Comprehensive analyses of immune tumor microenvironment in papillary renal cell carcinoma

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ABSTRACT

Background Papillary renal cell carcinoma (pRCC) is the most common non-clear cell RCC, and associated with poor outcomes in the metastatic setting. In this study, we aimed to comprehensively evaluate the immune tumor microenvironment (TME), largely unknown, of patients with metastatic pRCC and identify potential therapeutic targets.
Methods We performed quantitative gene expression analysis of TME using Microenvironment Cell Populations-counter (MCP-counter) methodology, on two independent cohorts of localized pRCC (n=271 and n=98). We then characterized the TME, using immunohistochemistry (n=38) and RNA-sequencing (RNA-seq) (n=30) on metastatic pRCC from the prospective AXIPAP trial cohort.
Results Unsupervised clustering identified two "TME subtypes", in each of the cohorts: the "immune-enriched" and the "immune-low". Within AXIPAP trial cohort, the "immune-enriched" cluster was significantly associated with a worse prognosis according to the median overall survival to 8 months (95% CI, 6 to 29) versus 37 months (95% CI, 20 to NA, p=0.001). The two immune signatures, Teff and JAVELIN Renal 101 Immuno signature, predictive of response to immune checkpoint inhibitors (CPI) in clear cell RCC, were significantly higher in the "immune-enriched" group (adjusted p<0.05). Finally, five differentially overexpressed genes were identified, corresponding mainly to B lymphocyte populations.
Conclusion For the first time, using RNA-seq and immunohistochemistry, we have highlighted a specific immune TME subtype of metastatic pRCC, significantly more infiltrated with T and B immune population. This "immune-enriched" group appears to have a worse prognosis and could have a potential predictive value for response to immunotherapy, justifying the confirmation of these results in a cohort of metastatic pRCC treated with CPI and in combination with targeted therapies.
Trial registration number NCT02489695.

INTRODUCTION

Papillary renal cell carcinoma (pRCC) is the most common non-clear cell RCC (nccRCC) and represents up to 15% of renal cell carcinoma (RCC).[1][2] The denomination nccRCC comprises a heterogeneous group of tumors

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Papillary renal cell carcinoma is the most common non-clear cell RCC, and associated with poor outcomes in the metastatic setting. We aimed to comprehensively evaluate, by RNA sequencing and immunohistochemistry (IHC), the immune tumor microenvironment (TME), largely unknown, to identify potential therapeutic targets.

WHAT THIS STUDY ADDS

⇒ The identification of TME subtypes, and notably the "immune-enriched" group, could be done by IHC, and in particular by the CD3 marker, a reliable and inexpensive technique. This "immune-enriched" feature appears to be correlated with a poor prognosis but could indicate a potential predictive value for response to immunotherapy, alone or in combination, a treatment not currently recommended.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study could lead to the development of predictive biomarker-driven clinical trials in these rare tumors.

with distinct histological and molecular characterization.[3] Patients with metastatic pRCC (mpRCC) have significantly lower response rates, lower median progression-free survival (PFS) and overall survival (OS) than those with clear cell renal cell carcinoma (ccRCC).[4][5] Based on the pathological assessment according to the WHO 2016 classification, pRCC have been routinely classified in two subtypes: type 1, commonly associated with multiple or bilateral small tumors with a favorable prognosis and few metastatic development, type 2, commonly more aggressive and associated with a dismal prognosis, and unclassified pRCC.[6] Several studies aimed at investigating molecular events specific to pRCC subtypes.[2][7][8]

The therapeutic management of patients with metastatic nccRCC has historically been similar to metastatic ccRCC given the lack of dedicated trials.[1 9 10] A few phase 2 studies, evaluating targeted therapies, have been carried out specifically in mpRCC. Those investigated sunitinib (SUPAP[11]), everolimus (RAPTOR[12]), cabozantinib (PAPMET[13]) and axitinib (AXIPAP[14]) as first-line agents. Cabozantinib treatment resulted in significantly longer PFS (median 9.0 months, 95% CI, 6% to 12%) than in the sunitinib group (5.6 months, 3% to 7%; HR for progression or death 0.60, 0.37 to 0.97, one-sided p=0.019). Response rate for cabozantinib was 23% versus 4% for sunitinib (two-sided p=0.010).[13] As well, axitinib demonstrated encouraging efficacy in patients with mpRCC, especially in type 2 pRCC, with manageable toxicity. The progression-free rate at 24 weeks, primary end-point, was 45.2% (95% CI, 32.6% to not reached), the objective response rate (ORR) 28.6% (95% CI, 15.7% to 44.6%) including 7.7% in type 1 and 35.7% in type 2. Median OS was 18.9 months (95% CI, 12.8 to not reached).[14] The clinical efficacy of immunotherapy, monotherapy or in combination, has since been established in metastatic RCC with a clear cell histologic component.[15 16] However, these pivotal studies of immune checkpoint inhibitors (CPI) excluded nccRCC. Small retrospective cohorts report discordant results regarding nccRCC response to CPI.[17–22] The activity of CPI, as a single agent appears variable in patients with metastatic pRCC, with ORR ranging from 8% to 25%. Indeed, in monotherapy, the KEYNOTE-427 cohort B study remains the largest prospective data set to date, showing promising antitumor activity with first-line pembrolizumab monotherapy in metastatic pRCC. At a median follow-up of 11.1 months (range 0.9–21.3), median PFS was 4.1 months and the median OS has not yet been reached with 72% of patients alive at 1 year in the entire nccRCC cohort. In 118 patients with mpRCC, ORR was 25.4% (95% CI, 17.9% to 34.3%). In combination, the preliminary analysis of KEYNOTE-B61, a single-arm, phase 2 study (NCT04704219) evaluating pembrolizumab and lenvatinib as first-line treatment for nccRCC, showed increased antitumor activity. The 6-month PFS rate was 72.3% (95% CI, 60.7% to 81.0%) and the 6-month OS rate was 87.8% (95% CI, 78.5% to 93.2%). In 51 patients with mpRCC, ORR was 52.9% (95% CI, 38.5% to 67.1%).[23] Further activity could be gained from combinations, but optimal partners still need to be investigated.[24]

CPI therapy appears to be more effective in patients with pre-existing antitumor immune activation. According to Charoentong et al,[25] immunogenicity can be represented by cytotoxic lymphocyte activity,[26] being the ultimate effector mechanism of the antitumor immune response. Lymphocyte infiltration has been described in pRCC but seems to be less present than in ccRCC.[25] Papillary RCC may have a different immunogenicity, and thus a different response to CPI than ccRCC. An heterogeneous expression of the immune checkpoint programmed death-ligand 1 (PD-L1) was described in pRCC.[27 28] Characteristics

of the immune infiltrate have also been described with limited information in pRCC,[25 29] highlighting two predominant profiles: inflammatory cluster with elevated *Th17* and *Th1* genes, or depleted lymphocyte cluster with a more prominent macrophage signature, associated with a different prognosis.[30] An immune signature based on the expression of *Th2* genes, was also described in a rare pRCC subtype with the worst prognosis and harboring a Cytosine preceding Guanine (CpG) island methylator phenotype, questioning the potential role of CPI in this context.[31] While further data is awaited from prospective trials, these studies constitute growing evidence that the immune tumor microenvironment (TME) may have a key role in pRCC and support the need to investigate the role of CPI in patients with metastatic pRCC.

Immune infiltration and expression of immune checkpoints may be critical factors to select patients, but the microenvironment of pRCC is still to be described.[26 32–35]

In ccRCC, several transcriptomic signatures were associated with predictive value of response to CPI,[36 37] as *Teff* signature,[38–42] or *myeloid* signature,[38–40 42] or *JAVELIN Renal 101 Immuno* signature.[40 42] Prospective studies are ongoing to validate these predictive transcriptomic signatures but their use in routine practice will represent a major challenge.[43]

Therefore, in this study, we comprehensively evaluated the TME of patients with pRCC, and identified key genes and B and T-cell subsets that are closely related to the TME of patients with pRCC and could be used as immunotherapeutic targets or predictive biomarkers.

MATERIALS AND METHODS
Study cohorts
We performed quantitative explorations of the immune infiltration gene expression on two independent cohorts of localized pRCC, as a discovery set.

We downloaded the gene expression RNA-sequencing (RNA-seq) data and clinical phenotype of kidney papillary cell carcinoma (KIRP) from The Cancer Genome Atlas (TCGA) portal (https://gdc.cancer.gov/). The transcriptome profiling of RNA expression was obtained by RNA-seq according to the GRCh38 reference genome annotation, Ensembl V.80 of May 2015 (BiomaRt package). HTSeq-count data were converted to transcripts per million (TPM) (TCGAbiolinks package). Log2-based transformation was used for the normalization of RNA expression profiles (TPM+1). Clinical data were extracted from Ricketts et al.[31] After the data were preprocessed, and the samples without clinical data excluded, 271 pRCC were enrolled. Updated survival data were extracted from Liu et al,[44] with OS available for 266 patients. The same TCGA barcode structure is used for both clinical data and molecular data, enabling integrated analysis of patient-based clinical data and sample-based molecular data.

Gene expression microarray data were also obtained from an independent cohort of frozen tissue samples

from 98 localized pRCC, with 47 type 1 pRCC, 45 type 2 pRCC and 6 unclassified pRCC.[45] We performed our analyses using the already normalized data.

Then, we performed a post hoc analysis of the AXIPAP trial.[14] This multicenter, single-arm, phase II trial enrolled patients with locally advanced or metastatic specifically confirmed pRCC, in first-line treatment. The study was registered on ClinicalTrials.gov. Fifty-six patients were screened, and 44 included (13 type 1, 30 type 2 and 1 non-specified, according to the WHO 2016 classification). For our ancillary study, clinical data cut-off was February 28, 2021, for the final analysis. The pRCC samples were all confirmed by expert central pathology review. Only formalin-fixed paraffin-embedded (FFPE) samples with sufficient materials were included.

Procedures in AXIPAP trial cohort
Immunohistochemistry
Paraffin blocks of pRCC have been centralized at the Department of Pathology (NRL, Rennes Hospital, France). They were sent with original pathology reports by different Departments of Pathology in France. An Hémalun Eosine Safran (HES) slide has been performed from each block and all the HES-stained slides were reviewed by a uropathologist (NRL).

The following pathological data were collected: histological type (according to the WHO 2016 classification), International Society of Urological Pathology (ISUP) nucleolar grade; presence of necrosis and sarcomatoid and/or rhabdoid component.

From each paraffin block, unstained sections were obtained for immunohistochemistry (IHC), and slides were labeled using the BenchMark ULTRA/Roche.

For angiogenesis analysis, the following antibodies were used: the anti-Vascular Endothelial Growth Factor (VEGF) antibody (clone SP28-M3281, Spring/Biosciences), and the anti-CD31 antibody (Clone JC70A; titer 1:500; DakoCytomation). We described two angiogenic phenotypes one and two according to the following publication in Human Pathology,[46] and we defined the micro vessel density as the vessel sections per mm^2.

For the immune microenvironment, we used the following antibodies: CD3 (MAb rabbit 2GV6, Roche), CD8 (clone C8/144B, M7103, DAKO), CD68 (clone PG-M1, Dako), and PD-L1 (E1L3N, Cell Signaling). We evaluated the tumor infiltrating lymphocytes (CD3, CD8) and macrophages (CD68) at the invasive margin defined as a 1 mm wide zone centered on the border of the malignant cells with the host tissue, and in the central tumor defined as the central tumor tissue surrounded by this zone. We scored the CD3/CD8/CD68 positive cells according to the proposal of the International ImmunoOncology Biomarkers Working Group,[47] using a manual semi quantitative 4-point scale: score 0 (no CD3/CD8/CD68+ cells or very rare positive cells), score 1 (rare diffuse or focal positive cells), score 2 (diffuse numerous positive cells), and score 3 (diffuse and numerous positive

cells with some aggregates). For PD-L1, we evaluated the % of positive tumor cells.

On HES, we also evaluated the tumor infiltrating mononuclear cells (lymphocytes, and plasma cells) both at the invasive margin and in the central tumor as previously described.

RNA-sequencing
RNA extraction was performed with COVARIS ME220 Focused-ultrasonicator, to allow a high quantity and high quality of RNA extracted. RNA libraries were prepared with the SureSelectXT RNA Direct Library Preparation kit and the SureSelectXT Human All Exon V6+UTR probes from Agilent. All libraries were sequenced on an Illumina NextSeq550 in paired-end mode (2×75 bp) with a target depth of 20 million fragments per sample.

Sequenced reads were trimmed with fastp V.0.20.1 and mapped to GRCh38 using HISAT2 V.2.1.0 both with default parameters. Reads overlapping genomic features were counted with featureCounts V.2.0.0 from the Subread package and Ensembl V.99. Only uniquely mapped and not duplicated reads were counted. Multiple overlaps of unique genomic feature were not counted. The counts data were converted to TPM. Log2-based transformation was used for the normalization of RNA expression profiles (TPM + 1).

The R package "DESeq2" was applied to screen differentially expressed messenger RNAs (mRNAs) between different groups. Next, the p value was calculated by the false discovery rate (FDR)-corrected method. The mRNAs with | log2 fold-change | >2 and p adjusted <0.01 were filtrated as differentially expressed genes.

Identification of clusters
Based on transcriptomic markers, using Microenvironment Cell Populations-counter (MCP-counter)-methodology,[48 49] that assesses the proportion of 10 immune and stromal cell populations in the TME, we applied unsupervised clustering by using pheatmap package and Ward-d2 distance method.

We applied the same unsupervised clustering method to IHC immune markers.

Gene expression analyses
From the output of MCP-counter, we performed exploratory quantitative analyses to characterize the TME. We analyzed immune cell populations and immune markers including *LAG3, TIGIT, CTLA4, PD-1* and *PD-L1*. Multiomics analyses were performed using available data from Chen *et al*[50] and Ricketts *et al*.[31] We analyzed the Th2 prognostic signature (PMCH, AHI, PTGIS, CXCR6, EVI5, IL-26, MB, NEIL3, GSTA4, PHEX, SMAD2, CENPF, ANK1, ADCY1, LAIR2, SNRPD1, MICAL2, DHFR, WDHD1, BIRC5, SLC39A14, HELLS, LIMA1, CDC25C, CDC7, GATA3)[31 51] in the TCGA cohort. Moreover, we analyzed three transcriptomic signatures described in ccRCC,[38–40] between each cluster, in each cohort: *angiogenesis* (VEGFA, KDR, ESM1, PECAM1, ANGPTL4, CD34)

signature, predictive of tyrosine kinase inhibitors (TKI) response, and *Teff* (CD8A, EOMES, PRF1, IFNG, CD274) and *JAVELIN Renal 101 Immuno* (CD3G, CD3E, CD8B, THEMIS, TRAT1, GRAP2, CD247, CD2, CD96, PRF1, CD6, IL7R, ITK, GPR18, EOMES, SIT1, NLRC3, CD244, KLRD1, SH2D1A, CCL5, XCL2, CST7, GFI1, KCNA3, PSTPIP1) signatures, predictive of response to CPI.

Statistical analysis

Data processing and statistical analyses were performed using R programming language (V.4.0.3). The heatmaps were generated by applying R package "pheatmap". The data, RNA-seq and IHC, were normalized by the scale function, before clustering. The Kruskal-Wallis test was employed to compare differences between clusters. In all data analyses, a two-tailed p<0.05 was considered statistically significant, with p adjusted for Benjamini-Hochberg correction (*p<0.05 **p<0.01, ***p<0.001). Boxplots were drawn using the ggplot2 package. Kaplan-Meier survival analysis between different clusters was performed using the R survival and survminer packages. The group comparisons were performed with the logrank test with p<0.05, considered statistically significant.

RESULTS

Gene expression analysis of localized pRCC reveal two distinct immune landscapes

The clinical characteristics of 271 patients from TCGA KIRP cohort were presented in table 1.

Discovery of TME subtypes in pRCC

Using MCP-counter methodology,[48 49] we estimated abundances of immune and stromal cell population from cell-specific transcriptomic markers. We applied unsupervised clustering to the abundance scores and identified two different clusters: 21% (n=56) of patients featured an "immune-enriched" tumor and 79% (n=215) an "immune-low" tumor, more heterogeneous (figure 1A). Notably, the "immune-enriched" cluster was significantly characterized by higher abundances of cytotoxic T cells, B cells and natural killer cells (online supplemental figure S1A). There was no significant difference between our two clusters in the number of mutations (data not shown), but there were significantly more copy number alterations of chromosome 7, corresponding to mesenchymal epithelial transition factor (MET) gene, in the "immune-low" cluster (p<0.01) (online supplemental figure S1B). Gene signature mRNA-based concerning the cell cycle, hypoxia, NRF2/ARE, TFE3 fusion, were significantly greater in expression in the "immune-enriched" cluster (p<0.001) (online supplemental figure S1C).

We performed the same analysis in an independent cohort of 98 patients with localized pRCC. We confirmed the presence of "immune-enriched" and "immune-low" TMEs (8% vs 92%, respectively) (online supplemental figure S2A).

Table 1 Characteristics of the localized papillary renal cell carcinoma population, from The Cancer Genome Atlas kidney papillary cell carcinoma cohort

Characteristic	N=271*
Age	61 (53–69)
Unknown	23
Gender	
Female	67 (25)
Male	204 (75)
Type	
Type 1 papillary RCC	159 (59)
Type 2 papillary RCC	78 (29)
Unclassified papillary RCC	34 (13)
Stage	
Stage I	163 (66)
Stage II	21 (8.5)
Stage III	48 (19)
Stage IV	15 (6.1)
Unknown	24
Vital_status	
Alive	231 (85)
Dead	40 (15)
Survival_time (days)	742 (403–1,465)
Unknown	1

*Median (IQR); n (%).
RCC, renal cell carcinoma.

pRCC TME subtypes are associated with immune checkpoints and prognosis

Interestingly, in two independent cohorts, the immune checkpoints markers, *LAG3, TIGIT, CTLA-4, PD-1, PD-L1* were significantly enriched in the "immune-enriched" cluster (adjusted p<0.001) (figure 1B and online supplemental figure S2B). Additionally, the three predictive signatures for response to TKI (*angiogenesis*) and CPI (*Teff* and *JAVELIN Renal 101 Immuno*) in ccRCC were significantly greater in the "immune-enriched" cluster (adjusted p<0.001) (figure 1C and online supplemental figure S2C).

The "immune-enriched" component was associated with a worse prognosis (median OS was 68 months (95% CI, 43.5 to NA) vs not reached, and 12-month OS=87% vs 97%, in "immune-enriched" vs "immune-low" clusters, respectively, p=0.002) (figure 1D). Similarly, the Th2 signature, associated with a poor prognosis in the literature,[31] was significantly higher in the "immune-enriched" cluster (adjusted p<0.001) (online supplemental figure S1D).

Based on these exploratory results in the localized pRCC cohort, we investigated the immune infiltrate in a cohort of metastatic pRCC with treatment data, as a validation cohort.
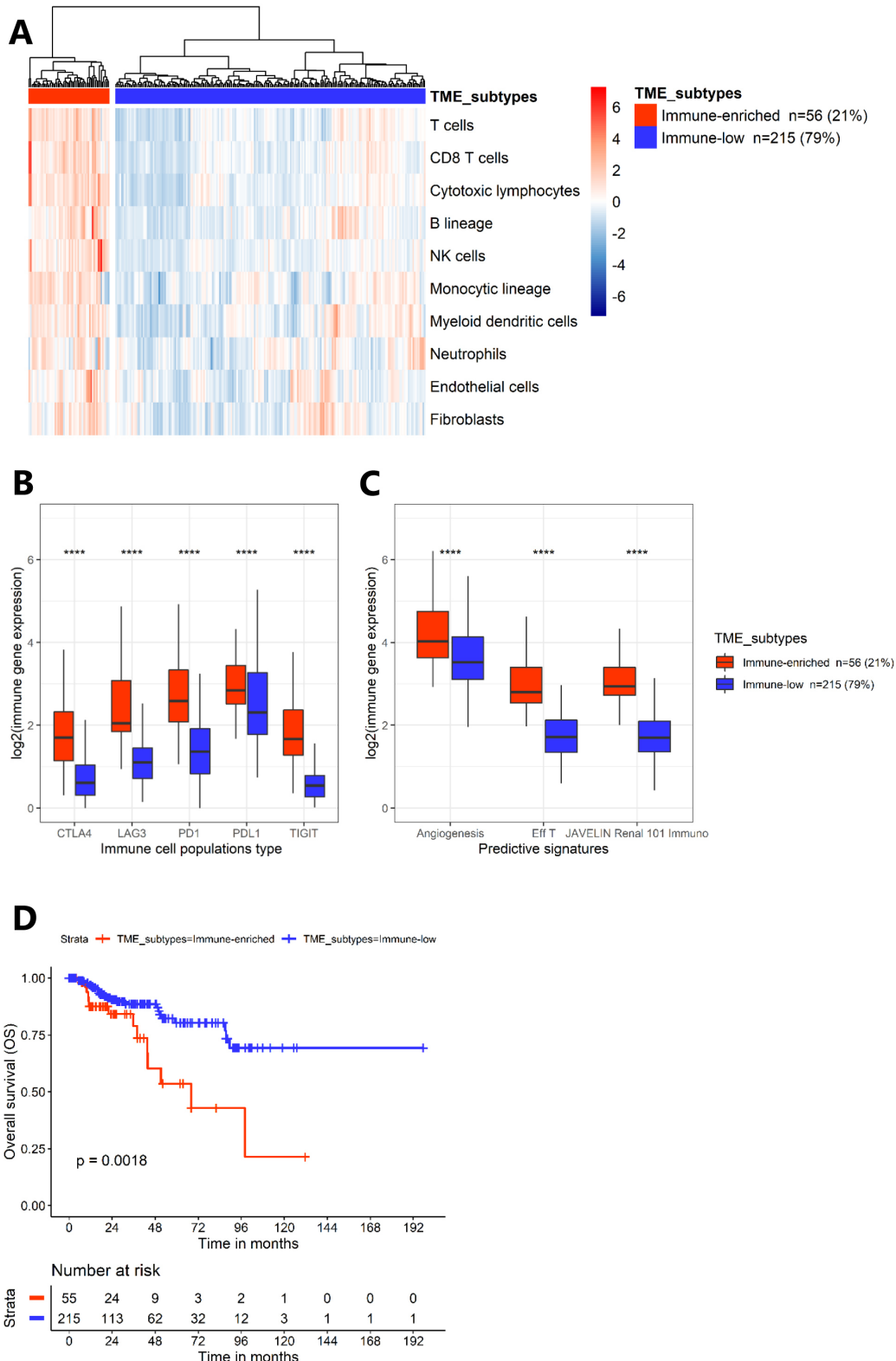
Figure 1 Transcriptomic analyses from The Cancer Genome Atlas kidney papillary cell carcinoma cohort (n=271). (A) Heatmap representing unsupervised analysis from MCP-counter on normalized transcriptomic expression data (k=2), identifying the TME subtypes. (B) Boxplots representing the transcriptomic expression of five checkpoint markers according to clusters from the MCP-counter analysis. (C) Boxplots representing the three predictive gene signatures of response to treatment described in the clear-cell renal cell carcinoma (angiogenesis, effector T-cell (Eff T), JAVELIN Renal 101 Immuno), according to the clusters from MCP-counter. P values were obtained using the two-sided Mann-Whitney test (***p<0.001). (D) Kaplan-Meier survival curves (overall survival (OS)) according to clusters from MCP-counter analysis. CTLA-4, cytotoxic T-lymphocyte–associated antigen 4; LAG-3, lymphocyte activation gene-3; MCP, Microenvironment Cell Populations; NK, natural killer; PD-1, programmed death 1; PD-L1, programmed death-ligand 1; TIGIT, T cell immunoglobulin and ITIM domain; TME, tumor microenvironment.
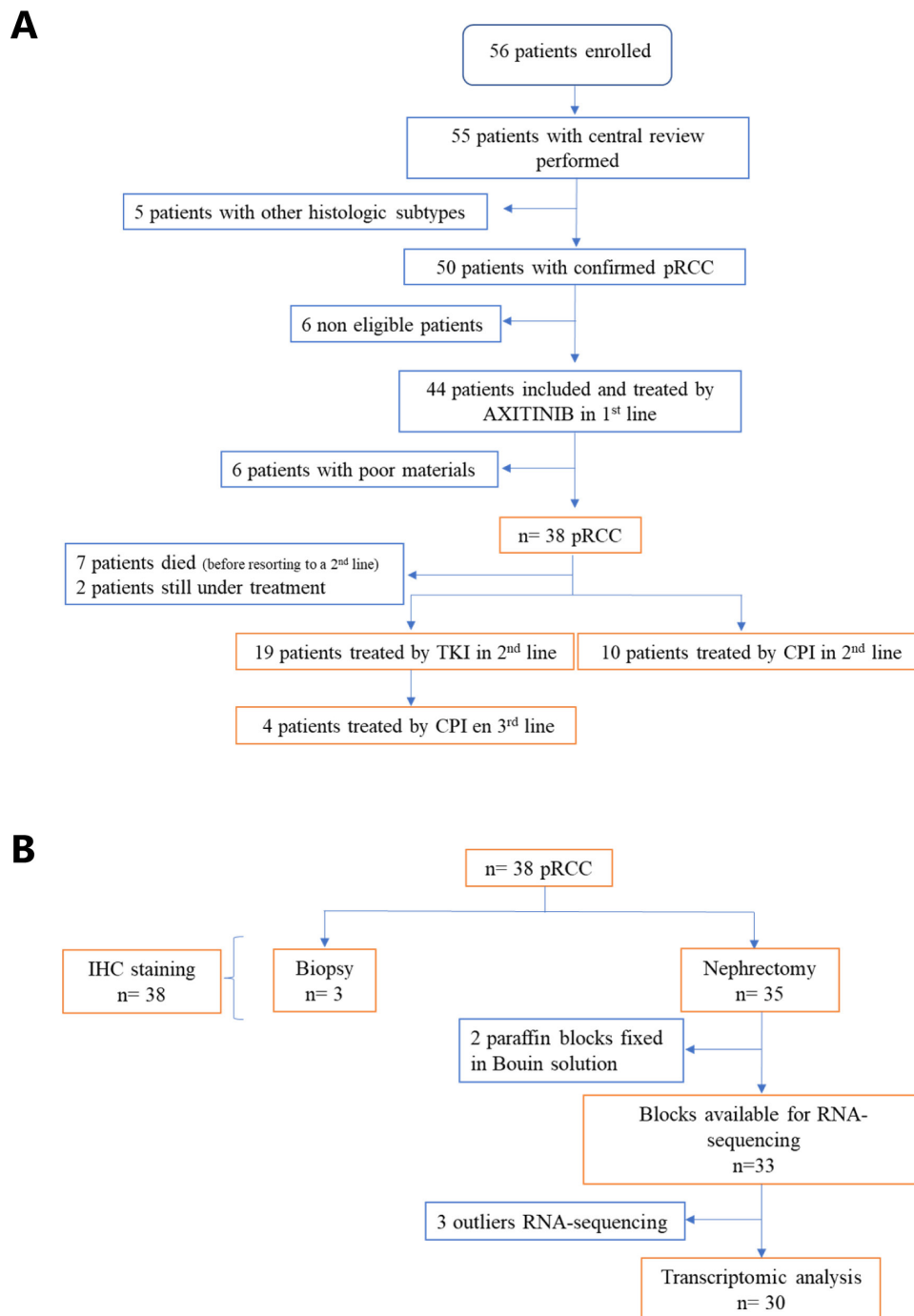
Figure 2 (A) Flow chart of patients included in AXIPAP and their follow-up (clinical data cut-off was February 28, 2021) and (B) with materials available for IHC and RNA-seq analysis. CPI, checkpoint inhibitors; IHC, immunohistochemistry; pRCC, papillary renal cell carcinoma; RNA-seq, RNA-sequencing; TKI, tyrosine kinase inhibitors.

Validation in patients with metastatic pRCC treated by axitinib

We performed a post hoc analysis of the AXIPAP trial[14] from 38 patients with available tissue samples and clinically annotated treatment response on first line systemic therapy, second and subsequent line, represented in flow chart (figure 2A). Fourteen patients were treated with immunotherapy in second or third line including 12 with nivolumab (in the French national AcSé program). The clinical characteristics of these 38 patients were presented

in table 2. Among the 38 cases, 1 (3%) case was from metastatic sites and 37 (97%) cases were primitive pRCC.

We performed IHC on 38 tumor samples, among which 30 samples had RNA of sufficient quality for downstream analyses (figure 2B).

Table 2 Characteristics of the metastatic papillary renal cell carcinoma population, from AXIPAP trial cohort

Characteristic	N=38*
Age	62 (53–69)
Gender	
Female	6 (16)
Male	32 (84)
Type	
Type 1 papillary RCC	12 (32)
Type 2 papillary RCC	26 (68)
Prior nephrectomy	35 (92)
ECOG	
0	19 (50)
1	19 (50)
IMDC score	
Favorable risk group	8 (22)
Intermediate risk group	16 (44)
High-risk group	12 (33)
Unknown	2
Vital_status	
Alive	12 (32)
Dead	26 (68)
Survival_time (months)	18 (8–35)

*Median (IQR); n (%).
ECOG, Eastern Cooperative Oncology Group; IMDC, International Metastatic RCC Database Consortium; RCC, renal cell carcinoma.

Protein analyses of pRCC TME subtypes in metastatic patients treated by axitinib

Using IHC, we scored T lymphocytes (CD3, CD8), macrophages (CD68) (from 0 to 3) and tumor-infiltrating lymphocytes (TILs) scoring, at the invasive front and intratumoral (IT), and the immune checkpoint ligand PD-L1 (% of cells). Based on unsupervised clustering, we identified two groups: 47% (n=18) of patients appeared to have an "immune-enriched" tumor and 53% (n=20) had an "immune-low" tumor (figure 3A). The proportion of histological type 2 pRCC appeared to be higher in the "immune-enriched" subgroup (72% vs 65%, χ^2 test p=0.63). Vascular density was similar in both groups (online supplemental figure S3).

Interestingly, most samples with CD3 IT>1 marker were in the "immune- enriched" group (36/38, 95%).

pRCC TME subtypes "immune enriched" is associated with worse OS and lower response rates to axitinib

As identified with RNA-seq analyses, the IHC "immune-enriched" component featured a worse prognosis. Median OS was 8 months (95% CI, 6 to 29) versus 37 months (95% CI, 20 to NA), and 12-month OS was 33% versus 90%, in "immune-enriched" versus "immune-low" groups, respectively) (p=0.001) (figure 3B).

Similarly, the PFS was significantly worse in this same group with p=0.0007. Median PFS was 5 months (95% CI, 2 to 9) versus 11 months (95% CI, 7 to 26), and 12-month PFS was 6% versus 45%, in "immune-enriched" versus "immune-low" groups, respectively) (figure 3C). ORR to axitinib appeared to be lower in the IHC "immune-enriched" group (11% vs 30%, Fisher's exact test p=0.2) (online supplemental figure S4A). Despite the limited data on responses to second and third line immunotherapy (n=14) in the AXIPAP cohort, we observed that the only partial response corresponded to a patient identified in the "immune-enriched" group. In the same group, we observed one stable disease and one progressive disease. In the "immune-low" group, we identified two stable diseases and seven progressive diseases. The ORR to immunotherapy was better in the "immune-enriched" group (33% vs 0%, Fisher's exact test p=0.16) (online supplemental figure S4B). The two gene expression immune signatures, predictive of response to CPI in ccRCC, were significantly higher in the "immune-enriched" group (adjusted p<0.05) (online supplemental figure S4C). These results suggest that pRCC patients from the "immune-enriched" TME subtype could potentially benefit from immunotherapy.

Transcriptomic characterization of the pRCC TME subtypes defined by IHC

To identify the transcriptomic programs driving the pRCC TME subtypes, we performed differential expression analyses. Under the condition of log2 (fold-change) >2 and p adjusted <0.01, five differentially overexpressed genes were identified between the two groups identified by IHC in the AXIPAP cohort (figure 4A): *IGHV4-61, CXCL13, FDCSP, ADAMTS14, EPHA3*. Three of these genes directly indicate that B cells and tertiary lymphoid structures (TLS) are likely drivers of the pRCC "immune enriched" subtype. *IGHV4-61,* immunoglobulin heavy variable 4–61, is coding for the variable region of an immunoglobulin which indicates involvement of B cells. Further, *CXCL13* (C-X-C motif chemokine ligand 13) is a chemo-attractant for B cells and T follicular helper cells. Finally, *FDCSP* (follicular dendritic cell secreted protein) is indicative of the presence of follicular dendritic cells, key component of mature TLS.

The prognosis of patients with a "high transcriptomic signature", defined as a higher expression of these five genes, seemed to be associated with a dismal prognosis, even if the difference in survival was not significant with p=0.11. Median OS was 10 months (95% CI, 7 to NA) versus 26 months (95% CI, 18 to NA), and 12-month OS was 47% versus 80%, in "high signature" versus "low signature", respectively (figure 4B).

According to the Gene Ontology terms analyses, and in particular biological process, similarly to the TCGA cohort (online supplemental figure S5A), the "immune-enriched" group was significantly associated with the immune-response and B-cell activation pathways (online supplemental figure S5B). Furthermore, from the top
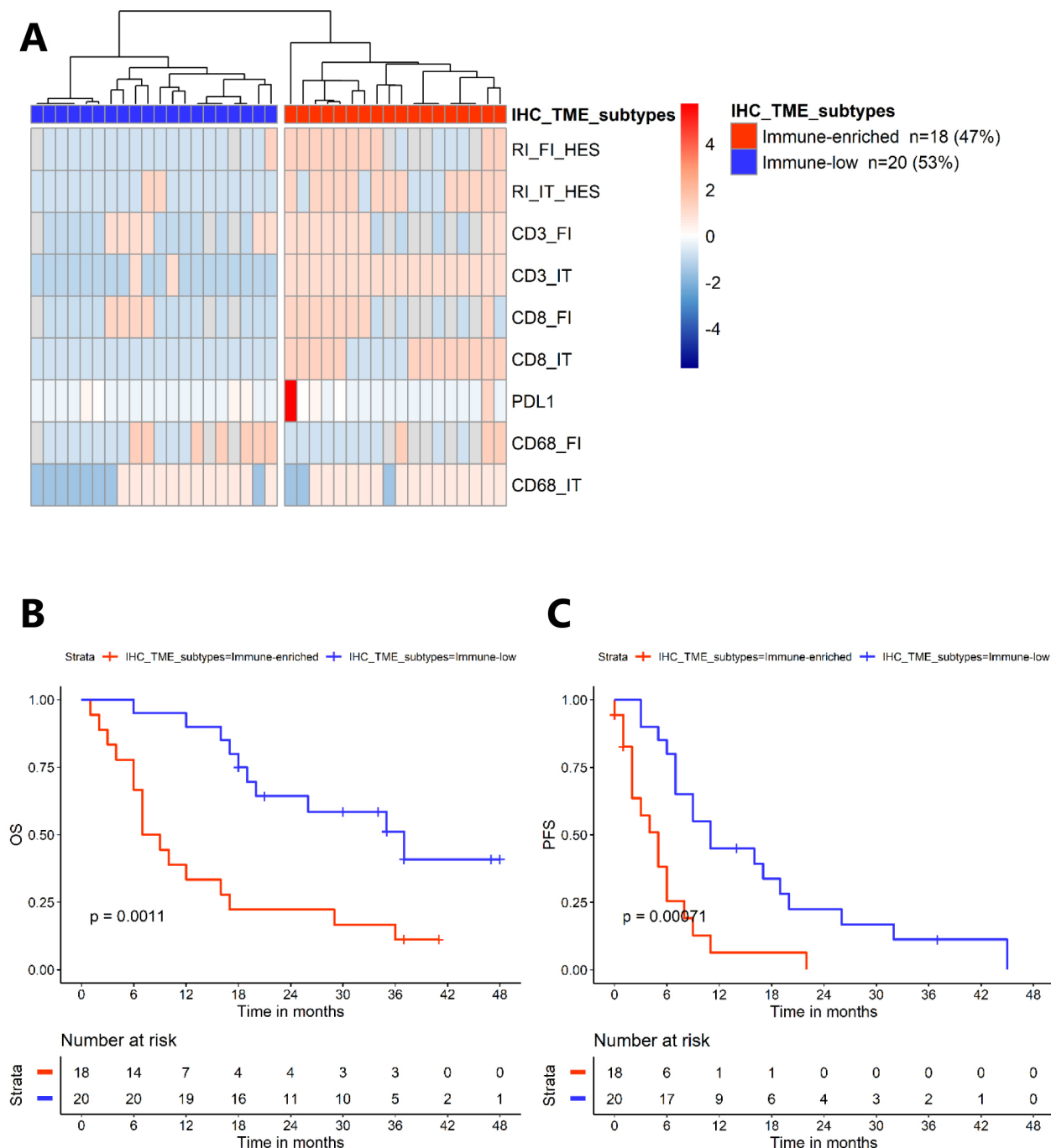
Figure 3 Immunohistochemistry (IHC) analyses from AXIPAP trial cohort (n=38). (A) Heatmap representing unsupervised analysis based on the immune markers performed in IHC (k=2), identifying the "IHC TME subtypes" with the "immune-enriched" group and the "immune-low" group. (B) Kaplan-Meier survival curves (overall survival (OS)) according to subgroups from the unsupervised analysis based on IHC immune markers. (C) Kaplan-Meier survival curves (progression-free survival (PFS)) according to subgroups from the unsupervised analysis based on IHC immune markers. FI, invasive front; HES, Hémalun Eosine Safran; IT, intratumoral; PD-L1, programmed death-ligand 1; TME, tumor microenvironment.

50, 42 pathways were common between the "immune-enriched" and "immune-low" clusters in the TCGA cohort and the "immune-enriched" and "immune-low" groups in the AXIPAP cohort.

DISCUSSION

We report the first comprehensive characterization of immune infiltration in pRCC in both localized and metastatic patients.

Using an unsupervised analysis on localized pRCC data, we identified distinct subsets of pRCC, including one population enriched in immune infiltrate. We confirmed this result in an independent localized pRCC cohort.[45]
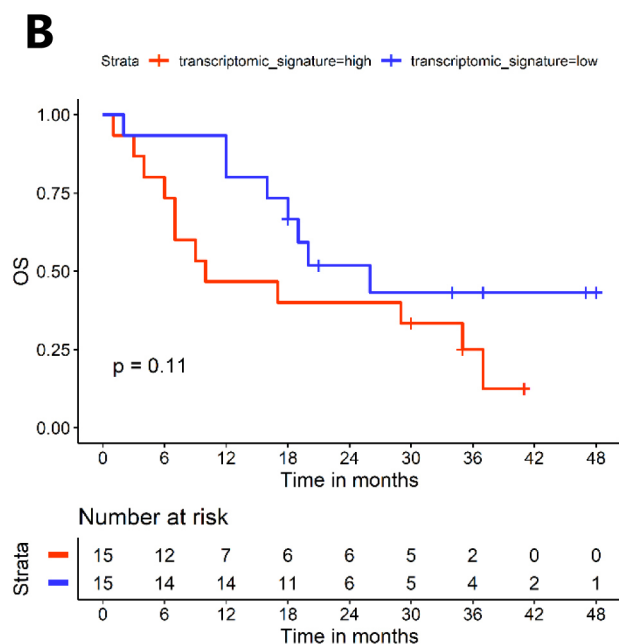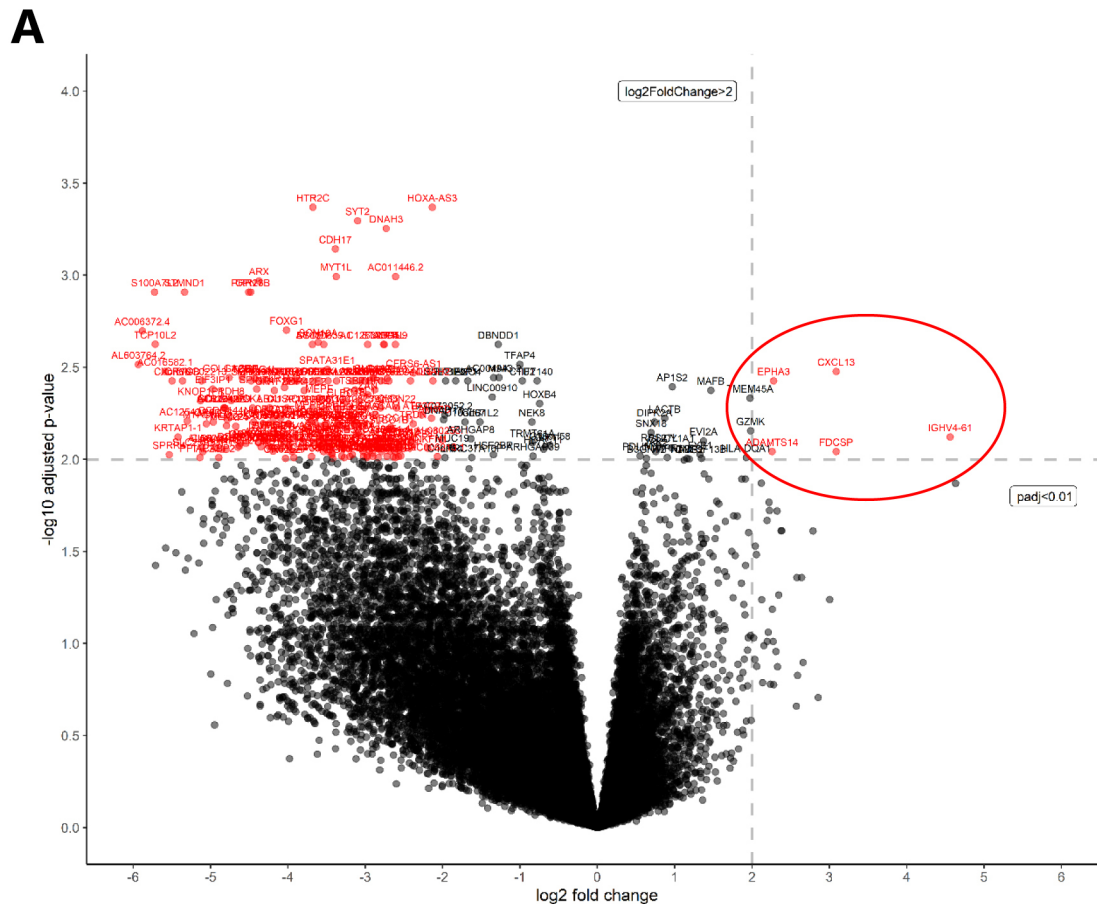
Figure 4 Transcriptomic characterization of the papillary renal cell carcinoma tumor microenvironment subtypes defined by IHC. (A) Volcano plot representing the messenger RNAs differential-expression analysis (=32,378 genes) from DESeq2, between the two groups identified from IHC in the AXIPAP trial cohort (n=30). Under the condition of log2 (fold-change) >2 and p adjusted <0.01, five differentially overexpressed genes were identified between the two groups (red circle). (B) Kaplan-Meier survival curves (overall survival (OS)) according to subgroups with a "high transcriptomic signature" and "low transcriptomic signature", defined as a median of the five significant differentially overexpressed genes. IHC, immunohistochemistry.

Given that the immune infiltrate may differ between localized and metastatic disease,[31 52] we subsequently characterized the TME of patients with metastatic pRCC. We conducted a comprehensive evaluation of immune infiltration, by IHC and RNA-seq, in a prospective metastatic pRCC trial cohort, annotated with systemic therapy treatment.[14]

In the metastatic cohort, we also identified an "immune-enriched" subgroup, by IHC, with the CD3 marker being a routinely applicable IHC staining. Our results provide further granularity to the previously presented analyses from the KEYNOTE-427-B cohort[53] performed in 136 nccRCC, showing that the T-cell–inflamed gene expression profile signature was significantly associated with ORR, which may be predictive of a response to CPI.[54] However, this signature remains based on RNA-seq, therefore limiting its clinical implementation in a daily practice.

In addition to identifying an "immune-enriched" subgroup pRCC, we highlighted its prognostic value, which was significantly worse than that of the identified "immune-low" population, both in RNA-seq and IHC. These results are similar to those of the ccRCC well described in literature.[55 56]

Our results as part of the AXIPAP clinical trial cohort, indicated that both ORR and PFS under axitinib appeared to be lower in the "immune-enriched" group. These findings suggest that other treatment options, such as immunotherapy, may be considered in this subgroup. We previously reported on CPI therapies limited activity in an unselected pRCC population,[24] highlighting the need to identify predictive biomarkers to better select patients more likely to respond to CPI. In our analysis, the "immune-enriched" cluster was characterized by significantly higher gene expression of CPI markers. Interestingly, the study by Şenbabaoğlu et al showed that enriched in T cells ccRCC tumors and responding to immunotherapy had also high expression levels of immune response-related genes, including immune checkpoint genes such as *PD-1, PD-L1 and CTLA-4*.[57] Moreover, the two predictive signatures for response to CPI (*Teff* and *JAVELIN Renal 101 Immuno*), described in ccRCC, were significantly greater in the "immune-enriched" cluster. Despite the limited data on responses to second and third line immunotherapy in our AXIPAP cohort, the ORR to immunotherapy appeared numerically higher in the "immune-enriched" group. Taken altogether, these results suggest that the "immune-enriched" feature may have potential predictive value for a favorable response to immunotherapy.

Furthermore, in order to characterize the difference between the two groups identified in IHC, we performed mRNAs differential expression analysis with strict significance criteria. We highlighted five differentially over-expressed genes in the "immune-enriched" group. The gene IGHV4-61 is part of B lymphocyte populations. The gene CXCL13, corresponds to chemokine (C-X-C motif) ligand 13 or BLC (B-lymphocyte chemoattractant) or BCA-1 (B cell-attracting chemokine 1). His role is selective chemotaxis for B cells, a product of follicular helper CD4+T cells and a contributor to TLS.[58] The gene FDCSP, follicular dendritic cell secreted protein, is able to specifically bind to activated B cells and is intimately connected to chemokine pathways, particularly with the CXCL13.[59] Follicular dendritic cells are found in the center of the germinal center of mature tertiary lymphoid structures[60] where they are likely to present antigen to B cells. The gene ADAMTS14, a disintegrin and metalloproteinase with thrombospondin motifs 14, is mainly involved in extracellular matrix assembly and degradation. It is highly associated with several immune cells in ccRCC, such as activated dendritic cell, central memory CD8 T cell, central memory CD4 T cell, and activated CD4 T cell.[61] Finally, the gene EPHA3, Eph receptor tyrosine kinases, formerly known as HEK, contribute to tumor development, modulating cell–cell adhesion and survival during invasion, neo-angiogenesis and metastasis.[62] Therefore, the gene EPHA3 represents a potential source of tumor-specific antigens recognized on tumor cells that express human leukocyte antigens (HLA) class II molecules. It appears to function as a tumor-suppressor in ccRCC as in the tumor stromal microenvironment with mesenchymal stromal cells (MSCs).[63] All these genes are related to the B population and TLS, recently described in the literature. Notably, tumors with mature TLS, a high density of B cells and plasma cells, as well as the presence of antibodies to tumor-associated antigens are typically associated with favorable clinical outcomes and responses to immunotherapy compared with those lacking these characteristics.[60]

Our exploratory analysis of a transcriptomic signature, defined as a higher expression of these five genes, seemed to be associated with a dismal prognosis in OS. This result is consistent with the literature as these five genes are described in ccRCC, each of which is associated with a poor prognosis and decreased survival of patients.[64–67] IT CXCL13+CD8+T cells abundance was associated with immune-evasive contexture. The abundance of CXCL13+CD8+T cells was shown to be an independent prognosticator and a potential immunotherapeutic target marker for ccRCC treatment.[68] Luo et al reports that four TME-related genes (CD79A, CXCL13, IL-6 and CCL19) were identified as biomarkers for pRCC prognosis in localized pRCC from the TCGA cohort.[69]

Beyond their prognostic value, data from the recent literature showed that the B and TLS populations have a potential predictive value for response to immunotherapy in various cancers,[66 70] demonstrating significantly higher expression of B-cell-related genes in responders versus non-responders.[71] CXCL13 expression, as a surrogate for tumor TLS, is a relevant candidate predictive biomarker of response to CPI for patients with advanced-stage bladder cancer.[72] Similarly, in the NIVOREN cohort, Carril-Ajuria et al, demonstrated for the first time, that a pre-existing high number of circulating baseline unswitched memory B cells is associated with higher probability of response to

nivolumab and longer PFS and OS in patients with metastatic ccRCC.[73]

Our study has several limitations. As pRCC are rare tumors, our analyses were performed on a limited number of available samples. We can note the differences in clustering proportions between cohorts, which can be explained by metastatic tumors, known to be more inflammatory and aggressive than localized pRCC,[52] the heterogeneity of techniques between RNA-seq, microarray, and IHC, and also a potential lack of power due to a limited number of available samples. Moreover, sequencing techniques, especially on paraffin blocks, are recent, and may generate biases in the results. Similarly, it would be interesting to be able to confirm the possibility of identifying the "immune-enriched" group from the CD3 marker in another independent cohort. Finally, the potential predictive value of response to immunotherapy of our identified "immune-enriched" group can only be formulated as a hypothesis since we have limited data from further CPI treatment. It will be necessary to validate our results and hypotheses in a prospective cohort of metastatic pRCC treated with CPI alone and in combination with a TKI.

CONCLUSION

In summary, for the first time, based on a comprehensive analysis, using RNA-seq and IHC, we identified a specific immune TME subtype of metastatic pRCC, significantly more infiltrated with cytotoxic T and B immune populations. The identification of this group could be done by IHC, and in particular by the CD3 marker, a reliable and inexpensive technique. This "immune-enriched" feature, with its defining markers highlighted, appears to be correlated with a poor prognosis but could indicate a potential predictive value for response to immunotherapy. This, however, requires a confirmation in metastatic pRCC treated with CPI alone and in combination with a TKI.

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