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Expression of T-cell Exhaustion Molecules and Human Endogenous Retroviruses as Predictive Biomarkers for response to Nivolumab in Metastatic Clear Cell Renal Cell Carcinoma

Miriam Ficial^{1,2}, Opeyemi A. Jegede³, Miriam Sant'Angelo^{1,2}, Yue Hou^{4,5}, Abdallah Flaifel^{1,2}, Jean-Christophe Pignon^{1,2}, David A. Braun^{2,5,6}, Megan Wind-Rotolo⁷, Maura A. Sticco-Ivins¹, Paul J. Catalano^{2,3}, Gordon J. Freeman^{2,5}, Arlene H. Sharpe⁸, F. Stephen Hodi^{2,9}, Robert J. Motzer¹⁰, Catherine J. Wu^{2,5,6}, Michael B. Atkins¹¹, David F. McDermott^{2,12}, Sachet A. Shukla^{4,6}, Toni K. Choueiri^{2,5}, Sabina Signoretti^{1,2,13}

¹Department of Pathology, Brigham and Women's Hospital, Boston, MA, 02115, USA

²Harvard Medical School, Boston, MA, 02215, USA

³Department of Data Sciences, Dana-Farber Cancer Institute, Boston, MA, 02215, USA

⁴Translational Immunogenomics Lab, Dana-Farber Cancer Institute, Boston, MA, 02215, USA

⁵Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, 02215, USA

⁶Broad Institute of MIT and Harvard, Cambridge, MA, 02142, USA

⁷Bristol-Myers Squibb, Princeton, NJ, 08540, USA

⁸Department of Immunology, Blavatnik Institute, Harvard Medical School, Boston, MA, 02215, USA

⁹Center for Immune-Oncology, Dana-Farber Cancer Institute, Boston, MA, 02215, USA

¹⁰Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, 10065, USA

¹¹Georgetown-Lombardi Comprehensive Cancer Center, Washington, DC, 20057, USA

¹²Medical Oncology, Beth Israel Deaconess Medical Center, Boston, MA, 02215, USA

¹³Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA, 02215, USA

Abstract

PURPOSE: We sought to validate levels of CD8⁺ tumor-infiltrating cells (TIC) expressing PD-1 but not TIM-3 and LAG-3 (IF biomarker) (Pignon et al, 2019) and to investigate human endogenous retroviruses (hERVs) as predictors of response to anti-PD-1 in a randomized trial of nivolumab (nivo) versus everolimus (evero) in patients with metastatic clear cell renal cell carcinoma (mccRCC) (CheckMate-025).

EXPERIMENTAL DESIGN: Tumor tissues (nivo: n=116, evero: n=107) were analyzed by multiparametric immunofluorescence (IF) and qRT-PCR. Genomic/transcriptomic analyses were performed in a subset of samples. Clinical endpoints included objective response rate (ORR), progression-free survival (PFS), overall survival (OS), and durable response rate (DRR, defined as CR or PR with a PFS ≥ 12 months).

RESULTS: In the nivo (but not evero) arm, patients with high-IF biomarker density (24/116, 20.7%) had higher ORR (45.8% vs 19.6%, $p=0.01$) and DRR (33.3% vs 14.1%, $p=0.03$) and longer median PFS (9.6 vs 3.7 months, $p=0.03$) than low-IF biomarker patients. By RNA-seq, several inflammatory pathways ($q<0.1$) and immune-related gene signature scores ($q<0.05$) were enriched in the high-IF biomarker group. When combined with the IF biomarker, tumor cell (TC) PD-L1 expression (≥ 1%) further separated clinical outcomes in the nivo arm. ERVE4 expression was associated with increased DRR and longer PFS in nivo-treated patients.

CONCLUSION: High levels of CD8⁺ TIC expressing PD-1 but not TIM-3 and LAG-3 and ERVE4 expression predicted response to nivo (but not to evero) in mcrRCC patients. Combination of the IF biomarker with TC PD-L1 improved its predictive value, confirming our previous findings.

Introduction

Immune checkpoint inhibitors (ICIs) have revolutionized the management of several cancer types, including advanced clear cell Renal Cell Carcinoma (ccRCC). Nivolumab (an anti-PD-1 ICI) (nivo) attained FDA-approval as a second line therapy in advanced ccRCC(1), while the combination of nivo and ipilimumab (an anti-CTLA4 ICI) was FDA-approved as a first line therapy in patients with advanced ccRCC based on the results of the CheckMate-214 trial(2). However, only a minority of patients achieve durable responses when treated with ICIs and predictive biomarkers of response are urgently needed to identify patients who are more likely to benefit from ICI therapy(3).

Results from mouse models show that CD8⁺PD-1⁺ cells expressing additional inhibitory receptors are more dysfunctional, e.g., CD8⁺PD-1⁺TIM-3⁺ cells are more dysfunctional than CD8⁺PD-1⁺TIM-3⁻ cells(4,5). Our group previously showed that levels (measured as either percentage or density) of CD8⁺ tumor infiltrating cells (TIC) expressing PD-1 but not TIM-3 and LAG-3 (CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC) predict response to nivo in patients with metastatic ccRCC(6).

Data from different groups recently suggested that CD8⁺ PD-1⁺TCF7⁺ progenitor or stem-like exhausted TIC are the major population that provides the proliferative burst after PD-1 ICI(7) and their presence is associated with response and better survival in ICI-treated melanoma patients(8–10), while scarcity of MHC-II immune niches enriched in CD8⁺TCF7⁺ cells predicted progressive disease in a small cohort of ccRCC(11).

Human endogenous retroviruses (hERVs) expression correlates with high levels of immune infiltration, increased cytolytic activity and immune checkpoint expression (PD-1, PD-L1, CTLA-4, CD80, BTLA, HVEM, LAG3) in ccRCC (12–14) and has been associated with response to ICI in two small ccRCC cohorts(12,13). Of note, HERV-E/ERVE4 (from now

referred to as ERVE4) and hERV4700-derived epitopes elicit a tumor-restricted CD8⁺ T cell-mediated immune response(13,15,16) and a phase 1 trial is currently evaluating the safety and efficacy of the infusion of ERVE4 TCR transduced CD8⁺/CD34⁺ enriched T cells ([NCT03354390](#)).

Here, we aimed to validate CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC as a biomarker of response to nivolumab and also investigate the predictive value of tumor cell (TC) PD-L1, CD8⁺TCF7⁺ TIC and hERV expression in a randomized trial of nivo versus everolimus (evero) in advanced ccRCC(1).

Patients and Methods

Study design and Patients

We analyzed tumor tissue samples from patients enrolled in the CheckMate 025 clinical trial (CM-025)(1). This was a randomized, multicenter, phase III study comparing nivo to evero in patients with advanced or metastatic ccRCC and measurable disease according to the Response Evaluation Criteria in Solid Tumors (RECIST version 1.1)(17), who had received one or two previous anti-angiogenic therapy regimens. Archival FFPE tissue sections were collected by the study sponsor at time of enrollment and transferred to the investigators upon approval of a material transfer agreement. Written informed consent to study participation, tissue collection and tissue analyses was provided by all patients before enrollment, according to the principles of the Declaration of Helsinki.

Clinical Endpoints

Objective response rate (ORR) was investigator-assessed and defined as the proportion of randomized patients with a complete response (CR) or a partial response (PR) per RECIST 1.1. Durable response rate (DRR) was defined as the proportion of randomized patients with a CR or PR with responses being progression-free for 12 months. Progression-free survival (PFS) was defined as the time from randomization to the first documented tumor progression per RECIST 1.1 or death from any cause (patients were censored at date of last disease assessment), while overall survival (OS) was defined as the time from randomization to the date of death (patients alive were censored at the date of last contact).

Immunohistochemistry assay

Tissue slides previously stained with Dako PD-L1 immunohistochemical (IHC) assay(1) were acquired by the study sponsor at 20x magnification using the Aperio AT2 Scanner (Leica Biosystems), exported as .svs files and transferred to the investigators.

The percentage of PD-L1 positive tumor cells (TC) was independently scored by two pathologists (M.F. and M.S.) using a cutoff of 1% for membranous staining. All cases considered positive by at least one pathologist were independently reviewed by an expert GU pathologist (S.S.). An agreement on the percentage of positive TC was found for discordant cases. All pathologists were blinded to the patient clinical outcomes.

Multiplex immunofluorescence assay and image analysis

A 6-plex immunofluorescence (IF) assay was optimized on a Bond RX Autostainer (Leica Biosystems) using the Opal multiplex IHC system (PerkinElmer/Akoya Biosciences Cat# NEL871001KT) and the BOND Polymer Refine Detection Kit (Leica Biosystems Cat# DS9800; see Supplementary Materials and Methods and Supplementary Table S1; DOI: [dx.doi.org/10.17504/protocols.io.bjbzkip6](https://doi.org/10.17504/protocols.io.bjbzkip6)).

Multiplex IF slides were acquired as whole slide multispectral images at 10x magnification using Vectra 3.0 (PerkinElmer) and five or more intra-tumoral CD8⁺T cells-enriched regions of interest (ROI; area of each ROI = 669 μ m x 500 μ m) were identified for each case and scanned at 20x magnification. ROI multispectral images were imported into InForm v2.2.0 (PerkinElmer, RRID:SCR_019161) and deconvoluted using a multispectral library. Image analysis was performed using HALO v2.1.1637.18 (Indica Labs, RRID:SCR_018350).

Details of the image acquisition and image analysis workflow are provided in the Supplementary Materials and Methods.

Genomic and transcriptomic data analysis

RNA sequencing (RNA-seq) and whole exome sequencing (WES) data were obtained as previously described(18). Among these, 71 patients had both IF and RNA-seq data and 117 patients had both IF and WES data available (see Supplementary Materials and Methods).

Real-time PCR for hERV expression

RNA was extracted from micro-dissected tumor-enriched areas from 4- μ m-thick unstained FFPE slides using the AllPrep DNA/RNA FFPE Mini Kit (Qiagen Cat# 80234). RNA concentration was assessed using the NanoDrop ND-1000 (Thermo Fisher Scientific).

Quantitative real-time PCR was performed using reverse transcribed RNA extracted from FFPE tumor tissues to assess levels of pan-ERVE4 and hERV4700-ENV and the reference genes 18S and HPRT (see Supplementary Materials and Methods). A list of forward primers, reverse primers, and probes used for the assay is provided in Supplementary Table S2.

Statistical analysis

All statistical analyses are described in the Supplementary Materials and Methods. Unless indicated otherwise, 1-sided p-values are presented and values ≤ 0.05 were considered statistically significant (see Supplementary Materials and Methods for methodology details).

Results

Patient Characteristics

Expression of PD-1, TIM-3, LAG-3, and TCF7 on CD8⁺ TIC was studied by multiparametric IF in 304 (nivo = 145, evero = 159) patients (Supplementary Figure S1) and evaluable IF data were obtained in 223 patients (nivo = 116, evero = 107) (see Supplementary Figure S2a for CONSORT diagram). Among the 304 available tissue samples, 191 (62.9%) were from primary tumors and 91 (29.9%) were from metastatic sites;

in 2 cases (0.7%) information about the sample type was not available. The majority of patients (62.9%) had received only one line of prior therapies and the median time from tissue collection to enrollment in the trial was 23.2 months. Detailed clinical information for patients with and without available tissue is provided in Supplementary Table S3.

There was no imbalance among the patients with any available tissue ($n = 304$) and those without ($n = 499$) with regards to gender, age, treatment arm, study crossover, clinical outcomes, clinical scores, number of prior therapies and time from tissue collection to randomization (Supplementary Table S3). However, the group of patients with available tissue was enriched for metastatic lesions as compared to patients without available tissue (29.9% vs 21.2%, p -value 0.018). Among the subset of patients with IF data ($n = 223$), patients in the nivo arm ($n = 116$) showed higher ORR (25% vs 1.9%, $p < 0.001$), higher DRR (18.1% vs 0.9%, $p < 0.001$), longer median PFS (4.2 vs 3.9 months, $p = 0.030$) and longer median OS (24.0 vs 16.2 months, $p < 0.001$) than patients in the evero arm ($n = 107$).

Association between tumor-infiltrating CD8⁺ cells expressing PD-1 but not TIM-3 and LAG-3 and clinical outcomes

We first used IF data to validate our previous finding that levels of CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC are a determinant of response to nivo in ccRCC(6). In the nivo arm, both density and percentage of CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC (as continuous variables) were linearly associated with higher ORR (OR = 1.43, $p = 0.028$ and OR = 9.22, $p = 0.020$, respectively). However, only density of CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC was associated with higher DRR (OR = 1.54, $p = 0.023$) and tended to be associated with longer PFS (HR = 0.87, $p = 0.058$) in the nivo arm. In the evero arm, there was no association between the tested biomarkers and any clinical outcomes (Table 1). Similar results were obtained when controlling for MSKCC and IMDC groups using stratified tests (Supplementary Table S4). Of note, a significant interaction between treatment and density of CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC was seen for both PFS and OS (2-sided $p = 0.024$ and 2-sided $p = 0.079$, respectively; significance determined as 2-sided $p < 0.15$) (Supplementary Table S5). The interaction was such that the higher the density of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC, the longer the PFS and OS in the nivo arm but not in the evero arm (Supplementary Figure S3).

To further explore the value of CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC as a predictive biomarker, we optimized a cutoff by maximizing sensitivity and specificity (Youden's Index approach(19)) for ORR in the nivo arm. At the optimized cutoff, nivo-treated patients with high density of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC (24/116, 20.7%) had higher ORR (45.8% vs 19.6%, $p = 0.011$) and DRR (33.3% vs 14.1%, $p = 0.035$) and longer median PFS (9.6 vs 3.7 months, $p = 0.032$) than patients with low IF biomarker (Figure 1a; Supplementary Table S6). High density of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC showed no association with PFS in the evero arm (Figure 1a) and no difference in OS was observed between patients with high and low IF biomarker in either arm (Figure 1b).

Since TCF7 expression on either CD8⁺ or CD8⁺PD-1⁺ TIC has been associated with response to anti-PD-1 in melanoma patients(8–10), we tested whether the expression of TCF7 (alone and in combination with immune checkpoints) on CD8⁺ TIC could help

predict responses to nivo in ccRCC patients. In our cohort, the percentage of CD8⁺ cells expressing TCF7 ranged from 0.00% to 51.01% (mean: 6.61% $\pm$ 7.66%; median: 4.17%). Among CD8⁺PD-1⁺ cells, TCF7 expression was more frequent in TIM-3-negative cells than in TIM-3-positive cells ($p < 0.001$) but did not significantly vary depending on LAG-3 expression. However, the density of CD8⁺PD-1⁺TCF7⁺TIM-3⁻LAG-3⁻ cells was higher than the density CD8⁺PD-1⁺TCF7⁺ cells expressing either TIM-3 or LAG-3, indicating that the majority of CD8⁺PD-1⁺ cells expressing TCF7 were negative for both TIM-3 and LAG-3 (Supplementary Figure S4).

In our analyses, density/percentage of CD8⁺TCF7⁺, CD8⁺PD-1⁺TCF7⁺ or CD8⁺PD-1⁺TCF7⁺TIM-3⁻LAG-3⁻ TIC (measured as continuous variables) were not associated with any clinical outcomes in either the nivo or the evero arm (Supplementary Tables S7-S8). However, there was a significant interaction between density of CD8⁺TCF7⁺, CD8⁺PD-1⁺TCF7⁺ or CD8⁺PD-1⁺TCF7⁺TIM-3⁻LAG-3⁻ TIC and treatment arm with respect to PFS ($p < 0.15$, Supplementary Table S9).

Association between CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC density and molecular tumor features

As our data show that tumors with high density of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC are more likely to respond to anti-PD-1 therapy, we sought to gain insights into the biology of these tumors by analyzing genomic and transcriptomic data available from 117 (nivo = 61; evero = 56) and 71 (nivo = 32; evero = 39) patients, respectively.

None of the most frequently mutated genes and copy number variations (CNVs) showed an association with density of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC (Supplementary Table S10, Supplementary Table S11).

By single sample gene set enrichment analysis (ssGSEA)(20) using the 50 Hallmark genes sets from the Molecular Signatures Database(21), multiple inflammatory pathways were enriched in the high CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density tumors as compared to the low density tumors (FDR $q < 0.1$) (Figure 1c). Consistent with this observation, immune-related gene signature scores (GSS) such as Tumor Inflammation Score (TIS) (22), IMmotion150_Teff(23), Cytolytic Activity(14), and JAVELIN(24) were also enriched in the high biomarker tumors (FDR $q < 0.05$) (Figure 1d). Transcriptomic inference of tumor-infiltrating immune populations (using CIBERSORTx(25)) showed increased CD8⁺ T cells, activated NK cells, follicular helper T cells and plasma cells in the high CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density tumors (FDR $q < 0.05$), while resting mast cells and resting CD4⁺ memory T cells were enriched in the low CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density cases (Figure 1e).

Patient stratification according to CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC density and TC PD-L1 status

Our previous study suggested that quantification of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC combined with TC PD-L1 expression can improve prediction of response to anti-PD-1 therapy in ccRCC patients(6). TC PD-L1 analysis performed by our group in cases with IF data showed that patients with TC PD-L1 $\geq 1\%$ had higher ORR ($p = 0.04$) and tended to

have higher DRR ($p = 0.14$) and longer PFS ($p = 0.07$) in the nivo arm but not in the evero arm (Supplementary Tables S12–S13; Supplementary Figure S5).

Similar to previous observations(6), when a maximum sensitivity cutoff was defined for the IF biomarker, combination with TC PD-L1 status further separated clinical outcomes in the nivo-treated patients with high CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC levels (92/111, 82.8%). In a combination of TC PD-L1 and CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density at the maximum sensitivity cutoff, we identified three groups of patients in the nivo arm with different clinical outcomes with regards to ORR (trend test p -value = 0.027), DRR (trend test p -value = 0.045) and PFS (trend test p -value = 0.019). Patients with both positive TC PD-L1 expression and high CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density had the most favorable outcomes, patients with negative TC PD-L1 expression and high CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density had intermediate outcomes and patients with negative/low for both markers had the poorest outcomes. Separation of PFS was not seen in the evero arm (Table 2; Figure 2). Similar results were obtained when controlling for MSKCC and IMDC groups using stratified tests (Supplementary Table S14).

Association between hERV levels and CD8⁺ PD-1⁺TIM-3⁻LAG-3⁻ TIC density, molecular tumor features, and clinical outcomes

Based on the published literature(12–14), we hypothesized that tumors expressing high levels of hERVs might have increased levels CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC and would display better outcome on nivo.

ERVE4 and hERV4700-ENV mRNA expression data was obtained for 224 patients (nivo = 112; evero = 112) (Supplementary Figure S2b for CONSORT diagram). ERVE4 and hERV4700-ENV were expressed in 51% and 40% of patients, respectively. The observed ERVE4 expression rate was in line with previously published data (26). No data about the expression rate of hERV4700-ENV was found in the literature. When we assessed the association between CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density and levels of ERVE4 and/or hERV4700-ENV dichotomized using an optimized cutoff (Youden's Index approach(19)), no difference in the distribution of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density was observed between hERV-positive and hERV-negative cases (Supplementary Figure S6).

ERVE4 expression as a continuous measure was associated with DRR (OR = 4.05, p -value = 0.042) and PFS in the nivo arm (HR = 0.42, p -value = 0.020) and showed an interaction with treatment with regard to PFS (2-sided $p = 0.080$, $p < 0.15$). The interaction was such that the higher the ERVE4 expression score, the better the PFS in the nivo arm but not in the evero arm. No association of continuous ERVE4 with ORR and OS was observed (Table 3). Similar results were obtained when controlling for MSKCC and IMDC groups using stratified tests (Supplementary Table S15). At the optimized cutoff, nivo-treated patients expressing ERVE4 had higher DRR ($p = 0.042$) and a significantly longer PFS (5.3 vs 3.6 months, $p = 0.017$) than patients not expressing ERVE4 (Supplementary Table S16; Figure 3a). No difference in ORR and OS was observed between ERVE4-positive and ERVE4-negative nivo-treated patients (Supplementary Table S17; Figure 3b). In the evero arm, ERVE4 status was not associated with clinical outcomes. No difference in gene mutations and CNVs was detected according to ERVE4 status (Supplementary Table S18–

S19). By RNA-seq data analysis, ERVE4 (X2256_chr6.89371970.89380600) was the only differentially expressed ERV transcript between ERVE4-positive and ERVE4-negative cases (FDR $q < 0.25$, Figure 3c). Tumor Inflammation Score (TIS)(22), IMmotion150_Teff(23), Cytolytic Activity(14), and JAVELIN(24) GSS were upregulated in ERVE4-positive tumors (FDR $q < 0.25$, Figure 3d). ssGSEA(20) (Hallmark gene sets) and CIBERSORTx(25) analyses showed no significant difference between ERVE4-positive and ERVE4-negative cases (Supplementary Figures S7).

hERV4700-ENV as a continuous measure showed both an association with OS in the nivo arm (HR = 0.59, p-value = 0.050) and an interaction with treatment with regard to OS (2-sided $p = 0.113$, $p < 0.15$). The interaction was such that the higher the hERV4700-ENV expression score, the better the OS in the nivo-treated patients but not in the evero-treated ones. Continuous hERV4700-ENV showed no association with ORR, DRR and PFS in either arm (Table 3). Similar results were obtained when controlling for MSKCC and IMDC groups using stratified tests (Supplementary Table S15). When split at the optimized cutoff (Youden's Index(19)), hERV4700-ENV-positive patients showed a trend towards better PFS in both arms ($p = 0.096$ and $p = 0.054$ in the nivo and evero arm, respectively), (Supplementary Tables S16–S17; Supplementary Figure S8).

Discussion

There are currently no approved predictive biomarkers that can guide treatment decision for patients with metastatic RCC. A major challenge in the development of clinical biomarkers is that promising results obtained in individual studies are not reproduced by investigations conducted in independent patient cohorts. Our previous study of a phase 2 trial of nivolumab in metastatic ccRCC(6) suggested that levels (measured as either percentage or density) of CD8⁺ TIC expressing the therapy target PD-1 but negative for other exhaustion molecules (TIM-3 and LAG-3) are associated with response to anti-PD-1 treatment. Here, we independently confirmed these findings by analyzing tumor specimens from the randomized CM-025 trial and demonstrated that the predictive value of our IF biomarker is specific to patients treated with anti-PD-1 therapy. High levels of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC likely identify inflamed tumors harboring mildly exhausted tumor-specific T cells, which can be effectively re-invigorated by anti-PD-1 monotherapy. In line with this hypothesis, we found that tumors with high CD8⁺ PD-1⁺TIM-3⁻LAG3⁻ TIC were enriched for several inflammatory pathways, immune-related gene signature scores and immune cell populations such as CD8⁺ T cells, NK activated T cells, plasma cells and helper T cells. Of note, we showed that baseline CD8⁺ cell infiltration and immune-related gene signature scores were not associated with improved response or survival in a large cohort of metastatic ccRCC treated with anti-PD-1 monotherapy(18), indicating that more detailed analysis of CD8 subsets is necessary for predictive biomarker development.

Here, we also validated that combination of CD8⁺ TIC immunophenotyping with TC PD-L1 status stratifies metastatic ccRCC patients in three groups with significantly different outcome on nivo but not on the control treatment (evero). Combining CD8⁺ TIC immunophenotyping with TC PD-L1 status is particularly effective in identifying subsets of

patients with high likelihood (ORR: 50%, DRR: 33%) of experiencing durable responses to anti-PD-1 and could help to select patients who can be treated with PD1 monotherapy.

While analyses of melanoma cohorts recently demonstrated that CD8⁺TCF7⁺ progenitor/stem-like exhausted TIC can be frequently detected in this tumor type and might represent a determinant of response in ICI-treated patients(8–10), our data show that in metastatic ccRCC TCF7 expression on CD8⁺ TIC is relatively rare (mean 6.61% ± 7.66%). Although we confirmed that the majority of CD8⁺TCF7⁺ cells lacked the expression of exhaustion markers (TIM-3 and LAG-3)(7,8,10,11), we did not detect any definitive association between TCF7 expression of CD8⁺ TIC and outcome on anti-PD-1 therapy in our metastatic ccRCC cohort. Overall, these findings suggest that relative to melanoma, metastatic ccRCC displays a more terminally exhausted phenotype characterized by depletion of progenitor exhausted CD8⁺ T cells that were critical for response to immunotherapy in melanoma. Our results may provide biological insight as to why, compared to melanoma, response rates to ICI treatment are generally lower in metastatic ccRCC and why the development of adoptive cell therapies with tumor-infiltrating lymphocytes has been largely unsuccessful in this disease.

We also showed for the first time that ERVE4 expression, both as a continuous and as a binary variable, was associated with increased DRR and longer PFS in nivo-treated (but not in evero-treated) patients. In comparison to other tumor types, ccRCC has abundant CD8⁺ T cell infiltration but a modest mutation burden and tumor specific antigens recognized by immune cells remain largely unknown. Expression of ERVE4 is particularly high in ccRCC likely due to its direct regulation by HIF-2 α (upregulated by VHL inactivation) in concert with hypomethylation of proviral long terminal repeat (LTR) (26), and there is evidence that antigens derived from this provirus can be immunogenic and stimulate kidney cancer cell killing by cytotoxic T cells *in vitro* and *in vivo*(13–16). Our findings further support the role of ERVE4 as a kidney cancer antigen and therapeutic target for immunotherapy. Future large-scale analysis of the hERV landscape in metastatic ccRCC might help identify other immunogenic hERVs that could be helpful in enhancing the predictive value of ERVE4 for ICI therapy in this tumor type. Moreover, as ERVE4 expression is modulated by epigenetic events (26), our data supports the potential of combining DNA methylation inhibitors with immunotherapy agents in ccRCC (27).

This study has several limitations. As only one tumor sample was analyzed per patient, our study did not account for intratumor heterogeneity. However, it needs to be recognized that the same limitation applies when analyzing marker expression in the clinical setting. Moreover, in most cases the samples analyzed derived from primary tumors obtained prior to initial systemic therapy and might therefore not entirely reflect the biology of the VEGFR TKI resistant/modulated metastatic lesions which were the target of the systemic therapy. Second line IO cohorts could also be relatively depleted for patients with aggressive tumor types, such as sarcomatoid. By focusing on CD8⁺ TIC immunophenotyping, our study did not explore additional immune cell populations which might play an important role in determining response and resistance to ICI, such as B cells and myeloid cells(28–32). In addition, due to the low response rate in the evero arm, ORR and DRR were analyzed in the

nivo arm only limiting comparison between the two treatment arms to PFS and OS. Finally, the retrospective nature of the study represents a further limitation by itself.

In conclusion, here we provided evidence that high levels of CD8⁺ PD1⁺TIM3⁻LAG3⁻TIC and ERVE4 expression predict response to nivo (but not to control evero) in metastatic ccRCC patients. Combination of CD8⁺ PD1⁺TIM3⁻LAG3⁻TIC with TC PD-L1 status further improves its predictive value, confirming our previous findings(6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. *N Engl J Med* 2015;373(19):1803–13 doi 10.1056/NEJMoa1510665. [PubMed: 26406148]
2. Motzer RJ, Tannir NM, McDermott DF, Aren Frontera O, Melichar B, Choueiri TK, et al. Nivolumab plus Ipilimumab versus Sunitinib in Advanced Renal-Cell Carcinoma. *N Engl J Med* 2018;378(14):1277–90 doi 10.1056/NEJMoa1712126. [PubMed: 29562145]
3. Havel JJ, Chowell D, Chan TA. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat Rev Cancer* 2019;19(3):133–50 doi 10.1038/s41568-019-0116-x. [PubMed: 30755690]
4. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 2009;10(1):29–37 doi 10.1038/ni.1679. [PubMed: 19043418]
5. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 2010;207(10):2187–94 doi 10.1084/jem.20100643. [PubMed: 20819927]
6. Pignon JC, Jegede O, Shukla SA, Braun DA, Horak CE, Wind-Rotolo M, et al. irRECIST for the Evaluation of Candidate Biomarkers of Response to Nivolumab in Metastatic Clear Cell Renal Cell Carcinoma: Analysis of a Phase II Prospective Clinical Trial. *Clin Cancer Res* 2019;25(7):2174–84 doi 10.1158/1078-0432.CCR-18-3206. [PubMed: 30670497]
7. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* 2016;537(7620):417–21 doi 10.1038/nature19330. [PubMed: 27501248]
8. Sade-Feldman M, Yizhak K, Bjorgaard SL, Ray JP, de Boer CG, Jenkins RW, et al. Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell* 2018;175(4):998–1013 e20 doi 10.1016/j.cell.2018.10.038. [PubMed: 30388456]

9. Siddiqui I, Schaeuble K, Chennupati V, Fuertes Marraco SA, Calderon-Copete S, Pais Ferreira D, et al. Intratumoral Tcf1(+)PD-1(+)CD8(+) T Cells with Stem-like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. *Immunity* 2019;50(1):195–211 e10 doi 10.1016/j.immuni.2018.12.021. [PubMed: 30635237]
10. Miller BC, Sen DR, Al Abosy R, Bi K, Virkud YV, LaFleur MW, et al. Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade. *Nat Immunol* 2019;20(3):326–36 doi 10.1038/s41590-019-0312-6. [PubMed: 30778252]
11. Jansen CS, Prokhnevskaya N, Master VA, Sanda MG, Carlisle JW, Bilen MA, et al. An intra-tumoral niche maintains and differentiates stem-like CD8 T cells. *Nature* 2019;576(7787):465–70 doi 10.1038/s41586-019-1836-5. [PubMed: 31827286]
12. Panda A, de Cubas AA, Stein M, Riedlinger G, Kra J, Mayer T, et al. Endogenous retrovirus expression is associated with response to immune checkpoint blockade in clear cell renal cell carcinoma. *JCI Insight* 2018;3(16) doi 10.1172/jci.insight.121522.
13. Smith CC, Beckermann KE, Bortone DS, De Cubas AA, Bixby LM, Lee SJ, et al. Endogenous retroviral signatures predict immunotherapy response in clear cell renal cell carcinoma. *J Clin Invest* 2018;128(11):4804–20 doi 10.1172/JCI121476. [PubMed: 30137025]
14. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* 2015;160(1–2):48–61 doi 10.1016/j.cell.2014.12.033. [PubMed: 25594174]
15. Takahashi Y, Harashima N, Kajigaya S, Yokoyama H, Cherkasova E, McCoy JP, et al. Regression of human kidney cancer following allogeneic stem cell transplantation is associated with recognition of an HERV-E antigen by T cells. *J Clin Invest* 2008;118(3):1099–109 doi 10.1172/JCI34409. [PubMed: 18292810]
16. Cherkasova E, Scrivani C, Doh S, Weisman Q, Takahashi Y, Harashima N, et al. Detection of an Immunogenic HERV-E Envelope with Selective Expression in Clear Cell Kidney Cancer. *Cancer Res* 2016;76(8):2177–85 doi 10.1158/0008-5472.CAN-15-3139. [PubMed: 26862115]
17. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009;45(2):228–47 doi 10.1016/j.ejca.2008.10.026. [PubMed: 19097774]
18. Braun DA, Hou Y, Bakouny Z, Ficial M, Sant' Angelo M, Forman J, et al. Interplay of somatic alterations and immune infiltration modulates response to PD-1 blockade in advanced clear cell renal cell carcinoma. *Nat Med* 2020;26(6):909–18 doi 10.1038/s41591-020-0839-y. [PubMed: 32472114]
19. Youden WJ. Index for rating diagnostic tests. *Cancer* 1950;3(1):32–5 doi 10.1002/1097-0142(1950)3:1<32::aid-cnrcr2820030106>3.0.co;2-3. [PubMed: 15405679]
20. Hanzelmann S, Castelo R, Guinney J. GSEA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* 2013;14:7 doi 10.1186/1471-2105-14-7. [PubMed: 23323831]
21. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* 2015;1(6):417–25 doi 10.1016/j.cels.2015.12.004. [PubMed: 26771021]
22. Danaher P, Warren S, Lu R, Samayoa J, Sullivan A, Pekker I, et al. Pan-cancer adaptive immune resistance as defined by the Tumor Inflammation Signature (TIS): results from The Cancer Genome Atlas (TCGA). *J Immunother Cancer* 2018;6(1):63 doi 10.1186/s40425-018-0367-1. [PubMed: 29929551]
23. McDermott DF, Huseni MA, Atkins MB, Motzer RJ, Rini BI, Escudier B, et al. Clinical activity and molecular correlates of response to atezolizumab alone or in combination with bevacizumab versus sunitinib in renal cell carcinoma. *Nat Med* 2018;24(6):749–57 doi 10.1038/s41591-018-0053-3. [PubMed: 29867230]
24. Choueiri TK, Albiges L, Haanen JBAG, Larkin JMG, Uemura M, Pal SK, et al. Biomarker analyses from JAVELIN Renal 101: Avelumab + axitinib (A+Ax) versus sunitinib (S) in advanced renal cell carcinoma (aRCC). *Journal of Clinical Oncology* 2019;37(15_suppl):101- doi 10.1200/JCO.2019.37.15_suppl.101.

25. Newman AM, Steen CB, Liu CL, Gentles AJ, Chaudhuri AA, Scherer F, et al. Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nat Biotechnol* 2019;37(7):773–82 doi 10.1038/s41587-019-0114-2. [PubMed: 31061481]
26. Cherkasova E, Malinzak E, Rao S, Takahashi Y, Senchenko VN, Kudryavtseva AV, et al. Inactivation of the von Hippel-Lindau tumor suppressor leads to selective expression of a human endogenous retrovirus in kidney cancer. *Oncogene* 2011;30(47):4697–706 doi 10.1038/onc.2011.179. [PubMed: 21602888]
27. Jones PA, Ohtani H, Chakravarthy A, De Carvalho DD. Epigenetic therapy in immune-oncology. *Nat Rev Cancer* 2019;19(3):151–61 doi 10.1038/s41568-019-0109-9. [PubMed: 30723290]
28. Cabrita R, Lauss M, Sanna A, Donia M, Skaarup Larsen M, Mitra S, et al. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. *Nature* 2020;577(7791):561–5 doi 10.1038/s41586-019-1914-8. [PubMed: 31942071]
29. Helmink BA, Reddy SM, Gao J, Zhang S, Basar R, Thakur R, et al. B cells and tertiary lymphoid structures promote immunotherapy response. *Nature* 2020;577(7791):549–55 doi 10.1038/s41586-019-1922-8. [PubMed: 31942075]
30. Petitprez F, de Reynies A, Keung EZ, Chen TW, Sun CM, Calderaro J, et al. B cells are associated with survival and immunotherapy response in sarcoma. *Nature* 2020;577(7791):556–60 doi 10.1038/s41586-019-1906-8. [PubMed: 31942077]
31. Petitprez F, Meylan M, de Reynies A, Sautes-Fridman C, Fridman WH. The Tumor Microenvironment in the Response to Immune Checkpoint Blockade Therapies. *Front Immunol* 2020;11:784 doi 10.3389/fimmu.2020.00784. [PubMed: 32457745]
32. Jahchan NS, Mujal AM, Pollack JL, Binnewies M, Sriram V, Reyno L, et al. Tuning the Tumor Myeloid Microenvironment to Fight Cancer. *Front Immunol* 2019;10:1611 doi 10.3389/fimmu.2019.01611. [PubMed: 31402908]

Statement of translational relevance

The anti-PD-1 antibody nivolumab is approved for patients with metastatic clear cell renal cell carcinoma (mccRCC) refractory to anti-angiogenic therapy, and is currently being evaluated in the front-line settings. Unfortunately, most patients derive no durable clinical benefit and predictive biomarkers are needed to establish the value of anti-PD-1 monotherapy in mccRCC.

By analyzing tumors for a randomized clinical trial of nivolumab versus everolimus in mccRCC, we independently validated our previous findings (Pignon et al, Clin Cancer Res 2019) that immunophenotyping of CD8+ tumor-infiltrating cells in combination with tumor cell PD-L1 status predicts response to anti-PD-1 (but not everolimus) treatment. Importantly, our results also establish a new link between expression of the human endogenous retrovirus ERVE4 and response to nivolumab, supporting ERVE4's role as a kidney cancer antigen and therapeutic target for immunotherapy. Overall, these findings represent an important step towards the development of clinically relevant predictive biomarkers for kidney cancer.

Figure 1.

Association of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density with clinical outcomes (PFS, OS) and with RNA-seq data. **a, b** Kaplan-Meier curves for PFS and OS per CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density at the optimized cutoff (Youden's Index approach) in the nivo and evero arm. **c**, ssGSEA Hallmark gene sets enriched in the CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC-High (orange) and CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC-Low (purple) tumors. Dotted lines indicate FDR q-value of 0.25 (light) and 0.1 (dark, significance level). **d**, Immune-related gene signature scores upregulated in CD8⁺PD-1⁺TIM-3⁻LAG-3⁻

TIC-High (orange) and CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC-Low (purple) tumors. Dotted lines indicate FDR q-value of 0.25 (light) and 0.05 (dark, significance level). **e**, Immune cell populations enriched in the CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC-High (orange) and CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC-Low (purple) cases by CIBERSORTx immune deconvolution of RNA-seq data. Dotted lines indicate FDR q-value of 0.25 (light) and 0.05 (dark, significance level).

Figure 2.

Kaplan-Meier curves for PFS per Tumor Cell (TC) PD-L1 expression levels (1%) combined with levels of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC density (High versus Low).

^a In the evero arm, one patient with PD-L1 High/CD8⁺ PD1⁺TIM3⁻LAG3⁻ TIC Low was combined with the PD-L1 Low/CD8⁺ PD1⁺TIM3⁻LAG3⁻ TIC High group.

Figure 3.

Association of ERVE4 status with clinical outcomes (PFS, OS) and integration with WES and RNA-seq data. **a**, **b** Kaplan-Meier curves for PFS and OS per ERVE4 status at the optimized cutoff (Youden's Index approach) in the nivo and evero arm. **c**, Most differentially expressed hERVs between ERVE4-positive and ERVE4-negative tumors using a database of about 1700 hERV transcripts. Horizontal red dotted line indicates FDR q-value of 0.25 (significance level). **d**, Immune-related gene signature scores upregulated in ERVE4-positive

(orange) and ERVE4-negative (purple) tumors. Dotted lines indicate FDR q-value of 0.25 (light) and 0.05 (dark, significance level).

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Table 1.

Association between density or percentage of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻TIC (measured as continuous variables) and clinical outcomes.

Arm	Biomarker	ORR			DRR			PFS			OS	
		beta ± SE	OR(LL 95% CI)	p-value	beta ± SE	OR(LL 95% CI)	p-value	beta ± SE	HR(LL 95% CI)	p-value	beta ± SE	HR(LL 95% CI)
Nivo	% CD8 ⁺ PD1 ⁺ TIM3 ⁻ LAG3 ⁻ TIC	2.22±1.08	9.22 (1.56)	0.020	1.8±1.19	6.07 (0.86)	0.065	-0.69±0.53	0.50 (0.21)	0.095	-0.21±0.55	0.81 (0.28)
	Log Density CD8 ⁺ PD1 ⁺ TIM3 ⁻ LAG3 ⁻ TIC	0.36±0.19	1.43 (1.05)	0.028	0.43±0.22	1.54 (1.08)	0.023	-0.14±0.09	0.87 (0.75)	0.060	-0.05±0.09	0.95 (0.79)
Evero	% CD8 ⁺ PD1 ⁺ TIM3 ⁻ LAG3 ⁻ TIC	NA ^a	NA ^a	NA ^a	NA ^a	NA ^a	NA ^a	0.29±0.47	1.33 (0.61)	0.728	0.25±0.47	1.28 (0.51)
	Log Density CD8 ⁺ PD1 ⁺ TIM3 ⁻ LAG3 ⁻ TIC	NA ^a	NA ^a	NA ^a	NA ^a	NA ^a	NA ^a	0.12±0.08	1.13 (0.995)	0.944	0.16±0.08	1.18 (1)

^a Association of the biomarkers with ORR and DRR was not assessed in the evero arm due to the low number of responders.

Abbreviations: SE, standard error; OR, odds ratio; LL, lower limit; CI, confidence interval; HR, hazard ratio; NA, not assessed.

Table 2.

ORR and DRR per TC PD-L1 (1%) and log density of CD8⁺PD-1⁺TIM-3⁻LAG-3⁻ TIC levels (High vs Low) in the nivo arm.

Marker Combination	Nivo (n = 111) ^a		
	n	ORR% (n)	DRR% (n)
PD-L1 High / CD8 ⁺ PD-1 ⁺ TIM-3 ⁻ LAG-3 ⁻ TIC High	12	50% (6)	33% (4)
PD-L1 Low / CD8 ⁺ PD-1 ⁺ TIM-3 ⁻ LAG-3 ⁻ TIC High	90	22% (20)	18% (16)
PD-L1 Low / CD8 ⁺ PD-1 ⁺ TIM-3 ⁻ LAG-3 ⁻ TIC Low	9	11% (1)	0% (0)
Trend test 1-sided p-value		0.027	0.045

^a Association of the biomarkers with ORR and DRR was not assessed in the evero arm due to the low number of responders.

Table 3.

Association between continuous ERVs and clinical endpoints.

ERV Marker	Nivo (OR/HR, p-value)					Evero (HR, p-value) ^a		
	n	ORR	DRR	PFS	OS	n	PFS	OS
ERVE4	n = 105	2.40, 0.119	4.05, 0.042	0.42, 0.020	0.70, 0.203	n = 104	1.24, 0.763	1.13, 0.635
hERV4700ENV	n = 82	1.28, 0.310	1.48, 0.237	0.79, 0.194	0.59, 0.050	n = 80	0.78, 0.196	0.96, 0.445

^a Association of the biomarkers with ORR and DRR was not assessed in the evero arm due to the low number of responders.

Abbreviations: OR, odds ratio; HR, hazard ratio.