Published in final edited form as:

Cancer Immunol Res. 2013 December ; 1(6): 378–385. doi:10.1158/2326-6066.CIR-13-0042.

Inverse Association between Programmed Death Ligand 1 and Genes in the VEGF Pathway in Primary Clear Cell Renal Cell Carcinoma

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Abstract

Increased angiogenesis and tumor-induced immune evasion are two mechanisms by which clear cell renal cell carcinoma (ccRCC) proliferate and metastasize; however, the relationship between these pathways in human ccRCC is poorly understood. We conducted a nested case–control study

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Disclosure of Potential Conflicts of Interest

E. Kwon has ownership interest (including patents) and is a consultant/advisory board member of Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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using 98 archived tumor samples from patients diagnosed with primary ccRCC between 1990 and 2006, half of which were identified by immunohistochemistry (IHC) as either programmed death ligand 1 (PDL-1)-positive or PDL-1-negative. RNAs were extracted from the formalin-fixed paraffin-embedded tumor slides and the expression of the VEGFA, VEGFR1, VEGFR2, and PDL-1 genes was quantified. We assessed the presence of tumor-infiltrating lymphocytes (TIL) by IHC for CD3, and then analyzed the relationship among VEGFA, VEGFR1, VEGFR2, CD3, and PDL-1. When analyzed as a continuous variable, PDL-1 protein expression by IHC inversely correlates with the expression of the three VEGF-related genes: *VEGFA* ($r = -0.23$; $P = 0.01$), *VEGFR1* ($r = -0.34$; $P < 0.001$), and *VEGFR2* ($r = -0.23$; $P = 0.01$). When dichotomized, the PDL-1-positive cohort trended toward a lower expression of VEGFA (fold change = 0.72; $P = 0.056$) and VEGFR1 (fold change = 0.69; $P = 0.057$). In addition, there was a significant and positive relationship between the presence of TIL as assessed by IHC for CD3 and PDL-1 by IHC ($r = 0.25$; $P = 0.015$), and there was a trend toward an inverse relationship between TIL and VEGFA gene expression ($r = -0.18$; $P = 0.089$). In conclusion, this is the first demonstration of an inverse association between the angiogenesis and PDL-1 pathways in tumor samples from primary ccRCC, and this relationship may be related to the immunosuppressive effects of VEGF signaling.

Introduction

Despite the approval of multiple agents to treat metastatic clear cell renal cell carcinoma (ccRCC), only a very small minority of patients remains alive at 5 years (1). An improved understanding of the molecular underpinnings that support the pathogenesis of ccRCC will lead to improved clinical outcomes. Two potential mechanisms by which ccRCC tumors proliferate and metastasize are increased angiogenesis and tumor-induced immune evasion. Although much is known about the individual roles of these potential mechanisms in ccRCC pathogenesis, the potential relationship between these two pathways in human ccRCC is poorly understood.

In ccRCC, tumor angiogenesis is triggered by the activation of the VEGF pathway resulting in improved blood supply to and enriched source of nutrients for the tumor (2–5). VEGF binds VEGF receptors 1 and 2 (VEGFR1 and VEGFR2, respectively) with a net effect of increasing angiogenesis, cellular proliferation, survival, and migration (6). Anti-VEGF therapies revolutionized the care for patients with metastatic ccRCC, induced response rates of 30% to 40% and improved overall survival (7, 8). Despite the success of anti-VEGF agents, virtually all patients develop resistance, and strategies are needed to improve the rate and duration of response to anti-VEGF therapies.

ccRCC can evade immune surveillance by the expression of programmed death ligand 1 (PDL-1), which binds PD-1 on the surface of activated T and B cells, thus negatively regulating the immune system (9, 10). We and others have reported a strong association between higher tumor-based PDL-1 expression and adverse clinical outcomes for patients with ccRCC (11–13). Blockade of the PDL-1/PD-1 interaction has shown durable responses in approximately 30% of patients with ccRCC. These responses seemed to be related to tumor PDL-1 expression (14, 15), which is at least partly driven as an adaptive response to immune cell infiltrates (16).

Although VEGF is primarily known for increased angiogenesis, there is mounting evidence that VEGF has immunosuppressive properties and that augmenting tumor vasculature may increase the efficacy of immunotherapies (17). We hypothesized that tumors with increased VEGF expression would possess an immunosuppressive tumor microenvironment, decreased immune cell infiltrates, and therefore less PDL-1 expression. To test whether an association between PDL-1 and VEGF exists, we analyzed Affymetrix-based gene expression of a set of 30 primary and patient-matched metastatic ccRCC samples and noted an inverse association between PDL-1 expression and multiple genes in the VEGF pathway. We leveraged the data and tissue resources available in the Mayo Clinic Nephrectomy Registry to conduct a nested case-control study to directly explore the association of VEGF-related genes in PDL-1-positive and -negative ccRCC tumors. Our primary endpoint was to study the association of PDL-1 protein by immunohistochemistry (IHC) with gene expression of VEGFA, VEGFR1, and VEGFR2 in primary human ccRCC tumor samples. The secondary endpoints included: the association between tumor-infiltrating lymphocytes (TIL) as assessed by IHC for CD3 and PDL-1, and the expression of genes in the VEGF pathway; the association of PDL-1 protein by IHC and PDL-1 gene expression; the association of VEGFA with VEGFR1 and VEGFR2 with each other. Here, we report the first evidence of an inverse association between PDL-1 expression and the expression of VEGFA, VEGFR1, and VEGFR2 in human primary ccRCC samples.

Materials and Methods

Patient selection

Under an Institutional Review Board-approved protocol, we queried the Mayo Clinic Nephrectomy Registry to identify all patients undergoing nephrectomy to treat sporadic, unilateral, and noncystic ccRCC between 1990 and 2006. Immunohistochemical staining for PDL-1 expression on tumor samples from these patients was available from a previous investigation (13). Using these existing data, we selected 50 patients with the highest levels of IHC-based PDL-1 (PDL-1-positive patients). When the blocks of the 50 PDL-1-positive tumors were reviewed, a pathologist identified that two specimens were from metastatic lymph nodes that were replaced by ccRCC, and these samples were removed from the analysis. We then identified 50 PDL-1-negative ccRCC tumors that were matched on Mayo SSIGN (a score that encompasses stage, size, grade, and tumor necrosis) score with the PDL-1-positive patients (18, 19).

We abstracted the following demographic and clinicopathologic features from the Nephrectomy Registry: age at surgery, sex, symptoms at presentation, Eastern Cooperative Oncology Group (ECOG) performance status, histologic subtype, 2002 American Joint Committee on Cancer (AJCC) primary tumor classification, regional lymph node involvement, distant metastases, 2002 AJCC tumor-node-metastasis (TNM) stage groupings, tumor size, nuclear grade, and presence of coagulative tumor necrosis.

Quantifying tumor PDL-1 and CD3 expression by IHC

IHC-based expressions of PDL-1 in samples from these patients were available from a previous investigation (13). The membranous staining pattern of PDL-1 was quantified as

the percentage of positive tumor cells in 5% increments as determined by a genitourinary pathologist (J. Cheville). To quantify the expression of both the tumor and the microenvironment, we did not carry out a microdissection. We also used IHC-based expression of CD3 to quantify tumor T-cell infiltrates. Two pathologists (K. Wu and J. Jiang) quantified CD3 expression as the percentage of positive cells within the tumor in 5% increments.

RNA isolation and generation of cDNA from formalin-fixed paraffin-embedded samples

We isolated total RNA from tumor-rich areas of formalin-fixed paraffin-embedded (FFPE) tissue blocks using the Qiagen AllPrep DNA/RNA FFPE Kit reagents (Qiagen) following vendor's standard protocols. Three slides per sample at 10 mm each were used for RNA extraction. Isolated FFPE RNA was treated with DNase (Qiagen) and RNA concentration and purity were determined spectrophotometrically ($OD_{260/280} > 1.9$; Nano-Drop 2000 spectrophotometer; Thermo Scientific). One microgram of FFPE tissue RNA was reverse transcribed using ABI High capacity RNA to cDNA kit (Applied Biosystems) as described in the manufacturer's instructions, and the resulting cDNA was diluted at 1:15 in molecular grade RNase/Dnase-free H₂O and stored at -20°C before use.

FFPE RNA quantification via RT-PCR

FFPE-compatible primer sets were designed with amplicon sizes more than 80 bp. All quantitative PCR (qPCR) reactions were assembled in triplicate using the ABI TaqMan Fast PCR Reagents Kit (Applied Biosystems) and run on ABI 7900HT Fast Real-Time PCR System and quantification was carried out using Sequence Detection Systems software v2.4 (Applied Biosystems) to quantify gene expression of PDL-1 (Hs01125301), VEGFA (Hs00900054), VEGFR1 (Hs00176573), VEGFR2 (Hs00911708), and POLR2a (Hs00172187). All primer probes were purchased from Life Technologies. The standard replicates for each qPCR assay were examined for amplification efficiencies between 90% and 105% and all standards and sample replicate data were analyzed for product-specific melt curves. If sample or standard replicates did not conform to these parameters, we tested a different dilution, and the cDNA was diluted with a range of 1:10 to 1:15. We assessed the RNA quality within each group post reverse-transcription by POLR2a expression.

Statistical analysis

We used the Mann–Whitney U test or Fisher exact test, as appropriate, to compare demographic and clinicopathologic characteristics between the PDL-1–positive and –negative tumors. Gene expression data from the reverse-transcription PCR (RT-PCR) were normalized to that of the control gene *POLR2a*. Specifically, the C_T values for each gene were averaged across the triplicate and the average C_T value for *POLR2a* was subtracted from the average C_T value for each gene in each sample. We used a Spearman rank correlation to evaluate the continuous association of PDL-1 protein expression with the expression of PDL-1- and VEGF-related genes, CD3 protein expression with the expression of VEGF-related genes, and VEGF-related genes with each other. We used a linear mixed effects model to compare normalized gene expression of *VEGFA*, *VEGFR1*, and *VEGFR2* between PDL-1–positive and –negative tumors.

Results

Clinical and pathologic characteristics

Of the 50 PDL-1-positive tumors we selected from the Mayo Clinic Renal Registry, two tumor samples came from blocks that were metastatic lymph nodes and were therefore excluded from the study. The clinical and pathologic characteristics are summarized in Table 1. PDL-1-positive tumors ($n = 48$) had the following distribution of PDL-1 positivity: $n=18$ with 5%, $n = 10$ with 10%, $n = 11$ with 15% to 50%, and $n = 9$ with more than 50%. Because they were matched on the Mayo SSIGN score, there were no differences in key pathologic characteristics between PDL-1-positive and -negative tumors. Fifty-eight percent of the patients were men, with a median age at surgery of 67.1 years, and a median follow-up of 10.3 years. In the study cohort, 33 patients (i.e., 34%) developed metastatic disease and 32 of 33 of these patients (i.e., 97%) died from RCC.

Association of gene expression of VEGFA, VEGFR1, VEGFR2, and PDL-1 with protein expression of PDL-1 by IHC

We could quantify transcripts of *VEGFA* in 100%, *VEGFR1* in 99%, and *VEGFR2* in 98% of tumor samples. We could quantify *PDL-1* gene expression in 64 of 98 (65%) samples with 36 of 48 (75%) in PDL-1-positive tumors and 23 of 50 (46%) of the PDL-1-negative tumors. When we analyzed PDL-1 IHC as a continuous variable, we observed a significant inverse correlation between the expression of PDL-1- and VEGF-related genes as follows (Fig. 1): *VEGFA* ($r = -0.201$; $P = 0.027$), *VEGFR1* ($r = -0.31$; $P = 0.001$), and *VEGFR2* ($r = -0.23$; $P = 0.015$). As expected, there was also strong positive correlation between PDL-1 protein and RNA expression (0.36 ; $P = 0.003$).

We compared RNA expression of *VEGFA*, *VEGFR1*, and *VEGFR2* (Fig. 2A) between the 50 PDL-1-negative (0% by IHC) and 48 PDL-1-positive tumors (5% by IHC). In the PDL-1-negative cohort, there was a trend of higher RNA expression of *VEGFA* (fold change=0.72; $P=0.06$) and *VEGFR1* (fold change = 0.67; $P = 0.06$), whereas there was no observed difference in *VEGFR2* expression between the two cohorts (fold change = 0.73; $P = 0.16$).

Finally, with the rationale that PDL-1 positivity has been defined as more than 0% cells (11) as well as more than 5% cells (12), as an exploratory analysis, we also compared VEGF-related genes between subjects with PDL-1 values 0% to 5% ($n = 68$) versus PDL-1 more than 5% ($n = 30$) by IHC (Fig. 2B). Using this cutoff for PDL-1, all VEGF-related genes were significantly higher in the PDL-1 0% to 5% cohort versus PDL-1 more than 5% cohort as follows: *VEGFA* (fold change = 1.82; $P < 0.001$), *VEGFR1* (fold change = 2.63; $P < 0.001$), and *VEGFR2* (fold change = 2.13; $P = 0.001$).

Association of TILs with VEGFA, VEGFR1, VEGFR2, and PDL-1

To determine the relationship among TIL, angiogenesis, and PDL-1 expression, we assessed all tumors for CD3 by IHC and analyzed expression as a continuous variable versus the gene expression of *VEGFA*, *VEGFR1*, *VEGFR2*, and PDL-1 protein expression by IHC (Fig. 3).

We found a trend of an inverse relationship between CD3 and VEGFA ($r = -0.18$; $P = 0.089$) and a significant positive association between CD3 and PDL-1 ($r = 0.25$; $P = 0.015$).

Association of VEGF-related genes with each other

Because we found that VEGFA, VEGFR1, and VEGFR2 all inversely correlated with PDL-1 by IHC, we compared the association of all three VEGF-related genes with each other (Fig. 4). We found a strong positive correlation between *VEGFA* and *VEGFR1* (correlation, 0.49; $P < 0.001$), between *VEGFA* and *VEGFR2* (correlation, 0.24; $P = 0.011$), and between *VEGFR1* and *VEGFR2* (correlation, 0.59; $P < 0.001$).

Discussion

Given the existing clinical benefits and limitations of anti- VEGF therapies, the prognostic significance of PDL-1 expression, and the promising results of PDL-1/PD-1 blockade, a logical next step to advance the field and help in informing the next generation of clinical studies for ccRCC is to enhance our understanding of the relationship between VEGF and PDL-1 expression in ccRCC samples. Indeed, while the individual roles of angiogenesis and immune evasion in ccRCC development and progression are well described, to date, very little is known about how these pathways are associated in clinical samples. To our knowledge, this is the first demonstration of an inverse relationship among angiogenesis, TIL, and PDL-1 expression in human ccRCC clinical samples.

Although activation of the VEGF pathway is most commonly associated with increased angiogenesis, there is emerging evidence that increased angiogenesis leads to an immunosuppressive tumor microenvironment (17, 20, 21), and VEGF inhibition can lead to an increase in TILs (22). For this study, we hypothesized that tumors with increased VEGF expression would have decreased immune infiltrates and less adaptive pressure to express PDL-1. Our data show a trend for an inverse relationship between the expression of VEGF and the presence of CD3-positive TIL; however, we note that the relationship has multiple outliers with some tumors having both low TIL and VEGF expression. We acknowledge that VEGF expression and by proxy angiogenesis is likely to be one of multiple mechanisms that may impact the tumor immune microenvironment.

The two leading hypotheses of why tumors express PDL-1 are the innate and adaptive models (23). In the innate model, tumor PDL-1 expression is independent of the tumor microenvironment and is influenced by intrinsic cell signaling pathways as shown in glioblastomas and ALK-positive lung cancers (24, 25). In the adaptive model of tumor PDL-1 expression, tumors are pressured to express PDL-1 in the presence of an immunostimulatory microenvironment, as exemplified by melanoma and merkel cell carcinoma in which all PDL-1–positive tumors had dense immune infiltrates and all PDL-1–negative tumors had no detectable immune infiltrates (16, 26). In the current study, we show that TIL positively associate with PDL-1 expression, albeit the relationship is not as strong as in melanoma and merkel cell carcinoma. We also note the presence of outliers that some ccRCC tumors express high levels of PDL-1 in the absence of TIL and conversely, some tumors do not express PDL-1 even in the presence of abundant TIL. In summary, it remains possible that both innate and adaptive responses could affect PDL-1 expression in ccRCC.

Although many groups quantify PDL-1 as a dichotomous variable (positive or negative), there is increasing evidence to suggest that PDL-1 should be quantified as a continuous variable. For example, not all patients with PDL-1–positive tumors respond to anti-PD-1 therapies, indicating that perhaps a certain threshold of PDL-1 positivity is necessary (14). In our study, when analyzed as a continuous variable, a strong inverse association between VEGF and PDL-1 was identified; however, when we dichotomized tumors into PDL-1–negative and –positive, the inverse association between PDL-1 and VEGF weakened. Given this difference, we believe that in future studies one should consider analyzing PDL-1 as both a continuous and dichotomous variable.

IHC can be used to assess relative quantities as in PDL-1 positivity and negativity but a more exact technique is needed to quantify PDL-1 expression. To our knowledge, this is the first study to both correlate PDL-1 protein expression by IHC with RNA expression from FFPE tissue and show a positive correlation between protein and RNA expression. There are three potential advantages for using RNA over using IHC to assess PDL-1 expression. First, RNA expression is more quantifiable than protein expression by IHC. Second, commercially available reagents can be used for RNA expression but PDL-1 protein expression requires the use of a proprietary antibody. Third, the sensitivity of using IHC to assess PDL-1 expression on FFPE tissues is reduced in comparison with frozen tissue. Despite the advantages of using RNA to assess PDL-1 expression, there are also several important drawbacks. First, we note that many of our PDL-1–negative tumors by IHC had detectable PDL-1 RNA, which raises two possibilities. The first possibility is that PDL-1–negative tumors by IHC do express PDL-1 at the RNA level. The second possibility is that because we did not microdissect the tumor from the stroma, we are in fact detecting PDL-1 RNA from the surrounding stroma rather than from the tumor itself.

Our case–control study was designed to evaluate the differential expression of VEGF-related genes in PDL-1–positive and –negative tumors. We selected cases on the basis of the expression of PDL-1, a known negative prognostic biomarker in ccRCC, and matched these cases on the Mayo SSIGN with PDL-1–negative tumors. Because the cases were selected with an adverse feature (PDL-1–positive), patients in this study cohort had a higher rate of ccRCC-related death than in the total Mayo Renal Registry (34% vs. 18%). However, there were no statistical differences in additional clinical and pathologic features between our cohort and all other ccRCC cases in the Mayo Renal Registry. Furthermore, the percentage of PDL-1–positive tumors in this study is higher than what is found in the total Mayo Renal Registry (50% vs. 30%, respectively). We acknowledge that biases of this study limit the applicability of our findings to ccRCC as a whole, and we are currently testing whether our findings hold up in a nonselected cohort of ccRCC tumors.

Both PDL-1 and VEGF expression are associated with an adverse prognosis in ccRCC (11, 27), and therefore the inverse relationship of these two markers is potentially concerning; however, we believe that the case–control design of the study can explain this apparent discrepancy. We selected the PDL-1–positive tumors first (cases) and then selected the matched PDL-1–negative tumors (controls). Both the PDL-1–positive and –negative cohorts have similar outcomes and a higher rate of ccRCC-related death relative to the total Mayo Renal Registry. As the disease outcomes between the cohorts are similar but the PDL-1 and

VEGF status differ, we hypothesize that there are at least two phenotypes that confer a poor prognosis: an immune evasive phenotype (VEGF^{low}PDL-1^{high}) and an angiogenic phenotype (VEGF^{high}PDL-1^{low}). Further studies in a random cohort of tumors are necessary to confirm these findings. Finally, given that not all PDL-1^{high} tumors were VEGF^{low} and *vice versa*, we believe that these biomarkers should not be used to guide treatment decisions at present. However, we do think that prospective analysis of VEGF and PDL-1 expression in upcoming combination trials of anti-VEGF and anti-PDL-1 therapies deserves consideration.

In summary, the mechanisms of angiogenesis and immune evasion are complex and perhaps inversely related in primary ccRCC tumor samples. Our results have indicated that future studies in larger and unselected populations of ccRCC are warranted. In addition, we believe that the relationship between VEGF and PDL-1 in other tumors also warrants consideration. Testing the impact of VEGF inhibition on PDL-1 expression could provide valuable insights when designing a clinical trial to either combine or sequence antiangiogenic and anti-PDL-1 therapies.

Acknowledgments

Grant Support

This work was supported by the AACR Judah Folkman Career Development Award in Antiangiogenesis.

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Figure 1.

The association of VEGFA, VEGFR1, and VEGFR2 gene expression with PDL-1 protein expression as assessed by IHC and as a continuous variable. Expression of PDL-1 by IHC inversely correlates with gene expression of VEGFA ($r = -0.201$; $P = 0.027$), VEGFR1 ($r = -0.311$; $P = 0.001$), and VEGFR2 ($r = -0.228$; $P = 0.015$). We quantified PDL-1 gene expression in 64% of all samples, in 38 of 50 (76%) cases, and in 23 of 50 (46%) controls. Predictably, there was a strong positive correlation between PDL-1 protein expression by IHC and PDL-1 RNA expression ($r = 0.36$; $P = 0.003$).

Figure 2.

Dichotomous analysis of VEGFA, VEGFR1, and VEGFR2 gene expression by RT-PCR and PDL-1 protein expression by IHC. A, PDL-1-negative versus -positive cohort. In the PDL-1-positive cohort, RNA expression of VEGFA (fold change=0.70; $P=0.036$) and VEGFR1 (fold change=0.67; $P=0.037$) was significantly lower than in the PDL-1-negative cohort, whereas there was no observed difference in VEGFR2 expression in the two cohorts (fold change=0.71; $P=0.13$). Solid horizontal line represents the median and the dotted horizontal lines represent the 95% confidence interval (CI). B, PDL-1 0% to 5% versus PDL-1 more than 5%. Using this cutoff for PDL-1, all VEGF-related genes were significantly higher in the PDL-1 0% to 5% cohort versus PDL-1 more than 5% cohort as follows: VEGFA (fold change=1.82; $P < 0.001$), VEGFR1 (fold change=2.63; $P < 0.001$), and VEGFR2 (fold change=2.13; $P=0.001$). Solid horizontal line represents the median, and the dotted horizontal lines represent the 95% CI.

Figure 3.

The association between CD3 TIL by IHC with gene expression of VEGFA, VEGFR1, VEGFR2, and PDL-1 protein expression by IHC. We quantified TILs with IHC by counting percentage positive CD3 cells in 5% increments. Using a Spearman rank correlation, we found a significant relationship between CD3 IHC and PDL-1 IHC ($r = 0.25$; $P = 0.015$), and there was a trend for an inverse relationship between CD3 IHC and VEGF gene expression ($r = -0.178$; $P = 0.088$). We did not find a relationship between CD3 IHC and gene expression of VEGFR1 or VEGFR2.

Figure 4.

Correlation of VEGF-related genes with each other. We found a strong positive correlation between VEGFA and VEGFR1 (correlation, 0.49; $P < 0.001$), VEGFA and VEGFR2 (correlation, 0.24; $P = 0.011$), and VEGFR1 and VEGFR2 (correlation, 0.59; $P < 0.001$).

Table 1

Clinical and pathologic information

	PDL-1 ⁺ (n = 50)	PDL-1 ⁺ (n = 48)	Total (n = 98)	P
Male				0.5394
No	19 (38.0%)	22 (45.8%)	41 (41.8%)	
Yes	31 (62.0%)	26 (54.2%)	57 (58.2%)	
Age at surgery				0.1044
Median	65.9	68.9	67.1	
Range	(35.4–82.7)	(26.6–85.7)	(26.6–85.7)	
Tumor size				0.4969
Median	6.0	5.2	5.5	
Range	(1.5–18.0)	(2.0–16.0)	(1.5–18.0)	
Time to last follow-up				0.5109
Median	9.4	12.2	10.3	
Range	(0.0–22.1)	(0.1–21.8)	(0.0–22.1)	
Metastasis				1.0000
No	33 (66.0%)	32 (66.7%)	65 (66.3%)	
Yes	17 (34.0%)	16 (33.3%)	33 (33.7%)	
Time from surgery to metastasis, years				0.6268
Median	1.5	1.4	1.5	
Range	(0.1–12.3)	(0.1–14.6)	(0.1–14.6)	
Death				1.0000
No	12 (24.0%)	11 (22.9%)	23 (23.5%)	
Yes	38 (76.0%)	37 (77.1%)	75 (76.5%)	
Death from RCC				1.0000
Missing	2	2	4	
No	32 (66.7%)	30 (65.2%)	62 (66.0%)	
Yes	16 (33.3%)	16 (34.8%)	32 (34.0%)	
2010 Primary tumor classification				0.0906
1	24 (48.0%)	30 (62.5%)	54 (55.1%)	
2	6 (12.0%)	8 (16.7%)	14 (14.3%)	

	PDL-1 ⁺ (n = 50)	PDL-1 ⁺ (n = 48)	Total (n = 98)	P
3	20 (40.0%)	9 (18.8%)	29 (29.6%)	
4	0 (0.0%)	1 (2.1%)	1 (1.0%)	0.5919
Nuclear grade				
1	4 (8.0%)	7 (14.6%)	11 (11.2%)	
2	25 (50.0%)	23 (47.9%)	48 (49.0%)	
3	18 (36.0%)	13 (27.1%)	31 (31.6%)	
4	3 (6.0%)	5 (10.4%)	8 (8.2%)	
Coagulative tumor necrosis				0.4961
No	35 (70.0%)	37 (77.1%)	72 (73.5%)	
Yes	15 (30.0%)	11 (22.9%)	26 (26.5%)	
SSIGN category				0.2265
Missing	6	8	14	
0-3	25 (56.8%)	28 (70.0%)	53 (63.1%)	
4-7	16 (36.4%)	10 (25.0%)	26 (31.0%)	
8+	3 (6.8%)	2 (5.0%)	5 (6.0%)	
PDL-1 IHC (%)				<0.0001
0	50 (100.0%)	0 (0.0%)	50 (51.0%)	
5	0 (0.0%)	18 (37.5%)	18 (18.4%)	
10	0 (0.0%)	10 (20.8%)	10 (10.2%)	
15-50	0 (0.0%)	11 (22.9%)	11 (11.2%)	
>50	0 (0.0%)	9 (18.8%)	9 (9.2%)	