 (n= 69)	Univariate analysis, p-value	Multivariate analysis, p-value
Tumour stage (T3-T4)	8	34	**p=0.035**†	p=0.232
Lymph node status (N1-N2)	3	8	p=1‡	
Metastasis at diagnosis (M)	3	25	**p=0.010**†	p=0.166
Median tumor size (cm)	7.5	8.2	p=0.094§	
ISUP nucleolar grade (3-4)	13	50	**p=0.009**†	p=0.781
Tumour necrosis	12	41	p=0.102†	
Sarcomatoid component	1	14	**p=0.036**	
Granular component	12	39	p=0.171†	
Microvascular invasion	9	30	p=0.251†	
Dense lymphocyte infiltrate	2	12	p=0.220‡	
VEGF expression ≥ 30%	8	38	**p=0.006**†	p=0.891
CAIX expression ≥ 85%	25	49	p=0.110†	
Cytoplasmic PAR-3 expression	9	41	**p=0.010**†	p=0.835
Moderate or dense PD-1 expression	5	32	**p=0.007**†	**p=0.004**
Non inactivated *VHL* status	4	29	**p=0.007**†	**p=0.024**
Wild-type *VHL*	0	11	**p=0.030**‡	

Note: † Pearson chi2 test, ‡ Fisher's exact test, § Mann-Whitney tests

Figures

Figure 1. Intensity of PDL1 expression
A) Absence of PDL1 expression, IHC x100
B) Low expression of PDL1 in tumor cells, IHC x100
C) Moderate expression of PDL1 in tumor cells, IHC x100
D) High expression of PDL1 in tumor cells, IHC x100

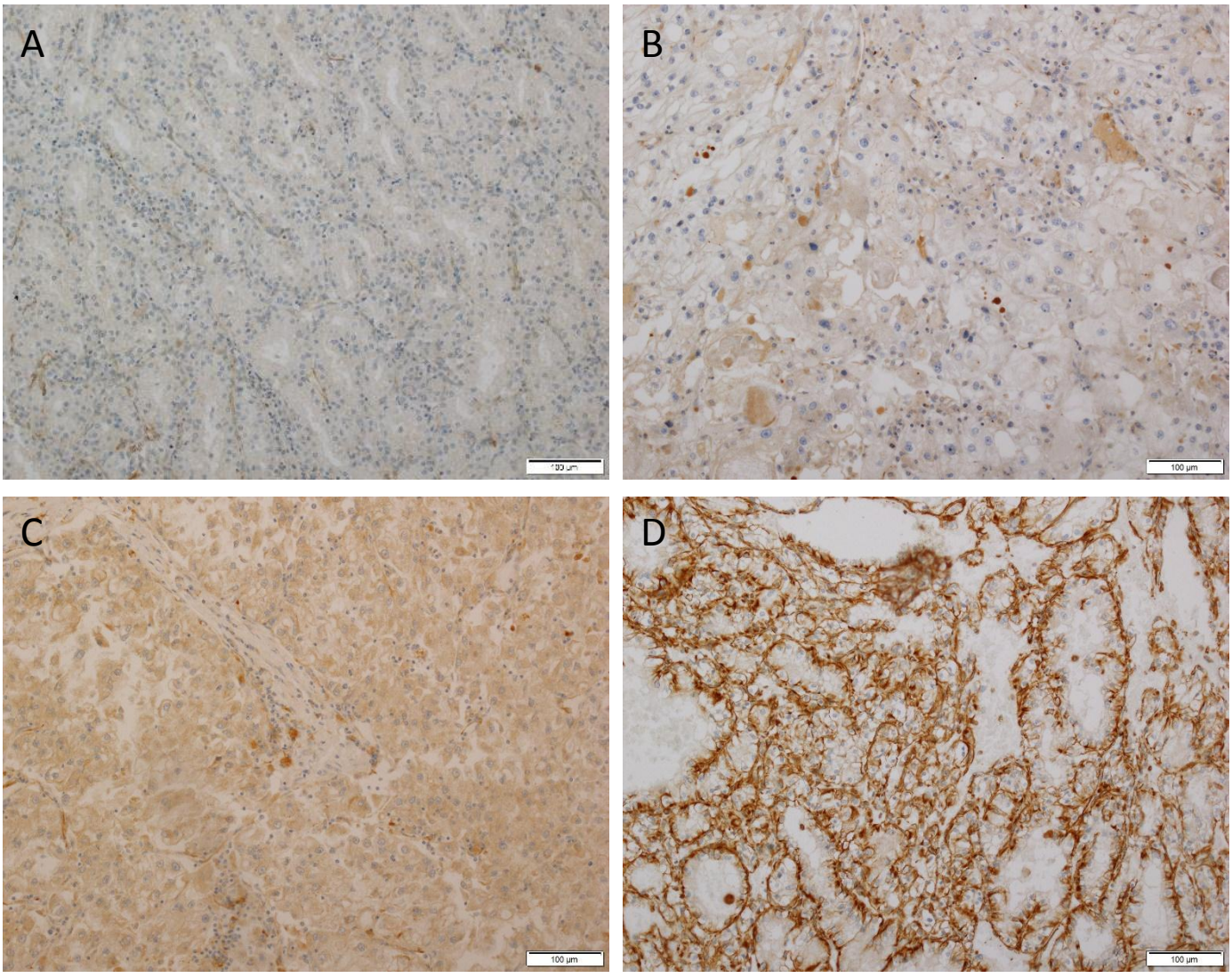

Figure 2. Pathological and immunohistochemical parameters associated with PD-L1 expression

A) Sarcomatoid component, HES x100

B) Cytoplasmic and membranous expression of PAR-3, IHC x100

C) Diffuse cytoplasmic expression of VEGFA, IHC x100

D) Dense PD-1 expression in tumor-infiltrating lymphocytes, IHC x100

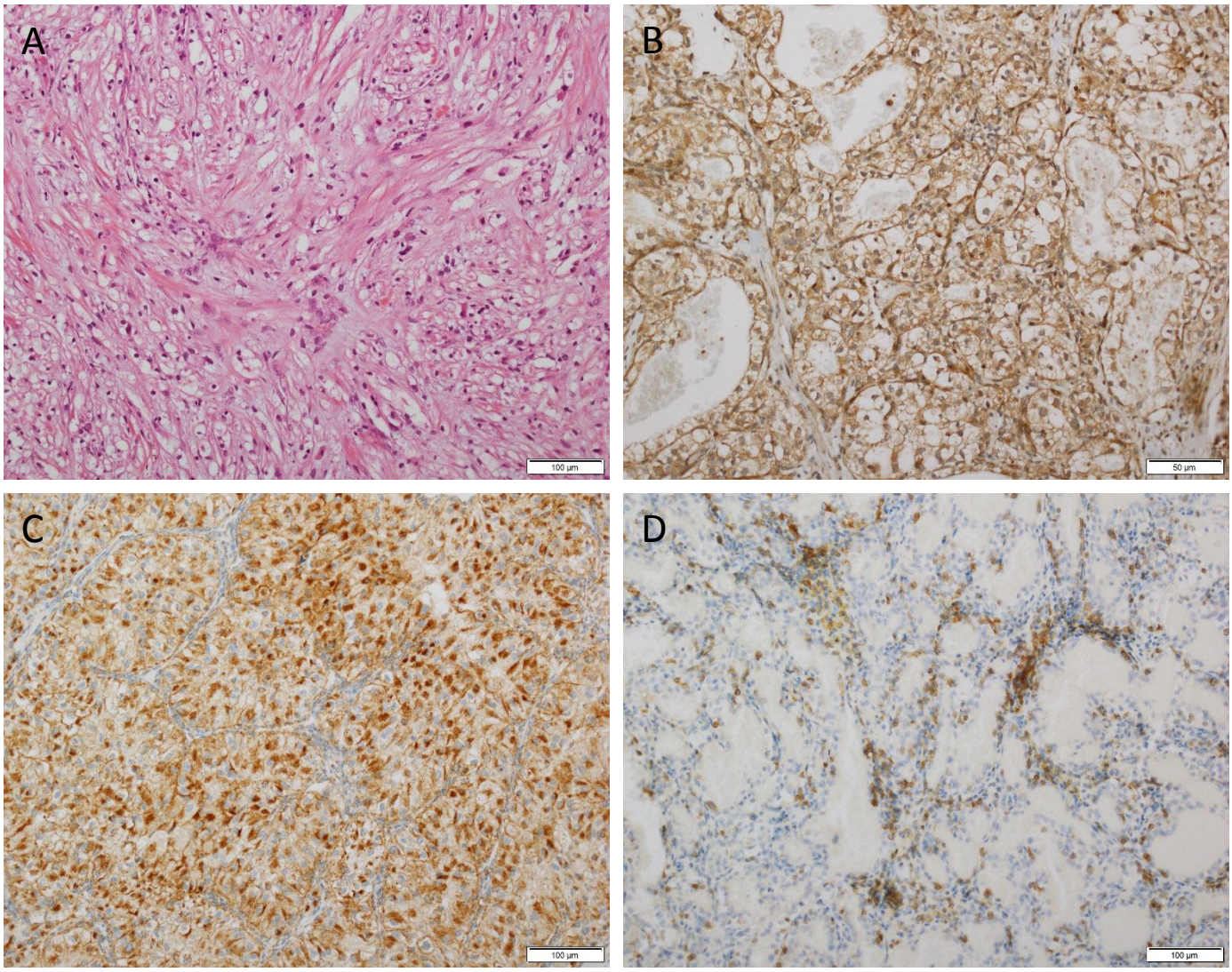

Figure 3. Kaplan-Meier curve representing cancer specific survival according to PD-L1 expression