Evaluation of programmed cell death protein 1 (PD-1) expression as a prognostic biomarker in patients with clear cell renal cell carcinoma

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ABSTRACT
Programmed cell death protein 1 (PD-1) immune checkpoint inhibitors have shown activity in patients with advanced renal cell carcinoma (RCC). However, the role of PD-1 expression in tumor-infiltrating lymphocytes (TILs) as a biomarker for poor outcome is not clear. In this study, we evaluated the prognostic value of TIL PD-1 expression in patients with clear cell RCC (ccRCC). 82 patients who underwent nephrectomy for localized or metastatic ccRCC and followed up for at least four years were searched from our database and retrospectively enrolled. Their fixed primary tumor specimens were stained with anti-PD-1 (NAT105). The specimens were classified as negative or positive for PD-1 expression, and the positive specimens were further scored in 10% increments. 37 (45.12%) patients were negative (~1% stained), 26 (31.71%) patients were low (~10 and 10%), and 19 (23.17%) patients were high (20–50%) for PD-1 expression. The prognostic value of TIL PD-1 expression was evaluated by univariate Cox proportional hazards regression on overall and recurrence-free survivals. Higher TIL PD-1 expression was not associated with increased risk of death (P = 0.336) or with increased risk of recurrence (P = 0.572). Higher primary tumor stage was associated with increased risk of recurrence (P = 0.003), and higher Fuhrman nuclear grade was associated with increased risk of death (P <0.001) and with increased risk of recurrence (P <0.001). Our study shows that TIL PD-1 expression by immunohistochemistry (IHC) does not correlate with poor clinical outcome in patients with ccRCC and is inferior to established prognosticating tools.

INTRODUCTION
Renal cell carcinoma (RCC) presents as regional or metastatic disease in a third of patients at the time of diagnosis.1 While those with clinically localized disease are surgically treated, the recurrence rate is 20–40%.2 In sum, approximately half of all RCC patients are eligible for systemic treatment at some point of the disease course. Immunotherapy is a promising treatment modality that bolsters host anti-tumor response and may accomplish durable remission. For example, high-dose interleukin-2 (IL-2) is the only agent to have demonstrated durable remission in RCC to date, albeit largely displaced by angiogenesis and mammalian target of rapamycin (mTOR) pathway inhibitors due to its modest efficacy and high toxicity.3

RCC is a promising candidate for immunotherapy due to its immunogenic nature. It is characterized by dense intra-tumor immune infiltrate that is associated with prognosis.4–8 Much research interest exists on these tumor-infiltrating immune cells and their altered phenotype—reduced proliferative capacity and effector function—as therapeutic target. This dysfunctional state of T cells, termed ‘exhaustion’ and reviewed extensively elsewhere, arises from chronic antigen exposure in the setting of chronic viral infection or cancer and has been proposed as a mechanism of immune evasion in RCC.9–13

Exhausted T-cells characteristically overexpress inhibitory co-receptors such as programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).9,14 The latest developments in cancer immunotherapy modulate these inhibitory pathways to reverse T cell exhaustion and reinvigorate immune response.10,11 The best known example is an anti-PD-1 antibody nivolumab that was approved in 2015 for RCC treatment on the basis of a phase III trial that showed improved survival, higher response rate, and less frequent adverse events compared to an mTOR inhibitor everolimus.15,16 An anti-CTLA-4 antibody ipilimumab is also being evaluated as an adjunct therapy with nivolumab in a phase III trial at the time of writing [NCT02231749].

While nivolumab unequivocally demonstrated its efficacy in clinical trials, there is a need for a better tool for patient selection. This is particularly important given modest objective response rates; 1% of the Phase III trial subjects showed complete response and 25% partial response,16 similar to figures reported with high-dose IL-2 treatment.3 This suggests that
both IL-2 and nivolumab may be highly effective only in a subset of ‘immune-responsive’ patients who we cannot yet reliably identify.\textsuperscript{17} Furthermore, its benefit in patients who show any less than complete response remains to be seen, extrapolating from the precedent high-dose IL-2 study in which all partial responders eventually demonstrated recurrence in contrast to 17% of complete-responders.\textsuperscript{3} Long-term benefit of nivolumab treatment is under active research.\textsuperscript{18}

A number of studies have associated PD-1 and Programmed death-ligand 1 (PD-L1) expression on immunohistochemistry (IHC) in RCC with clinical outcome\textsuperscript{19-30} and/or response to immunotherapeutic agent with conflicting results.\textsuperscript{16,19-32} While earlier studies reported a significant association between PD-1/ PD-L1 expression and survival or recurrence, later studies did not replicate those findings. In addition to prognostication, no study has demonstrated correlation between PD-L1 expression and treatment benefit, highlighting the need to elucidate the pathophysiological role of PD-1 and PD-L1.

In this study, we aimed to add to the current knowledge on prognostic value of tumor-infiltrating lymphocyte (TIL) PD-1 expression by evaluating its impact on overall survival (OS) and recurrence-free survival (RFS) in a single-center cohort with long-term clinical follow-up. We concurrently evaluated association between primary tumor stage (pT stage), Fuhrman nuclear grade (FNG)—two widely used prognostic tools—and clinical outcome to compare their prognostic value with that of TIL PD-1 expression.

Methods

Patient selection

Upon approval by Emory University Institutional Review Board, we reviewed the Emory Nephrectomy Registry to identify patients who underwent nephrectomy for treatment of clear cell renal cell carcinoma between 2005 and 2013. The enrolled patients had made full informed consent that permitted storage and analysis of surgically removed organ specimens and blood as well as access to medical records by authorized researchers. Enrollment into this study and procurement of the samples did not incur any risk to the patient beyond that of the actual surgery done as standard of care, and the results of the study did not affect the patient’s medical care in any way. Only patients over age of 18 at the time of surgery were reviewed. Inclusion in the study was limited to patients with 4 years of post-operative follow-up unless they had documented death during that period. 82 patients were identified who met the inclusion criteria and had archived formalin-fixed paraffin-embedded tumor tissue blocks available for study. Their primary tumor tissue blocks were retrieved from our nephrectomy tissue library irrespective of presence or absence of nodal disease or distant metastasis at the time of surgery. Medical records of the patients were accessed on November 2016 to obtain clinical information such as age at intervention, sex, and race as well as tumor pathology.

Tumor pathology and clinical follow-up information

Primary tumor stage and Fuhrman nuclear grade of the specimens obtained from anatomic pathology reports were re-evaluated and confirmed. Baseline presence or absence of metastatic disease was determined from pre-operative clinic notes and cross-sectional imaging studies (MRI or IV-contrasted CT) of the chest, abdomen, and pelvis. Serial post-operative clinic notes and radiology reports were accessed to document disease course. Standard of care post-operative surveillance at our institution consisted of symptom review and cross-sectional imaging studies every 3 months for the first year, spaced out to every 6 months until 5 years after the surgery. Patients without evidence of disease after 5 years were evaluated by renal ultrasound annually. Patient vital status was obtained from chart review, and if not documented in our institution’s medical records, from Social Security Death Index. Patients alive at the time of chart review were censored on the date of the most recent presentation to our institution or documented communication by phone. Date of recurrence was defined as the date of the first imaging study that showed tumor growth at the surgical site or at a distant site. Imaging findings that were concerning but not certain to be recurrent RCC were ascertained from biopsy reports. Patients with no evidence of recurrence throughout surveillance were censored on the date of the most recent imaging.

Immunohistochemistry and PD-1 quantification

Tumor sections were de-paraffinized and prepared into slides using standard immunohistochemistry techniques. Two slides were made per each tissue sample. One slide was stained with hematoxylin and eosin, and the other slide was stained for PD-1 using a commercially available anti-PD-1 antibody NAT105 (Abcam) at 1:100 dilution. The H&E sections of the tumors were stained. Finally, univariate Cox proportional hazards regression were conducted to evaluate the impact of TIL PD-1 expression to compare their prognostic power to that of TIL PD-1 expression.
of TIL PD-1 expression. Statistical significance was set at two-sided $P < 0.05$. All statistical analyses were performed using SAS 9.4.

**Results**

**Cohort description**

82 clear cell RCC patients who met the inclusion criteria were enrolled. Patient characteristics are summarized in Table 1. Median (range) age at intervention was 60 (26–89), and overall median (IQR) follow-up was 70.7 (48.2–83.8) months. 30 patients had died by the time of chart review with median (IQR) follow-up of 40.6 (16.9–66.3) months. 52 surviving patients had median (IQR) follow-up of 74.3 (63.0–91.8) months. Distribution of pT stage from pT1 to pT4 in ascending order was 30, 19, 32, and 1. The single pT4 patient was negative for PD-1 expression. Most covariates (age at surgery, race, pT stage, FNG, and presence of baseline metastasis) were not significantly associated with TIL PD-1 expression on any level: PD-1-low (HR 1.43; HR $P$-value 0.336). Kaplan-Meier survival curves by PD-1 expression levels are shown in Fig. 2.

**TIL PD-1 expression**

Representative photomicrographs of clear cell RCC tumor-infiltrating lymphocytes stained for PD-1 are shown in Fig. 1. The distribution of TIL PD-1 expression of the overall survival cohort (N = 82), measured in 10% increments, is shown in Table 2. By the 2-way stratification, 37 (45.12%) patients were negative and 45 (54.88%) patients were positive for PD-1 expression. Most covariates (age at surgery, race, pT stage, FNG, and presence of baseline metastasis) were not significantly associated with TIL PD-1 expression by ANOVA and $\chi^2$ tests except for patient sex (Table 1). By the 3-way stratification, 37 (45.12%) patients were negative, 26 (31.71%) patients were low, and 19 (23.17%) patients were high for PD-1 expression. The distribution of TIL PD-1 expression of the recurrence-free survival cohort (N = 67) is shown in Table 3. By the 2-way stratification, 32 (47.76%) patients were negative and 35 (52.24%) patients were positive. By the 3-way stratification, 32 (47.76%) patients were negative, 21 (31.34%) patients were low, and 14 (20.90%) patients were high for PD-1 expression. The specimens from female patients were likely to express less PD-1 than those from male patients in 3-way stratification ($\chi^2$ $P = 0.018$): they expressed 20, 9, and 3 in negative, low, and high PD-1 levels, in comparison to 17, 17, and 16 in those from male patients, respectively. Since PD-1 is known to be expressed in macrophages as well as in TILs, a separate experiment was done to verify that the PD-1 expression we scored was contributed by mostly TILs and not macrophages. Three tissue slides were cut from 19 randomly selected primary ccRCC samples. One set was stained with H&E, the other with CD68, an immune marker of macrophages, and the last with PD-1. All 19 samples demonstrated scattered patterns of CD68. 17 samples stained positive for PD-1, and 2 were negative. Comparing the areas of heavy PD-1 positivity with the same areas on the CD68 stained slide showed that the staining pattern differed between the two stains, showing that lymphocytes, not macrophages, are preferentially stained by PD-1 (data not shown).

**Impact of TIL PD-1 expression on overall survival**

Univariate Cox proportional hazards regression on overall survival (N = 82) was performed by TIL PD-1 expression level (Table 2). In the 2-way analysis, PD-1-positive patients did not have a significantly increased risk of death compared to PD-1-negative patients (HR = 1.25; 95% CI 0.61–2.55; HR $P$-value = 0.548). Subdivision of the PD-1-positive patients into PD-1-low and PD-1-high patients did not reveal a significant association between survival and TIL PD-1 expression on any level: PD-1-low (HR = 0.58; 95% CI 0.24–1.43; HR $P$-value = 0.241), PD-1-high (HR = 1.21; 95% CI 0.51–2.89; HR $P$-value = 0.669), and overall (log-rank $P = 0.336$). Kaplan-Meier survival curves by PD-1 expression levels are shown in Fig. 2.

**Table 1. Patient characteristics and tumor pathology by TIL PD-1 positivity (N = 82).**

| Variable                  | Total (N = 82) | PD-1 negative (N = 37) | PD-1 positive (N = 45) | Parametric P-value |
|---------------------------|---------------|------------------------|------------------------|--------------------|
| Age at surgery (yrs)      |               |                        |                        |                    |
| Median (range)            | 60 (26–89)    | 61 (45–83)             | 58 (26–89)             | 0.229              |
| Male                      | 50 (60.98)    | 17 (45.95)             | 33 (73.33)             | 0.011              |
| Female                    | 32 (39.02)    | 20 (54.05)             | 12 (26.67)             |                    |
| Race                      |               |                        |                        |                    |
| Caucasian                 | 57 (76)       | 27 (81.82)             | 30 (71.43)             | 0.296              |
| Other                     | 18 (24)       | 6 (18.18)              | 12 (28.57)             |                    |
| pT stage                  |               |                        |                        |                    |
| pT1                       | 30 (36.59)    | 11 (29.73)             | 19 (42.22)             | 0.484              |
| pT2                       | 19 (23.17)    | 9 (24.32)              | 10 (22.22)             |                    |
| pT3–4                     | 33 (40.24)    | 17 (45.95)             | 16 (35.56)             |                    |
| FNG                       |               |                        |                        |                    |
| 2                         | 33 (40.24)    | 14 (37.84)             | 19 (42.22)             | 0.578              |
| 3                         | 41 (50)       | 18 (48.65)             | 23 (51.11)             |                    |
| 4                         | 8 (9.76)      | 5 (13.51)              | 3 (6.67)               |                    |
| Baseline metastasis       |               |                        |                        |                    |
| Yes                       | 13 (15.85)    | 3 (8.1)                | 10 (22.2)              | 0.128              |
| No/Localized              | 69 (84.15)    | 55 (94.59)             | 19 (42.22)             |                    |

Values expressed as N (%). Considered PD-1 positive if stained ≥ 1%. A single pT4, PD-1-negative patient was merged with the pT3 group. There was no FNG 1 in the cohort. Significant P-value bolded. No covariates except patient sex was significantly associated with PD-1 status. pT stage: primary tumor stage by size; FNG: Fuhrman Nuclear Grade.
Impact of TIL PD-1 expression on recurrence-free survival

Univariate Cox proportional hazards regression on recurrence-free survival (N = 67) was performed by TIL PD-1 expression level (Table 3). In the 2-way analysis, PD-1-positive patients did not have an increased risk of recurrence compared to PD-1-negative patients (HR = 1.07; 95% CI 0.5–2.27; HR P-value = 0.866). Subdivision of the PD-1-positive patients into PD-1-low and PD-1-high patients did not result in a significant association between recurrence and TIL PD-1 expression on any level: PD-1-low (HR = 0.74; 95% CI 0.29–1.85; HR P-value = 0.512), PD-1-high (HR = 1.29; 95% CI 0.51–3.24; HR P-value = 0.588), and overall (log-rank P = 0.572). Kaplan-Meier analysis on recurrence-free survival by PD-1 expression levels are shown in Fig. 3.

Impact of primary tumor stage and Fuhrman nuclear grade on clinical outcome

Univariate Cox proportional hazards regression on overall survival (N = 82) and recurrence-free survival (N = 67) was performed by pT stage and FNG (Table 4). Higher pT stage trended with increased risk of death (N = 82): pT3–4 had a 2.28 times increased risk over pT1 (95% CI 0.98–5.3; HR P-value = 0.055; overall log-rank P = 0.053). Higher pT stage significantly correlated with risk of recurrence (N = 67): pT3–4 had 3.84 times increased risk over pT1 (95% CI 1.56–9.47; HR P-value = 0.003; overall log-rank P = 0.003). The correlation between Fuhrman nuclear grade and clinical outcome was more pronounced. Higher FNG significantly correlated with increased risk of death (N = 82) on all grades: FNG 3 (HR over FNG 2 = 3.75; 95% CI 1.4–10.04; HR P-value = 0.009), FNG 4 (HR over FNG 2 = 10.5; 95% CI 3.17–34.79; HR P-value <0.001), and overall (log-rank P <0.001). Similarly, Higher FNG significantly correlated with increased risk of recurrence (N = 67) on all grades: FNG 3 (HR over FNG 2 = 3.99; 95% CI 1.47–10.83; HR P-value = 0.007), FNG 4 (HR over FNG 2 = 16.2; 95% CI 4.58–57.28; HR P-value <0.001), and overall (log-rank P <0.001).

Discussion

Programmed cell death protein 1 was first identified in 1992 as a transmembrane receptor that induces cell death in murine T-cell hybridoma and lymphoid/myeloid progenitor cell lines. PD-1 receptor is expressed in multiple immunocytes such as thymocytes undergoing positive selection, mature T and B cells following activation, and macrophages. PD-1 pathway is crucial for immune homeostasis and prevention of autoimmunity; PD-1 deficient mice develop various immunopathologies such as glomerulonephritis, arthritis, and cardiomyopathy. In contrast, high PD-1 expression in T cells has been associated with good clinical outcome in autoimmune and inflammatory diseases and poor outcome/response to therapy in chronic viral infection and vaccination.

The observation that PD-L1 is expressed in a broad range of human cancers led to a hypothesis that it contributes to tumor

Table 2. Distribution of TIL PD-1 expression and its impact on risk of death.

| TIL PD-1 expression (%) | N | OS 2-way analysis (N = 82) | OS 3-way analysis (N = 82) |
|------------------------|---|---------------------------|----------------------------|
|                        |   | N | N | HR (95% CI; HR P) | N | HR (95% CI; HR P) | L-R P |
| <1                     | 37| 37| Reference              |                           |
| <10                    | 15| 45| 1.25 (0.61–2.55; 0.548) | 26| .58 (0.24–1.43; 0.241) |
| 10                     | 11| 8 |                           | 19| 1.21 (0.51–2.89; 0.669) |
| 20                     | 8 | 6 |                           |               |
| 30                     | 4 | 4 |                           |               |
| 40                     | 1 | 1 |                           |               |

TIL PD-1 expression was stratified negative (<1%) / positive (≥1%) or negative (<1%) / low (<10 and 10%) / high (20–50%). Univariate Cox proportional hazards regression was performed. No statistically significant relation between TIL PD-1 expression and risk of death was found. TIL: tumor-infiltrating lymphocyte; OS: overall survival; HR: hazard ratio; L-R: log-rank.
immune evasion from tumor-infiltrating lymphocytes. The hypothesis has been subsequently tested in various cell-lines and murine cancer models and resulted in multiple immune checkpoint inhibitors being developed. PD-1 blockade in particular has produced positive clinical results in treatment of advanced melanoma, non-small cell lung carcinoma, renal cell carcinoma, and metastatic bladder cancer. However, response rate and treatment benefit have been modest. The role of PD-1 and PD-L1 expression as a determinant of responsiveness to therapy or prognostic marker is not established across different cancers, including renal cell carcinoma.

In this study, we evaluated the association between tumor-infiltrating lymphocyte PD-1 expression and clinical outcome in 82 clear cell renal cell carcinoma patients with long clinical follow-up (>4 years). We did not find TIL PD-1 expression on immunohistochemistry to correlate with risk of death or recurrence, whether stratified in a binary (negative/positive) or in a semi-quantitative (negative/low/high) manner. However, we did find primary tumor stage and Fuhrman nuclear grade to correlate with clinical outcome; primary tumor stage significantly correlated with risk of recurrence, and Fuhrman nuclear grade significantly correlated with both risk of death and risk of recurrence. This confirmed the utility of the two established prognostic markers over TIL PD-1 expression.

Several prior studies have correlated PD-1/PD-L1 expression with clinical outcome (Table 5). However, substantial inter-study variability exists among the conclusions of the studies even when the comparison is confined to clear cell subtype. Among the studies that quantified PD-1 expression, Thompson, et al., Kang, et al., and Giraldo, et al. reported significant association with clinical outcome, with a caveat that the last study noted inconsistent staining between invasive margin and tumor core. In contrast, Shin, et al. found only a trend between PD-1 positivity and cancer-specific and progression-free survivals. Among the studies that quantified PD-L1 expression, Thompson, et al., Choueiri, et al., Shin, et al., and Abbas, et al. reported significant association. Giraldo, et al. found borderline association, while Leite, et al. found it to correlate with pathological features such as higher FNG (P = 0.021) and microvascular invasion (P = 0.039) but no other prognostic factors.

The inter-study discrepancies stem from technical challenges of immunohistochemistry as well as intrinsic complexity of tumor biology. Arguably the largest sources of discrepancy are the lack of standards in PD-1/PD-L1 scoring methods and threshold for positivity as well as inter-rater reliability (Table 5). Among the listed studies, proportion of samples read as positive ranged from 18.8% to 43.2% for PD-1 positivity, and from 12.6% to 56.5% for PD-L1 positivity. Our study reports 54.88% of the cohort to be PD-1 positive. This value is higher than what others reported because the low threshold of 1% we used. Using a different cutoff for PD-1/PD-L1 dramatically changed the fraction of ‘positive’ patients; applying a threshold of 5–10% resulted in a value in line with other studies. Dramatic variation of percent positivity with different thresholds has been criticized as a design flaw in other studies of this kind. Further stratification of PD-1 expression into negative/low/high in this study was intended to partially mitigate this issue.

Table 3. Distribution of TIL PD-1 expression and its impact on risk of recurrence.

| TIL PD-1 expression (%) | N     | N     | HR (95% CI; HR P)  | N     | HR (95% CI; HR P) | L-R P |
|------------------------|-------|-------|-------------------|-------|-------------------|-------|
| <1                     | 32    | 32    | 1.07 (0.52–2.27; 0.866) | 21    | 0.74 (0.29–1.85; 0.512) | 0.572 |
| <10                    | 12    | 35    |                  | 14    | 1.29 (0.51–3.24; 0.588) |
| 10                     | 9     |       |                  | 14    |                   |
| 20                     | 7     |       |                  | 14    |                   |
| 30                     | 5     |       |                  |       |                   |
| 40                     | 1     |       |                  |       |                   |
| 50                     | 1     |       |                  |       |                   |

Figure 2. Kaplan-Meier curves on overall survival by TIL PD-1 expression. A (left): TIL PD-1 expression stratified negative (<1% stained, blue) / positive (≥ 1% stained, red). B (right): TIL PD-1 expression stratified negative (<1% stained, green) / low (<10 and 10%), red) / high (20–50%, blue). Vertical tick marks represent censored subjects.
There are other technical challenges of immunohistochemistry that need to be addressed. Except the earliest studies that used fresh-frozen tissue, most studies used fixed tissue, because it was conducive to large retrospective studies. It has been suggested that fixation process may compromise antigen staining and underestimate the prevalence of PD-L1 expression. Furthermore, several different antibody clones with presumably different affinities have been used. The prevalence of PD-L1 expression were shown to vary significantly when using different antibodies. Another technical issue is that, while it is known that PD-1 and PD-L1 are expressed in multiple cell types ranging from tumor cells to stromal cell elements and various immune cell types, investigators have not employed methods such as double staining to control which cell type is counted, nor do we know which cell population is clinically relevant for predicting prognosis or treatment benefit.

Tumor heterogeneity further complicates the matter. A multi-region sequencing study showed that spatially separated samples from the same RCC tumor underwent a number of convergent and divergent mutations, some of which are signatures of good prognosis and some of poor prognosis. Supporting that finding, studies on variability of PD-L1 expression within tumor and between primary and metastatic sites have been inconclusive, neither showing dependence or independence. It remains to be determined whether sampling multiple sites adds value to clinical decision making or where in the tumor spatially (invasive margin versus tumor core) best represents the tumor microenvironment. A way to potentially circumvent the tumor heterogeneity issue is to measure systemic markers circulating in the blood, and to that end, an association between level of soluble PD-L1 circulating in serum and survival has been reported in clear cell RCC patients.

Lastly, cellular PD-1/PD-L1 expression seems to occur in a spectrum rather than simple on/off. Flow cytometric studies have reported different tiers of cellular PD-1 expression that would not be distinguishable using immunohistochemistry techniques. Blackburn et al. identified two subsets of exhausted CD8 T cells from mice with chronic LCMV infection and demonstrated that the T cells expressing intermediate level of PD-1 could be reinvigorated with PD-1 blockade while the T cells expressing high level of PD-1 could not, representing a more terminally differentiated T cell population. It is known that PD-1 is transiently expressed in effector T cells and chronically expressed in exhausted T cells, and these two T cell subsets have opposite prognostic implications. To our knowledge, no IHC study has analyzed differential PD-1 expression and considered its clinical impact as it relates to prognosis or response to anti-PD-1 therapy. In this regard, immunohistochemistry may be lacking the quantitative resolution needed to study PD-1 and PD-L1 expression. Recent studies have quantified PD-L1 on cell lines and mouse models using quantitative RT-PCR or flow cytometry and may represent future directions.

This study shares the limitations listed above with other studies; therefore, inter-study comparison must be done with caution. Other limitations of this study include a relatively small cohort of retrospective nature that can introduce selection bias. We only performed univariate analysis with PD-1 expression and not adjust for pT stage and FNG for two reasons: first, we wanted to compare the prognostic value of PD-1 positivity to those of pT stage and FNG, and second, the univariate correlations were already extremely weak that multivariate analysis would not bring forth statistical significance. In regard to the choice of antibody, the anti-PD-1 antibody

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**Table 4. Distribution of pT stage and FNG and their impact on risk of death and risk of recurrence.**

| Variable | OS (N = 82) | RFS (N = 67) |
|----------|-------------|-------------|
|          | N | HR (95% CI; HR P) | L-R P | N | HR (95% CI; HR P) | L-R P |
| pT stage |   |                  |       |   |                  |       |
| pT1      | 30 | Reference | 0.053 | 28 | Reference | 0.003 |
| pT2      | 19 | 0.9 (0.29–2.77; 0.858) |       | 16 | 1.28 (0.41–4.05; 0.669) |       |
| pT3–4    | 33 | 2.28 (0.98–5.3; 0.055) |       | 23 | 3.84 (1.56–9.47; 0.003) |       |
| FNG      |   |                  |       |   |                  |       |
| 2        | 33 | Reference | <0.001 | 29 | Reference | <0.001 |
| 3        | 41 | 3.75 (1.4–10.04; 0.009) |       | 33 | 3.99 (1.47–10.83; 0.007) |       |
| 4        | 8  | 10.5 (3.17–34.79; <0.001) |       | 5  | 16.2 (4.58–57.28; <0.001) |       |

Univariate Cox proportional hazards regression on overall survival and recurrence-free survival was performed by primary tumor stage and Fuhrman nuclear grade. Statistically significant relation in bold. pT stage and FNG were found to be significant predictors of adverse clinical outcome. pT stage: primary tumor stage by size; FNG: Fuhrman Nuclear Grade; OS: overall survival; RFS: recurrence-free survival; L-R: log-rank.
| 1st author | Year | Histology | Cohort | Tissue prep | Clone | Criteria for positivity | % positive | Follow-up | Outcome | Univariate impact (95% CI) | Multivariate impact (95% CI) |
|------------|------|-----------|--------|-------------|-------|-------------------------|------------|----------|---------|-----------------------------|-----------------------------|
| Thompson   | 2007 | CC        | 267    | FFr         | MIH4  | TIMC ≥ 5%               | 28.8%      | Median 2.9 yr (range 0–5.6) | CSS      | RR 2.24 (1.30–3.86); p = 0.004 | RR 1.66 (0.96–2.88); p = 0.071 |
| Kang       | 2013 | CC        | 199    | FFPE        | NAT105| ≥ 1 cell in 5 HPF (1 HPF = 0.238 mm²) | 43.2%      | Range 0.3–13 yr | DMR      | HR 12.371 (2.855–53.608); p = 0.015 | HR 6.146 (1.249–30.238); p = 0.026 |
| Giraldo    | 2015 | CC        | 80     | FFPE        | EH33  | Invasive margin; slide scanner cutoff for minimum p-value; ≥ 626 stained cells per mm² | 18.8%      | Not reported | DFS      | HR 5.79 (1.79–20.0); p = 0.005 | HR 6.40 (1.3–31.0); p = 0.02 |
| Shin       | 2015 | CC        | 214    | FFPE        | MRQ-22| ≥ 5 lymphocytes stained per mm² | 19.0%      | Median 77.5 mo (range 0–175) | OS       | HR 3.87 (2.27–6.87); p < 0.001 | HR 2.19 (1.13–4.24); p = 0.02 |
| Abbas      | 2016 | non-CC    | 63     | FFPE        | Unspec.| TIMC stained area > 5% | 30.9%      | Not reported | OS       | HR 2.87 (1.47–5.57); p = 0.002 | HR 1.94 (0.99–3.81); p = 0.055 |
| Erlemeier  | 2016 | Chr       | 81     | FFPE        | NAT105| TIMC stained area > 5% | 37.2%      | Median 40.5 mo (range 1–226) | OS       | HR 3.35 (1.75–6.43); p < 0.001 | HR 2.19 (1.13–4.24); p = 0.02 |
| Thompson   | 2005 | CC        | 196    | FFr         | SH1   | ≥ 10% tumor stained | 20.4%      | Median 2.7 yrs (range 0–4.4) | OS       | HR 2.87 (1.47–5.57); p = 0.002 | HR 1.94 (0.99–3.81); p = 0.055 |
| Thompson   | 2006 | CC        | 306    | FFPE        | SH1   | ≥ 5% tumor stained      | 23.9%      | Median 11.2 yrs (range 0–15) | OS       | HR 2.87 (1.47–5.57); p = 0.002 | HR 1.94 (0.99–3.81); p = 0.055 |
| Choueiri   | 2014 | non-CC    | 101    | FFPE        | 405SA11| ≥ 5% tumor stained      | 10.9%      | Median 5 yr (IQR 3.5–6.2) | OS       | HR 6.41 (2.17–18.88); p < 0.001 | HR 1.43; p = 0.028 |
| Choueiri   | 2014 | CC        | 453    | FFPE        | SH1   | Tumor staining score (0–3) % of cells > 55 | 56.4%      | Not reported | TTR      | HR 2.49 (0.86–7.2); p = 0.08 | HR 1.43; p = 0.028 |
| Giraldo    | 2015 | CC        | 80     | FFPE        | 405SA11| ≥ 5% tumor stained      | 27.5%      | Median 40.5 mo (range 1–226) | DFS      | HR 1.96 (1.0–4.0); p = 0.06 | HR 1.31 (0.68–2.52); p = 0.415 |
| Shin       | 2015 | CC        | 214    | FFPE        | E1L3N | ≥ 5% tumor stained      | 12.6%      | Median 58.7 mo (range 1.4–202.1) | OS       | HR 2.87 (1.2–7.1); p = 0.02 | HR 1.92 (1.04–3.54); p = 0.036 |
| Pap        | 201  |          |        |             |       |                        | 6.0%       |                        | PFS      | p = 0.363                    | p = 0.484                    |
| Leite      | 2015 | CC        | 115    | FFPE        | Unspec.| Tumor stain score > 0, graded 0–3 | 56.5%      | Mean 115.7 mo | CSS      | p = 0.014                    | p = 0.014                    |

(Continued on next page)
Table 5. (Continued)

PD-1 expression and clinical outcome

| 1st author | Year | Histology | Cohort | Tissue prep | Clone | Criteria for positivity | % positive | Follow-up | Outcome | Univariate impact (95% CI) | Multivariate impact (95% CI) |
|-------------|------|-----------|--------|-------------|-------|-------------------------|------------|-----------|---------|---------------------------|-----------------------------|
| Abbas      | 2016 | non-CC    | 56     | FFPE        | Unspec.| Tumor stained area > 5% | 46.4%      | Median 77.5 mo (range 0–175) | CSS | p = 0.08 | HR 1.26 (0.41–3.9); p = 0.68 |
| Abbas      | 2016 | CC        | 177    | FFPE        | Unspec.| Tumor stained ≥ 1%     | 20.9%      | Median 64.7 mo (range 0.3–188.8) | OS   | p = 0.08 | HR 2 (1.2–3.3); p = 0.005 |
| Erlmeier   | 2016 | Chr       | 81     | FFPE        | E1L3 N| Tumor stained area > 5% | 13.6%      | Median 40.5 mo (range 1–226) | OS  | p = 0.48 |

Some studies only reported log-rank P-value without risk ratio or hazard ratio. All studies set statistical significance at two-sided p ≤ 0.05. Significant values bolded. Different studies included different covariates for multivariate analysis. CC: clear cell; Pap: papillary; Chr: chromophobe; FF: Fresh frozen; FFPE: Formalin-fixed paraffin-embedded; TIMC: tumor-infiltrating mononuclear cell; HPF: High-power field; IQR: interquartile range; CSS: cancer-specific survival; OS: overall survival; DMR: distant metastatic relapse; RFS: relapse-free survival; DFS: disease-free survival; PFS: progression-free survival; TTR: time to recurrence; DM: distant metastasis; TR: tumor recurrence; RR: risk ratio; HR: hazard ratio.
employed in this study (clone NAT105) was not used in clinical trials but has been shown to produce a clear staining pattern with good effect in a preceding study.22

It is evident much research is needed before PD-1 blockade immunotherapy can be optimally directed to RCC patients. The biological link between PD-1 pathway and immune dysfunction must be better characterized in the tumor microenvironment, and in order to do so, a reliable and repeatable way to quantify tumor PD-1 expression is necessary. We argue that PD-1/PD-L1 immunohistochemistry in its current form is an inadequate tool for studying tumor PD-1 biology and is inferior to established tools such as primary tumor stage or Fuhrman nuclear grade in prognosticating. We suggest that an effort to standardize PD-1 scoring and validate the results using external and/or inter-institutional cohort is necessary before effective stratification of patients and clinical application of PD-1 blockade immunotherapy.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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