Immune dysfunction revealed by digital spatial profiling of immuno-oncology markers in progressive stages of renal cell carcinoma and in brain metastases

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ABSTRACT
Background The tumor microenvironment (TME) contributes to cancer progression and treatment response to therapy, including in renal cell carcinoma (RCC). Prior profiling studies, including single-cell transcriptomics, often involve limited sample sizes and lack spatial orientation. The TME of RCC brain metastases, a major cause of morbidity, also remains largely uncharacterized.

Methods We performed digital spatial profiling on the NanoString GeoMx platform using 52 validated immuno-oncology markers on RCC tissue microarrays representing progressive stages of RCC, including brain metastases. We profiled 76 primary tumors, 27 adjacent histologically normal kidney samples, and 86 metastases, including 24 brain metastases.

Results We observed lower immune checkpoint (TIM-3 and CTLA-4), cytolytic (GZMA and GZMB), and T cell activation (CD25) protein expression in metastases compared with primary tumors in two separate cohorts. We also identified changes in macrophages in metastases, with brain metastases-susceptible patients showing less M1-like, inflammatory macrophage markers (HLA-DR and CD127) in metastatic samples. A comparison of brain metastases to extracranial metastases revealed higher expression of the anti-apoptotic, BCL-2-family protein BCL-XL and lower expression of the innate immune activator STING in brain metastases. Lower TIM-3 and CD40 in the TME of brain metastases appear to be associated with longer survival, a finding that requires further validation.

Conclusions Compared with primary tumors, RCC metastases, including brain metastases, express lower levels of numerous markers of immune activation and current or investigational therapeutic targets. Our findings may have important implications for designing future biomarker and treatment studies and may aid in development of brain metastases-specific therapies.

BACKGROUND
Renal cell carcinoma (RCC) is relatively prevalent and potentially deadly, with over 70,000 new cases each year in the USA, 30%–40% diagnosed with advanced stage and nearly 14,000 deaths.1 RCC is also comparatively cerebrotropic; 10%–12% of patients with advanced RCC harbor brain metastases based on observational studies, although the actual incidence may be much higher.2

RCC is resistant to traditional chemotherapy. Treatment options for advanced RCC have included high-dose interleukin-2, interferon-alpha, tyrosine kinase inhibitors (TKIs) targeting the VEGF-pathway, and mTOR inhibitors.3 In recent years, numerous
drugs and regimens have been approved. These regimens generally consist of immune checkpoint inhibitors (ICIs) that target the PD-1/PD-L1 axis in combination with anti-CTLA-4 or VEGF-pathway TKIs4–7; hypoxia-inducible factor 2-alpha inhibitors are also being investigated.8 While these regimens represent substantial advances, most patients either have primary resistance or acquire resistance with time, and ultimately succumb to their disease.9 For patients otherwise responding to ICI-based therapies, the brain is a common site of progression.2 Although retrospective studies and case reports suggest intracranial activity of some of these regimens, including the TKIs and anti-PD-1 therapies, prospective studies are limited and efficacy of these regimens for RCC brain metastases remains unclear.10–13

The tumor microenvironment (TME) contributes to cancer progression and response to therapy, although effects and predictive value can vary by tumor type. For example, while generally a favorable prognostic marker in other tumor types, higher CD8+ T cell infiltration is associated with a worse prognosis in RCC.14 15 and response to ICIs is similarly not associated with broad T cell infiltration in RCC, although poor response to anti-PD-1 has been associated with high baseline myeloid infiltration.16 Tumor PD-L1 expression, while widely used as a predictive biomarker in other tumor types, does not have substantial predictive value in RCC.17 Single-cell RNA-sequencing studies have provided additional insight into the TME in RCC. Braun et al demonstrated enrichment of terminally exhausted CD8+ T cells, characterized by high expression of multiple immune checkpoints, including PD-1, CTLA-4, LAG3, TIM-3, and TIGIT, as well as other markers of terminal T cell exhaustion, in the primary tumors of patients who went on to develop more advanced disease.18 They also found concomitant increases in immunosuppressive, protumorigenic M2-like macrophages in these samples, and proposed the development of a dysfunctional immune circuit as RCC progresses. Bi et al studied ICI-treated RCC patients and found more intra-tumoral T cell differentiation towards terminally differentiated states, potentially derived from low-abundance progenitor-exhausted T cells, in ICI-responders.19 This state was associated with higher expression of immune checkpoints in T cells and expression of immunosuppressive transcriptional programs in tumor-associated macrophages and cancer cells, potential mechanisms of eventual ICI-resistance.

The TME of brain metastases has many distinct features relative to other metastatic sites. The central nervous system (CNS) has long been considered a relatively immune privileged site although the TME of brain metastases is infiltrated by macrophages derived from circulating monocytes and lymphocytes.20 21 Comparisons of the TME of RCC brain metastases and other sites have consisted of limited cohort studies looking at small groups of markers and have demonstrated an immunosuppressive brain microenvironment but have not shown significant differences in tumor infiltrating lymphocyte or CD68 cell composition or tumor cell PD-L1 expression.22–24

RCC has high interlesional and intralesional heterogeneity.25 26 We previously found weak correlations in expression of PD-L1 and other tumor-based drug targets between matched pairs of nephrectomy and metastatic specimens.27–29 Multiple biopsy cores were queried for each specimen, revealing substantial intratumor heterogeneity. We further observed a significantly lower CD8+ T cell density in metastases compared with primary tumors.30 These studies used tissue microarrays (TMAs), which permit the simultaneous and rapid assessment of large numbers of patient samples, including specimens with limited tissue, such as those collected by core needle biopsy. Concerns that individual TMA cores are not reflective of overall tissue heterogeneity because of their small size can be mitigated by the careful selection of the tissue region from which the core is extracted and by the use of multiple cores per source block.31

Digital spatial profiling (DSP) allows for quantitative assessment of multiplexed proteins or RNAs using oligonucleotide tags in predefined cellular compartments while preserving spatial orientation.32 33 DSP can be performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections, including those on TMAs, enabling a high-throughput workflow and use of archived samples. Molecularly defined cellular compartments can be interrogated individually using fluorochrome markers. Here, we aimed to characterize the TME of progressively advanced stages of RCC, including brain metastases, using DSP. We uncovered reduced levels of multiple immune checkpoints and T cell cytolytic and activation markers in metastases versus primary tumor samples, and lower inflammatory macrophage activation markers in metastatic samples of patients who develop brain metastases.

METHODS

Tissue microarrays

The three RCC TMAs used in this study have been described previously.28 34 35 Yale TMA-84 (YMA-84) consists of matched adjacent histologically normal kidney and primary tumor pairs from renal tumors resected between 1987 and 1999. YMA-166 was constructed using matched primary tumor and metastasis pairs (from a distinct cohort of RCC patients relative to YMA-84) with metachronous or synchronous metastatic disease treated between 1978 and 2011, with four 0.6 mm cores from different areas of each tumor specimen within the same FFPE block, placed in two TMA blocks. YMA-528 consists of all available archived, FFPE tumor specimens collected from 2002 to 2021, from all sites including primary tumors and brain metastases and extracranial metastases, exclusively from patients who developed brain metastases. Replicate cores were used to construct two master TMA blocks. An associated clinical database was constructed for YMA-528, including clinical
follow-up data on which survival and response to immunotherapy analyses were based, with censoring on May 1, 2022. During the creation of these TMA blocks, two independent pathologists reviewed and selected areas of tumor and adjacent normal kidney, when appropriate. Due to TMA usage since their construction, there was some depletion of tissue cores – only intact tissue cores were assessed.

**Digital spatial profiling**

We performed DSP on a GeoMx DSP instrument (NanoString Technologies) according to the manufacturer’s instructions and based on previously described methods.34 36 TMA slides were stained with three fluorescently labeled antibodies to define the cellular components: macrophages by CD68; leucocytes by CD45 positivity/CD68 negativity; and tumor/epithelial cells by pan-cytokeratin (CK). Of note, all RCC cells do not stain positive for CK although positivity thresholds were set to include cells with low CK staining levels. TMs were incubated with a panel of photocleavable oligonucleotide-conjugated antibodies validated by NanoString and directed towards 52 immuno-oncology markers, three housekeeping proteins (GAPDH, histone H3, ribosomal protein S6) and three negative controls (mouse IgG1, mouse IgG2a and rabbit IgG) (online supplemental table S1). For YTMA-84, only tumor spots with an intact matched adjacent normal kidney spot were analyzed, and each tissue specimen had n=1. For YTMA-166, all intact tumor cores were analyzed, including all replicates, with preference for cores with at least one intact matched sample (primary tumor to metastasis). For YTMA-528, all intact cores were analyzed. Images of two representative spots, including a H&E stain, the fluorescence patterns of the cellular-molecular compartment markers, and the compartment masks created by the GeoMx instrument, are shown in online supplemental figure S1A.

Quality control checks as specified by the manufacturer were performed and digital counts were first normalized to internal spike-in controls (External RNA Control Consortium). They were then normalized to the geometric mean of two housekeeping markers (histone H3 and ribosomal protein S6) within each compartment for a given region of interest. Expression levels of the three cellular-molecular compartment markers in each compartment validated the compartmentalization (online supplemental figure S1B).

**Statistical analysis**

The quantified protein counts within each compartment generated by the nCounter platform were used for all downstream analysis. For differential protein expression analysis, a mixed-effects model was used with false-discovery rate correction for multiple comparisons, except where specified. Matched-pair analysis was performed using the mean normalized marker count and the non-parametric Wilcoxon signed-rank test. Univariable and multivariable Cox proportional hazards regression analyses of survival were also performed on the data from YTMA-528 using the mean normalized marker count and based on an extensive clinical database that was constructed. For survival analyses, the median levels of a marker within a tumor type and compartment were used as the cut-point. Kaplan-Meier plots were generated, and log-rank tests were performed. Response to immunotherapy was determined retrospectively using Response Evaluation Criteria in Solid Tumors V.1.1, for patients whose tumors were included in YTMA-528. Overall response rate (ORR) was defined as the proportion of patients with a best overall response of complete or partial response. Disease control rate (DCR) was defined as the proportion of patients with a best response of complete response, partial response, or stable disease lasting at least 6 months. All statistical testing was performed with a two-sided p<0.05 considered significant. Statistical testing was performed using RStudio V.2022.07.1 and GraphPad Prism for Windows software V.8.0 (GraphPad Software, La Jolla, California, USA).

**RESULTS**

**Patients and cohorts**

Patients represented in the three RCC TMAs are described in table 1. Twenty-seven pairs of matched adjacent normal kidney and primary tumor were analyzed from YTMA-84. For YTMA-166, there were 14 matched pairs of primary tumors and metastases, with unmatched samples from an additional 10 patients. For YTMA-528, we profiled 95 tumor specimens from 59 unique patients with 24 matched primary tumor and metastases pairs. Tissue blocks were represented by 1–4 cores (table 1). Nearly all specimens (93%) were of clear cell histology. For the patients included on YTMA-528, 20% had brain metastases at the time of their initial cancer diagnosis and 69% received an immunotherapy-containing regimen. Ninety-five per cent of these regimens contained anti-PD-1 and over two-thirds consisted of single-agent anti-PD-1 or anti-PD-1 plus anti-CTLA-4 (online supplemental table S2).

**Marker-specific intratumoral heterogeneity**

YTMA-166, with up to four cores per tumor, provided an opportunity to extensively compare intratumor heterogeneity between primary and metastatic tumors. We used a previously described method to compute the composite median absolute deviation within each compartment (figure 1A).39 We found no significant overall differences in heterogeneity between primary and metastatic sites in each compartment. At the individual marker level, BCL-XL in the CD45 compartment and BCL-XL, BAD, and GZMA in the CD68 compartment displayed significantly more heterogeneity in primary tumors compared with metastases, while PD-L1 expression in the CD45 compartment was significantly more heterogenous in metastases relative to primary tumors (figure 1B and online supplemental table S3).
For YTMA-528, we used Pearson correlations to compare expression of all markers in each of the three compartments for tumor specimens with replicate cores, looking separately at primary tumors, extracranial metastases, and brain metastases (figure 1C). There was more intrallesional heterogeneity in marker levels in the immune compartments (CD45 and CD68) for all tissue sites compared with the tumor (CK) compartment. Within the CD45 and CD68 compartments, primary tumors and brain metastases displayed significantly more heterogeneity than extracranial metastases. In the tumor compartment, primary tumors demonstrated the most heterogeneity, and no significant differences were found between intracranial and extracranial metastases. Correlation scores for individual markers in each compartment and tissue type are shown in figure 1D.

Marker and cell population differences with advancing disease stage
We next identified markers with the largest differences in expression in progressively more advanced stages of RCC (figure 2 and online supplemental figures S2-S4). All marker counts had been normalized to the geometric mean of two housekeeping markers (histone H3 and ribosomal protein S6) within each compartment for a given region of interest (please see the Methods section). Compared with adjacent normal kidney, primary tumor samples were more infiltrated with macrophages (CD68) and had higher levels of B7-H3, a B7 family immune checkpoint protein with protumorigenic effects, in both the CD68 and CD45 compartments (figure 2A, online supplemental figure S2). Primary tumors had higher CD11c, implying higher infiltration with dendritic cells.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics</th>
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<td></td>
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<td>YTMA-166</td>
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<tr>
<td># of patients</td>
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</tr>
<tr>
<td># of samples</td>
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</tr>
<tr>
<td>1</td>
<td>54 (100)</td>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>15 (63)</td>
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<td>ccRCC</td>
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<tr>
<td>Primary</td>
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cCRCC, clear cell RCC; IMDC, International Metastatic RCC Database Consortium; nccRCC, non-clear cell RCC; RCC, renal cell carcinoma.
Figure 1  Marker expression heterogeneity in primary tumors and metastases. (A) Comparison of composite median absolute deviation (MAD) scores between primary tumors and metastases in the TMA composed of patient-matched primary and metastatic sites (YTMA-166), showing no clear differences in overall heterogeneity between primary versus metastatic tumors. Each point represents a single patient with more than one core for both primary tumor and metastatic samples. The central diagonal red line represents identical heterogeneity in primary and metastatic tumors; points below the diagonal line represent samples with greater heterogeneity within primary tumors and points above with greater heterogeneity within metastases. (B) Heatmap of the sign-rank score of differences in MAD values between primary tumors and metastases by compartment in YTMA-166. A positive (negative) value indicates higher (lower) heterogeneity in the primary tumor compared with the matched metastasis. The score represents the mean across all patients with paired specimens of the product of the rankings by marker of the absolute value of the difference in MAD scores and the sign. Markers with p values<0.05 are starred. Online supplemental table S3 lists all p values. For (A, B) statistical testing was performed using the Wilcoxon paired, two-sided signed rank test. (C) Boxplots of the Pearson correlation r between replicate cores for all markers within the specified tissue site and compartment, showing differences between primary and metastatic sites. Statistical testing was performed using the non-parametric Kruskal-Wallis test comparing the means of all groups within a compartment with correction for multiple comparisons with the Benjamini-Hochberg procedure. (D) Heatmaps of the Pearson correlation r between replicate cores for all markers within the specified tissue site and compartment. Non-macrophage immune cells appear to demonstrate greater heterogeneity of marker expression than tumor cells or macrophages. CK, cytokeratin; TMA, tissue microarray.
Figure 2  Differential marker expression by cancer stage and cellular compartment. Volcano plots of the log₂(fold-change) in marker expression on the x-axis and the −log₁₀(p value) on the y-axis for the relevant markers for each cellular compartment comparing: (A) normal kidney and primary tumors; (B, C) primary tumors and metastases in two patient cohorts; and (D) brain metastases and extracranial metastases. Fold-change values and statistical testing were performed using a mixed-effects model, with false-discovery rate (FDR) correction for multiple comparisons. For (A–C) markers with FDR<0.05 are represented with red dots. For (D) markers with p values<0.05 are shown in red, as this analysis was considered more exploratory. |Log₂(fold-change)|=0.5 and −log₁₀(p value)=2 axes are highlighted with a red-dashed line for visualization purposes.
There were no major differences in T cell (CD3, CD4, CD8, FOXP3, CD45RO), NK cell (CD56), B cell (CD20), monocyte (CD14), or neutrophil (CD66b) marker levels (online supplemental figure S2). In the CK compartment, adjacent normal kidney had higher levels of p53 and the apoptotic proteins BAD and BIM, and higher levels of PARP. Conversely, primary tumors had higher levels of B7-H3, PD-L1, Ki-67, HLA-DR, and IDO1.

In the TMAs with patient-matched primary and metastatic tumors (YTMA-166 and YTMA-528), which were analyzed separately, there was striking overlap in markers with the highest differential expression between primary and metastatic specimens in the CD45 compartment (figure 2B,C, online supplemental figure S3,4). The immune checkpoint TIM-3 was among the top differentially expressed marker in both arrays, with lower expression in metastatic samples. CTLA-4 levels were also significantly lower in metastases in both TMAs. To a lesser degree, LAG3 and PD-L2 levels were also lower, but only in YTMA-528. The other immune checkpoints in the panel (PD-1, PD-L1, B7-H3, and VISTA) were not significantly different between primary tumors and metastases. Some but not all markers of cytolytic activity and T cell activation were significantly lower in metastases in both arrays, particularly GZMA. Expression of both GZMB and CD25 was lower in metastases, more prominently in YTMA-528. Additionally, IDO1 and OX40L levels were notably lower in metastatic samples in both arrays.

Both arrays demonstrated lower immune cell infiltration in metastatic samples, with lower CD45 levels compared with primary tumors. Looking at specific immune populations, CD20 (B cells) and FOXP3 (Tregs) levels were reduced at metastatic sites (online supplemental figure S3,4). In YTMA-166, overall T cell (CD3) and CD8 levels were lower in metastatic sites, but this was not replicated in YTMA-528. There were no significant differences in other T cell population (CD4, CD45RO), NK cell (CD56), monocyte (CD14), or neutrophil (CD66b) marker levels in either array.

In the CD68 compartment, CD163 and ARG1, generally thought to be markers of M2-like, immunosuppressive macrophages, were expressed at significantly lower levels at metastatic relative to primary sites in YTMA-166 (figure 2B and online supplemental figure S3).38 39 Conversely, M1-like, inflammatory macrophage markers HLA-DR and CD127 were lower in metastatic samples in YTMA-528 (figure 2 and online supplemental figure S4). This suggests that the possibility of enrichment of different macrophage subsets in metastases depending on disease characteristics, such as the cerebrotropic nature of samples included on YTMA-528. In the tumor compartments of both arrays, primary tumors had higher levels of IDO1 and OX40L, various apoptotic markers (cleaved caspase 9, BAD, BIM, CD95/Fas), and the fibroblast marker FAP-alpha, compared with metastases. A summary of markers with significantly different expression between primary and metastatic specimens shared between the arrays is shown in online supplemental figure S5A. We performed additional matched-pair expression analysis on select markers (TIM-3, CTLA-4, GZMA, CD25, CD20, FOXP3) identified through the differential expression analysis using the mixed-effects model and observed continued significant expression differences (online supplemental figure S5B).

Marker and cell population differences in brain metastases versus extracranial metastases

We next investigated marker expression in brain metastases compared with extracranial metastases in the cohort from YTMA-528 and saw few significant differences in marker expression (figure 2D, online supplemental figure S6). We grouped all non-brain metastases together to have adequate sample sizes for comparison, recognizing that this is not a homogeneous group. STING levels were higher in extracranial metastases in all compartments, although more prominently in macrophages and tumor cells. In the CD45 compartment, CD11c levels were also higher in brain metastases.

Survival analyses in patients with brain metastases

We next explored whether there were features of the TME that could predict survival after brain metastases development based on the samples on YTMA-528. We performed univariable regression analyses of marker expression in brain metastases for each compartment using all 52 immuno-oncology markers and other clinical variables, including International Metastatic RCC Database Consortium risk score, tumor size, tumor grade, sex, age, and the presence of de novo brain metastases. Variables significant on univariable analysis were subjected to multivariable analysis (figure 3A). Only high TIM-3 (HR 3.79, adjusted p=0.016) and CD40 (HR 5.59, adjusted p=0.004) in the CD45 compartment remained significantly associated with shorter survival on multivariable analysis. Lower expression levels of TIM-3 and CD40 in brain metastases were associated with longer overall survival (OS) (figure 3B). There were no significant prognostic markers on multivariable analysis in the macrophage and tumor compartments.

We repeated this analysis based on marker expression in all metastases (brain and extracranial) and primary tumors separately from YTMA-528. High STING expression in leukocytes of primary tumors was strongly associated with better OS after brain metastases development (HR 0.08, adjusted p<0.001) (figure 3C,D), as was high PD-L2 (HR 0.45, adjusted p=0.011) in the tumor compartment of metastases (figure 3E,F).

We also sought to explore whether there were features of the TME from tissue specimens prior to the occurrence of brain metastases that could predict timing of brain involvement. We looked at primary tumors and extracranial metastases on YTMA-528 individually. In
Figure 3  Lower expression of TIM-3 and CD40 are associated with longer survival after development of brain metastasis. Multivariable Cox proportional hazards regression analyses of survival after diagnosis of brain metastasis for significant factors identified by univariable analysis for: (A) CD45 compartment of brain metastatic samples; (C) CD45 compartment of primary tumors; and (E) CK compartment of all metastatic samples. (B, D, F) Kaplan-Meier plots of survival post-brain metastasis, with the corresponding HRs and log-rank p values, based on dichotomized (by median) expression of the indicated marker in the given compartment and tissue type. All plots were cut-off at day 2000 for visualization purposes, with no meaningful changes to the data after this point. CI, confidence interval; CK, cytokeratin; HR, hazard ratio; IMDC, International Metastatic RCC Database Consortium.
primary tumors, B7-H3 levels in leucocytes (HR 2.78, adjusted p=0.038) and HLA-DR levels in macrophages (HR 0.26, adjusted p=0.007) were associated with time to development of brain metastasis on multivariable analysis (figure 4A–D). Higher STING levels in tumor cells of extracranial metastases (HR 0.17, adjusted p=0.002), and lower tumor grade and older patient age, were significantly associated with brain metastasis-free survival (figure 4E,F).

**Markers associated with potential benefit from immunotherapy**

As over two-thirds of the patients on YTMA-528 had received an immunotherapy-containing regimen at some point (online supplemental table S2), we assessed potential markers of progression-free survival (PFS) and OS with the first immunotherapy regimen, looking separately at primary tumors and metastases. Of note, 90% of the tumor specimens included in this analysis were of clear cell histology. In primary tumors, only higher STING expression in leucocytes was significantly associated with longer PFS (HR 0.22, adjusted p=0.013) and OS (HR 0.24, adjusted p=0.017) on multivariable analysis (figure 5A,B). For metastases (extracranial and brain), higher PD-L2 and lower Ki-67 in the tumor compartment were significantly associated with longer PFS (PD-L2: HR 0.31, adjusted p=0.002; Ki-67: HR 2.14, adjusted p=0.016) and OS (PD-L2: HR 0.25, adjusted p=0.003; Ki-67: HR 3.26, adjusted p=0.008) (figure 5C,D). Higher p53 expression was also associated with longer OS (HR 0.27, adjusted p=0.006). No markers in the leucocyte and macrophage compartments in metastases were associated with PFS or OS.

Although this analysis was not done in the context of a clinical trial with a non-immunotherapy treated arm to test whether these factors were truly predictive, we did compare OS from time of first treatment based on dichotomized expression of these factors in the patients on YTMA-528 that did not receive immunotherapy. Because of small group sizes, we could not analyze primary tumors and metastases separately, but instead grouped them together. STING levels in leucocytes and PD-L2 and Ki-67 levels in tumor cells were not associated with OS after treatment, suggesting that the prior associations seen may be specific to immunotherapy (online supplemental figure S7).

We next investigated whether expression of the significant markers correlated with immunotherapy ORR (complete response+partial response) and DCR (complete response+partial response+stable disease for >6 months). We assessed response for high and low marker expression in both primary and metastatic tumors. In primary tumors, the ORR was similar between high and low STING expressing tumors (in leucocytes) although the DCR was increased in high STING expressors (78% vs 64%) (online supplemental figure S8A). In metastases, low STING expression was associated with a slightly higher ORR, but a similar DCR.

In the tumor compartment, ORR did not seem to vary based on PD-L2 expression, whether in primary tumors or metastases (online supplemental figure S8B). However, high PD-L2 expressors in metastases had higher DCR than low expressors (80% vs 50%). For Ki-67, expression in metastases did not seem to predict ORR, whereas high Ki-67 in primary tumors had a threefold increase in ORR (75% vs 25%) (online supplemental figure S8C), consistent with the higher response rate to immunotherapy seen with more aggressive disease. DCY varied by Ki-67 expression in both primary tumors and metastases but in opposing directions.

**DISCUSSION/CONCLUSION**

In this study, we profiled the expression of 52 immunology related proteins in the leucocyte (CD45+), macrophage (CD68+), and epithelial (CK+) compartments of tissue specimens on TMAs representing progressively advanced stages of RCC. We found lower infiltration of certain immune populations in metastases relative to primary tumors, and lower expression of certain immune checkpoint and cytolytic and T cell activation markers. We also saw differences in macrophage polarization markers in more advanced stages. When comparing brain metastases to extracranial metastases, there were strikingly few differences, but STING levels were higher in extracranial metastases while BCL-XL levels were higher in brain metastases. We then used these data to explore prognostic features of the TME and found that STING levels, among other features, may have prognostic and predictive value in immunotherapy treated patients.

Primary RCC tumors are highly heterogeneous and differences between primary and metastatic tumors have previously been demonstrated for specific markers. This is important for selecting the biopsy site to determine the likelihood of response to certain therapies. Our analysis confirmed this, with low correlations in expression of many markers across replicate cores. However, the degree of heterogeneity depended on the cellular compartment and tissue site being queried, echoing our prior findings related to LAG3. Overall, expression was more variable in the immune compartments (CD45+ and CD68+) than in tumor cells. Within the immune compartments, primary tumors and brain metastases were more heterogeneous than extracranial metastases, while in tumor cells, the degree of heterogeneity was similar between brain metastases and extracranial metastases, with only primary tumors displaying higher heterogeneity. To our knowledge, this is the first study specifically looking at heterogeneity in the TME of RCC brain metastases, although others have noted the high spatial heterogeneity of brain metastases in other cancer types. These results suggest that consideration should be given not only to the specific biopsy/resection site (eg, primary tumor vs extracranial metastasis vs brain metastasis) but also the cellular compartment where the target of interest is expressed when deciding if a single biopsy specimen...
Figure 4  Lower expression of B7-H3 and higher expression of HLA-DR and STING are associated with longer time to development of brain metastases. Multivariable Cox proportional hazards regression analyses of time to development of brain metastases from initial diagnosis for significant factors identified by univariable analysis for: (A) CD45 compartment of primary tumors; (C) CD68 compartment of primary tumors; and (E) CK compartment of extracranial metastatic samples. (B, D, F) Kaplan-Meier plots of brain metastases disease-free survival, with the corresponding HRs and log-rank p values, based on dichotomized (by median) expression of the indicated marker in the given compartment and tissue type. All plots were cut-off at day 2000 for visualization purposes, with no meaningful changes to the data after this point. CI, confidence interval; CNS, central nervous system; HR, hazard ratio.
Figure 5  STING, PD-L2, and Ki-67 expression levels are associated with progression-free and overall survival after immunotherapy. Multivariable Cox proportional hazards regression analyses of progression-free survival and overall survival after the first immunotherapy drug administered for significant factors identified by univariable analysis for: (A) CD45 compartment of primary tumors; and (C) CK compartment of metastatic samples. (B, D) Kaplan-Meier plots of progression-free survival and overall survival after the first immunotherapy drug administered, with the corresponding HRs and log-rank p values, based on dichotomized (by median) expression of the indicated marker in the given compartment and tissue type. All plots were cut-off at day 2000 for visualization purposes, with no meaningful changes to the data after this point. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival.
or limited area from a resected tumor provides reliable information on expression of predictive biomarkers.

Our two TMAs containing pairs of primary and metastatic tumors both showed significantly lower expression of the immune checkpoints TIM-3 and CTLA4 and the cytolytic and T cell activation markers GZMA, GZMB, and CD25 in metastatic sites compared with primary tumors. Notably, TIM-3 was among the top differentially expressed marker in both TMAs. Lower levels of TIM-3 in metastases relative to primary tumors in RCC has been noted before in a study using IHC.

Our results must be interpreted within the confines of the technology—namely, the lack of single-cell resolution using DSP. We could only assess protein expression of cells within a molecularly defined compartment in aggregate, and thus could not discern heterogeneity in immune cell populations or determine if the comparisons between primary and metastases represent all cells or only certain subsets of cells. For example, it is possible that marker intensity per individual cell might be higher among certain cellular subsets in metastases compared with primary sites but lower on aggregate. Our study did take advantage of the higher-throughput nature of DSP relative to single-cell technologies and the ability to use archived FFPE tissue and encompassed 76 unique primary tumors specimens and 86 unique metastatic specimens, including 24 brain metastases, often with multiple replicate cores per specimen. Our large cohort sizes and the ability to include replicate cores would potentially allow us to capture more of the variability within and between tumors, although not within specific immune subsets.

Our findings cannot be directly compared with those in Braun et al, which almost exclusively compared primary tumor samples among patients that developed different disease stages and showed that samples from patients who developed more advanced disease are more highly enriched with terminally exhausted CD8+ T cell populations, characterized by high expression of multiple immune checkpoints. We compared primary tumors to metastatic samples among cohorts of patients that all developed advanced disease. In effect, our primary tumor samples were comparable to their advanced stage samples (ie, primary tumor samples from patients who would go on to develop more advanced disease) and indeed displayed high levels of multiple immune checkpoints relative to metastatic samples. Our findings are also supported by the lower overall levels of the cytolytic proteins GZMA and GZMB and the T cell activation marker CD25 that we observed in metastases. Alternatively, our results could be explained by an increased proportion of progenitor exhausted T cells, which have lower expression of immune checkpoints compared with terminally exhausted T cells, in metastases relative to primary tumors.

Our analysis of the CD68+ macrophage compartment revealed a divergence in expression of macrophage markers between primary tumors and metastases. Metastatic samples from patients with brain involvement expressed lower levels of the M1-like markers HLA-DR and CD127 relative to primary tumors, suggesting a tumor-permitting phenotype with more advanced stages of RCC. This is consistent with the single-cell transcriptomic findings and model of a dysfunctional immune circuit involving T cells and macrophages proposed in Braun et al. We did not see major differences in macrophage polarization markers when comparing brain metastases and extracranial metastases, suggesting that patients who develop brain metastases may have a more tumor-permitting TME at all sites. Another possible explanation is the enrichment of different macrophage populations in metastases depending on other intrinsic properties of the tumors, such as a propensity for cerebrotropism, as described by our group previously for melanoma. While other studies have investigated certain immune characteristics of RCC brain metastases, including increased CCR2+ macrophages in brain metastases compared with primary tumors, to our knowledge, ours is the first to investigate macrophage polarization specifically in RCC brain metastases. We were limited to the macrophage markers included in the immuno- oncology panel. Further studies, including whole transcriptomic profiling and single-cell analysis, are warranted to comprehensively characterize the functional and phenotypic state of these macrophages in brain metastases.

When we compared marker levels in brain metastases to extracranial metastases, we saw few significant differences, including for most markers of specific immune populations except for the dendritic cell marker CD11c, which was expressed at modestly higher levels in brain metastases. The other two exceptions were BCL-XL and STING. BCL-XL levels were higher in brain metastases in all compartments. Interestingly, BCL-XL was recently identified as a genetic dependency in kidney cancer cell lines that possessed a mesenchymal gene signature. Pharmacological or genetic inhibition of BCL-XL in dependent cell lines led to antitumor effects, and a ‘BCL-XL dependency’ signature was identified in a substantial proportion (~30%) of RCC patients and was associated with worse clinical outcomes, supporting the further exploration of BCL-XL inhibitors in RCC. Our data suggest that BCL-XL expression may be specifically enriched in RCC brain metastases. Further work is needed to determine if BCL-XL is required for RCC metastatic seeding of the CNS and is a brain metastases-specific vulnerability, especially as BCL-XL protein levels did not appear predictive of response to BCL-XL inhibitors in the aforementioned study.

STING levels were higher in extracranial metastases relative to brain metastases in all compartments, most prominently in CD45+ leukocytes. The cGAS-STING pathway can inhibit tumor growth by activating interferon signaling and immune surveillance, although it is increasingly recognized as also having protumorigenic and metastasis-promoting functions, depending on the context, such as more advanced stages of disease when cancer cells can become tolerant of chronic inflammatory signaling and even use it to drive metastasis.
functional significance of relatively reduced STING expression in brain metastases is unclear, and further work is needed to determine whether STING agonism in RCC brain metastases would have antitumorigenic or protumorigenic effects.

Of note, we had limited sample sizes at other specific metastatic locations (eg, bone, lung, lymph node) that precluded a more granular comparison of the TME at specific metastatic locations, which are likely not homogeneous. Future studies with adequate samples sizes should focus on more sitespecific comparisons.

STING also emerged as a potential prognostic and predictive biomarker. Higher STING expression in the CD45+ cells of primary tumors was associated with significantly longer OS after brain metastases development. This association may be indicative of a general tendency to a strong immune response and surveillance system at a stage of disease before the cancer becomes tolerant of chronic inflammation. Higher STING expression was also associated with significantly longer PFS and OS in immunotherapy-treated patients, which could be reflective of similar principles. Higher STING expression in the tumor compartment of extracranial metastases was associated with a longer time to developing CNS disease. Given the lower STING levels observed in brain metastases, this could also indicate that higher STING expression in other metastatic niches is a barrier to successful CNS spread.

We also observed that lower expression of TIM-3 and CD40 in the CD45+ leucocytes of brain metastases was associated with longer OS, suggesting that these proteins may be important modulators of the immune milieu in brain metastases. Antagonistic TIM-3 antibodies and agonistic CD40 antibodies are being investigated as immunotherapeutic agents, including for RCC, and disease evolution in the CNS should be observed closely.

Interestingly, we did not observe concordant prognostic associations between various immune checkpoints in different tumor locations (primaries, extracranial or intracranial metastases) in CD45+ cells. Indeed, lower expression of a different checkpoint, B7-H3, on the leucocytes in primary tumor samples was associated with longer time until brain metastases occurrence. These findings imply that the key driving checkpoint proteins may vary based on disease stage and anatomic location, perhaps not surprising given our and others’ findings about evolution of the TME over time and space in response to therapy. Further work is needed to verify and expand on these hypothesis-generating findings.

Although our analyses only included a small, single-institution TMA cohort of immunotherapy-treated patients who received various regimens, we did find an interesting association between higher PD-L2 tumor expression in metastases and longer immunotherapy-related PFS and OS and a higher DCR. PD-L2 may be expressed to a greater degree than PD-L1 in RCC and has previously been identified as a possible poor prognostic marker in clear-cell and chromophobe RCC. PD-L2 protein expression has been associated with response to anti-PD-1 therapy in other cancer types, including head and neck cancer, but to our knowledge has not previously been identified as a predictive biomarker for immunotherapy in RCC. A small retrospective study did find an association between PD-L2 mRNA levels and response to anti-PD-L1 therapy in four cancer types, including RCC. The PD-L2 finding also is in accord with the findings in Bi et al of an immunosuppressive program identified in the cancer cells of ICI-responders. However, we did not identify higher immune checkpoint expression on CD45+ cells in patients with a better response to immunotherapy. Validation of our finding in a separate cohort and with orthogonal approaches is needed.

In summary, we performed proteomic profiling of immuno-oncology-related proteins on a large cohort of RCC tumors representing progressively advanced stages of disease, including brain metastases and extracranial metastases. We were able to assess protein expression in more than one area of tumor for most specimens and found a greater degree of heterogeneity in the immune compartments of primary tumors and brain metastases, suggesting that single-point assessments of these areas for clinical decision-making may not be sufficiently informative. We also found that brain metastases have higher expression of BCL-XL and lower expression of STING, suggesting a possible therapeutic combination to explore further in preclinical models of RCC metastases. We used an extensive clinical database connected with the brain metastasis-focused TMA to start developing prognostic and predictive biomarkers. Lower B7-H3 and higher HLA-DR and STING may predict a longer time until the occurrence of brain metastases, and lower TIM-3 and CD40 in the TME of brain metastases predicts longer OS after brain metastases development. Lastly, higher STING in primary tumors and higher PD-L2 in metastases are associated with longer PFS and OS after receiving immunotherapy. These preliminary biomarker data need to be validated in larger cohorts and with the use of orthogonal technologies.

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