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# Potential impact of PD-L1 (SP-142) immunohistochemical heterogeneity in clear cell renal cell carcinoma immunotherapy

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ABSTRACT

Intratumor heterogeneity (ITH) detection remains a challenge in modern oncology because it can have a direct impact on the success of new therapies. Anti-PD-1/PD-L1 immunotherapy is an emerging treatment modality that is showing great promise for clear cell renal cell carcinoma (CCRCC) patients with advanced disease. Patient selection for such therapy relies upon the immunohistochemical detection of PD-1/PD-L1, however the degree of ITH for these markers among tumor cells and/or inflammatory mononuclear infiltrates remains unknown. Therefore, we analyzed PD-L1 (SP-142) expression in the tumor inflammatory cells of 22 CCRCC cases with the aim to define the pattern of PD-L1 expression, and to compare the reliability of current tumor sampling protocols (RS) with a multisite tumor sampling strategy (MSTS). While the RS protocol identified 5/22 (22.7%) of cases that were positive for PD-L1 expression, MSTS identified 10/22 (45.45%) of cases. This suggests that RS may miss a proportion of CCRCC patients that might benefit from immunotherapy. In addition, MSTS demonstrated that positive and negative regions of PD-L1 expression are very variable within each tumor.

## 1. Introduction

Renal cell carcinoma is included in the top-ten list of the most common malignancies in Western countries [1]. Clear cell renal cell carcinoma (CCRCC) is the most frequent renal malignancy, accounting for roughly 70%–80% of the cases [2]. CCRCC is an aggressive neoplasm with different molecular profiles influencing treatment response [3]. Despite all therapeutic efforts, however, only radical surgery and early diagnosis have had a significant influence on survival [4].

CCRCC is a paradigmatic example of intratumor heterogeneity (ITH) typically displaying both temporal and spatial differences at the morphological, immunohistochemical and molecular levels [5]. Importantly ITH is the cornerstone of many therapeutic failures, and many efforts are being made to achieve a full characterization of tumors that may eventually allow better personalized approaches [6]. Immune checkpoint inhibitors, alone or in combination with anti-angiogenic drugs, have emerged in recent years as promising new therapeutic options for advanced CCRCC [7]. As a consequence, the influence of the cancer immune microenvironment is attracting great interest [8].

The expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), and programmed death-1 (PD-1) on activated T-cells inhibits the immune-mediated attack on tumor cells. Checkpoint inhibitors, in particular anti-CTLA-4 and anti-PD-1 (and its ligand anti-PD-L1), show great promise for renal cancer patients, however not all patients receive a benefit from these therapies, and as a consequence there is great interest in finding effective predictive biomarkers [9,10].

Currently patient selection for anti-PD-L1 treatments relies on the identification of PD-1/PD-L1 by routine immunohistochemical protocols, and several anti PD-1/PD-L1 clones have been developed for this purpose. However, up to 17% of patients that respond to PD-L1 therapy

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appear not to express PD-L1 when tested using current methodologies [9]. This apparent contradiction suggests that either factors other than PD-L1 are involved in the therapeutic action of anti-PD-L1 treatment, or more likely that current protocols are suboptimal for detection of PD-L1 expression [10].

Recent evidence has shown that routine protocols may be insufficient to reliably detect ITH in large tumors [11,12]. We have developed a multi-site tumor sampling (MSTS) protocol that out-performs routine sampling (RS) protocols in detecting histological ITH, and prognostic biomarkers [13]. MSTS follows the rationale *the more you sample the more you find*, and it is based in the divide-and-conquer algorithm [14], a successful strategy to solve complex problems in physics [15] that has been successfully applied also in biology [16] and in medicine [17]. Here, we have applied this strategy to analyze the expression of PD-L1 in the microenvironment of CCRCC tumors.

## 2. Material and methods

The authors declare that all the analyses carried out in this study comply with current Spanish and European Union legal regulations. Samples from patients included in this study were obtained retrospectively from the archive of the Pathology Lab, Cruces University Hospital, Barakaldo, Spain. All patients gave written consent for the use of their samples in this study as approved by the Ethical and Scientific Committees of the Basque Health Service (Osakidetza) (CEIC-E PI2016096).

Twenty-two CCRCC were selected between November 2015 and February 2016. All cases were simultaneously sampled following two different protocols: RS [18], one large tumor sample per cassette for each centimeter of the tumor diameter (i.e., 22 tumors, 22 samples, 22 cassettes); and MSTS [13], six to eight small samples per cassette (22 tumors, 160 samples, 22 cassettes), as illustrated in Fig. 1. Note that both methods use the same number of cassettes.

Immunostaining was performed in a BenchMark Ultra (Ventana, Roche, AZ, USA) immunostainer following routine protocols and specific instructions of the manufacturer. Prediluted PD-L1 antibody (clone SP-142, Ventana, Roche, AZ, USA) was used for the analysis.

Microscopic evaluation of all samples in both sampling protocols was performed in a blind way by the same observer to guarantee objectivity. As suggested by the manufacturer, only immunostaining of the inflammatory mononuclear cells present in the tumor itself, or within the inner side of the tumor capsule, were considered positive (Fig. 2). Mimickers of PD-L1 immunostaining (namely, formaldehyde precipitation and hemosiderin deposition) were identified as such (Fig. 3). A cut-off of 1% positive tumor-associated inflammatory cells [19] was used as this cut-off has previously been associated with increased progression free survival in patients treated with atezolizumab, both alone or in association with bevacizumab [7].

Concordant positive and negative results between the two sampling protocols (RS and MSTS) were considered above and below 1% of positive inflammatory cells, respectively.

## 3. Results

The main clinicopathological data of the patients included in this study are depicted in Table 1. Most cases were male (16 M/6 F), and the average age of patients was 60 years (range 15–82). Radical nephrectomy was carried out on 20 patients, and partial nephrectomy in two patients. The average tumor diameter was 8.5 cm with a range between 3.5 and 15 cm. Eight cases were low-grade (G1/2) and fourteen cases high-grade (G3/4). Pathological staging revealed an equal distribution of organ-confined and non-organ-confined disease (11/11).

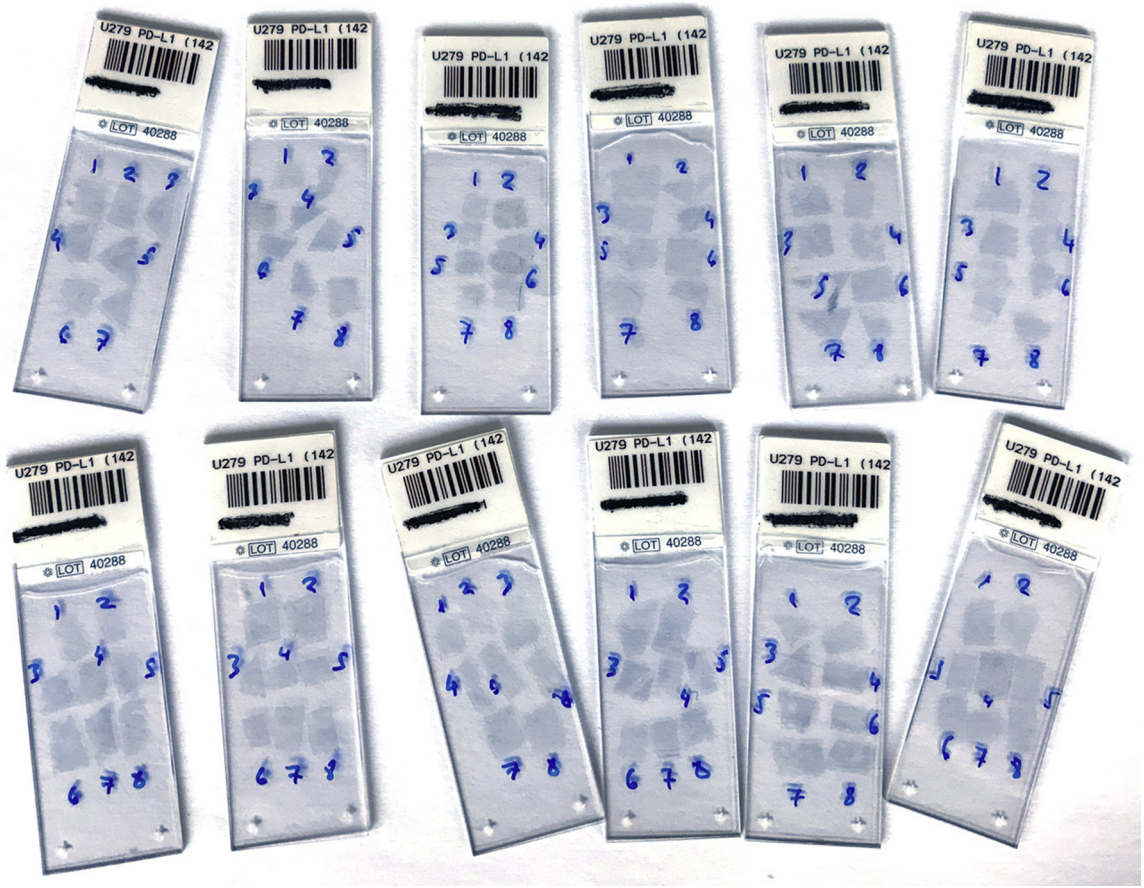


**Fig. 1.** Selection of the multisite tumor sampling protocol (6–8 samples per cassette) in 12 clear cell renal cell carcinomas ready for microscopic analysis.

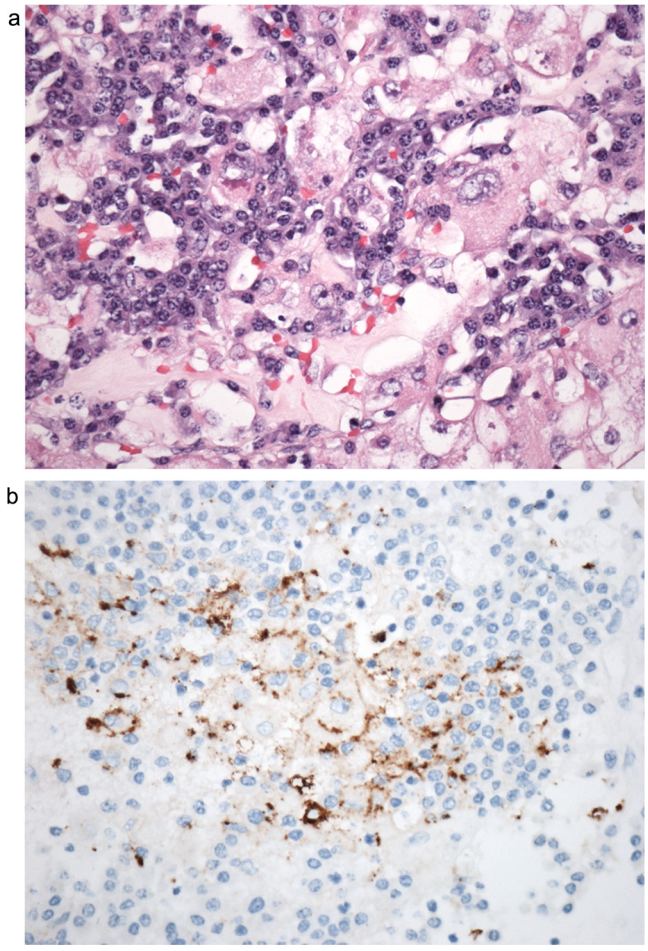


**Fig. 2.** High power view of a high grade clear cell renal cell carcinoma (A) with abundant mononuclear inflammatory cells expressing anti-PD-L1 (SP-142) (B).

The immunohistochemical data are depicted in Table 1. Using MSTS, ten of the cases (45.45%) were positive for PD-L1 expression. In contrast, RS detected only five PD-L1 positive cases (22.7%). No significant correlation was found between PD-L1 positivity and histologic grade (7 high vs. 3 low) or pathological staging (5 confined vs. 5 non-confined neoplasms).

Concordance (either positive or negative) between MSTS and RS was detected in 17 cases (77.3%), 6 of them being positive ($\geq 1\%$) and 11 negative ($< 1\%$). Discordant results were seen in 5 cases (22.7%), all of them corresponding to MSTS-positive/RS-negative staining for PD-L1.

Table 1 also demonstrates that PD-L1 expression was highly variable amongst the various regions tested in this experiment. Indeed, only 6 out of 22 CCRCC cases had homogeneously negative immunostaining across all their samples. The remaining 16 cases displayed some degree of heterogeneity between samples.

## 4. Discussion

RCC is a health problem of major concern in Western countries as well as a source of great interest to pathologists, with around 20 well-recognized clinical entities, and several emerging subtypes proposed in the last update of ISUP classification [20]. Traditionally, radio- and chemo-resistant CCRCC patients are treated with antiangiogenic drugs and checkpoint inhibitors, alone or in combination with radical surgery.

Drugs targeting the immune checkpoints (i.e., immunotherapy) represent a new frontier for modern oncology. US Food and Drug Administration (FDA) approved ipilimumab, an anti-CTLA-4 drug, for the treatment of advanced melanoma in 2011. Since then, anti-CTLA-4 or anti-PD-L1/PD-1 drugs have been approved for many other cancer types including non-small cell lung carcinoma, renal cell carcinoma and urothelial carcinoma [9]. Nivolumab, an anti-PD-1 monoclonal antibody, was approved for advanced renal cell carcinoma treatment as a second line therapy in 2015 [9], and atezolizumab, an anti-PD-L1 monoclonal antibody, is in the process of clinical validation for metastatic RCC [7].

Immunohistochemical detection of PD-L1 expression is the most common predictive biomarker of PD-1-based immunotherapy. The expression of PD-L1 either in tumor cells and/or accompanying inflammatory cells, depends upon the manufacturers' indications. However, standardization of this analysis is currently lacking. This study illustrates both the scarcity and unpredictability of PD-L1 (SP-142)-positive inflammatory cells across diverse regions of the same tumor and suggest serious concerns about the appropriateness of sampling methodology followed in routine assessments. We have recently

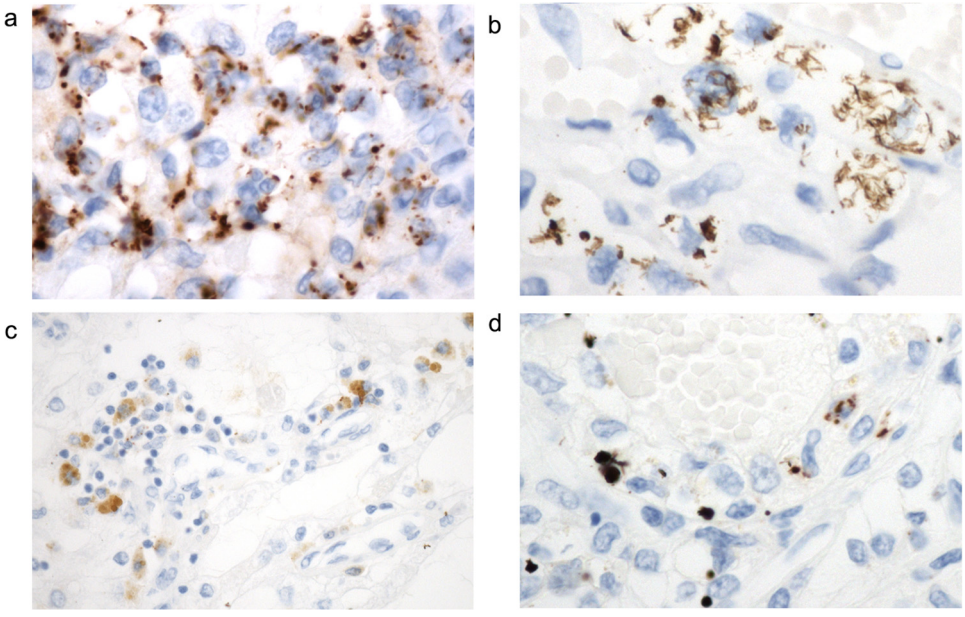


**Fig. 3.** High power detail of PD-L1 (SP-142) immunostaining and its mimickers in clear cell renal cell carcinomas. (A) Genuine PD-L1 expression showing the characteristic deep-brown lumpy spots located in inflammatory cells. (B) Formaldehyde precipitation displaying black, spiky/filamentous structures located everywhere. (C) Hemosiderin deposition showing orange or deep yellow powder or balls located in the interstitium and/or macrophages. (D) PD-L1 expression (right side) and formaldehyde precipitation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

**Table 1**
Clinical data of 22 clear cell renal cell carcinomas and values of PD-L1 (SP-142) across different tumor regions in multisite tumor sampling (MSTS) and comparison with their respective values in routine sampling (RS).

| Case | Age | Sex | Diam | Grade | Stage | R1 | R2 | R3 | R4 | R5 | R6 | R7 | R8 | MSTS | RS | Result |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 62 | 10 | 4 | pT2 | 0 | 1 | 0 | 0 | 0.5 | 0 | – | – | 1 | 1 | pos/pos |
| 2 | M | 72 | 6.7 | 3 | pT3a | 0 | 0 | 0 | 0.5 | 0.5 | 0 | – | – | 0.5 | 0 | neg/neg |
| 3 | F | 49 | 8 | 2 | pT2 | 0 | 1 | 1 | 0 | 0.5 | 0.5 | – | – | 1 | 1 | pos/pos |
| 4 | M | 64 | 7.4 | 3 | pT2 | 0 | 0 | 0 | 0 | 0 | 0 | – | – | 0 | 0 | neg/neg |
| 5 | M | 70 | 3.5 | 2 | pT3a | 0 | 0 | 0 | 0 | 0 | 0 | – | – | 0 | 0.5 | neg/neg |
| 6 | M | 46 | 9.5 | 3 | pT3a | 0 | 0 | 0 | 0 | 0 | 0 | – | – | 0 | 0 | neg/neg |
| 7 | M | 42 | 3.5 | 1 | pT1a | 0 | 0 | 0 | 0 | 0 | 0 | – | – | 0 | 0 | neg/neg |
| 8 | M | 71 | 8 | 2 | pT3a | 0 | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 0 | 0.5 | 0 | neg/neg |
| 9 | F | 41 | 5 | 3 | pT1b | 0 | 0 | 0 | 0.5 | 0 | 0 | 0 | 0 | 0.5 | 0 | neg/neg |
| 10 | M | 77 | 9.2 | 1 | pT2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | neg/neg |
| **11** | **M** | **52** | **7** | **2** | **pT2** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **pos/neg** |
| **12** | **M** | **66** | **15** | **4** | **pT4** | **0** | **1** | **0** | **0** | **0** | **0.5** | **0.5** | **0** | **1** | **0** | **pos/neg** |
| 13 | M | 67 | 5.5 | 4 | pT3a | 0 | 0.5 | 0 | 0 | 0 | 0.5 | 0 | – | 0.5 | 0 | neg/neg |
| 14 | M | 71 | 9 | 3 | pT3a | 0.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 | 0.5 | neg/neg |
| 15 | M | 82 | 7.5 | 4 | pT3a | 0.5 | 1 | 0 | 0.5 | 0 | 1 | 0.5 | – | 1 | 1 | pos/pos |
| 16 | F | 68 | 5.6 | 3 | pT4 | 0 | 5 | 1 | 0.5 | 10 | 10 | 5 | 5 | 10 | 5 | pos/pos |
| **17** | **M** | **62** | **6** | **4** | **pT3a** | **0.5** | **0** | **0** | **1** | **1** | **0.5** | **2** | **1** | **2** | **0.5** | **pos/neg** |
| **18** | **M** | **53** | **5.5** | **3** | **pT3a** | **1** | **0.5** | **0** | **1** | **1** | **1** | **1** | **0** | **1** | **0** | **pos/neg** |
| 19 | F | 14 | 5.8 | 3 | pT1b | 1 | 0.5 | 1 | 1 | 1 | 2 | 2 | 10 | 10 | 1 | pos/pos |
| 20 | F | 61 | 6.5 | 3 | pT1b | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 | neg/neg |
| **21** | **M** | **70** | **6.2** | **2** | **pT1b** | **0** | **0** | **0.5** | **0** | **5** | **0.5** | **0** | **0** | **5** | **0** | **pos/neg** |
| 22 | F | 61 | 4.3 | 2 | pT1b | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 | – | 0.5 | 0.5 | pos/pos |

R1 to R8: Different regions sampled in multisite tumor sampling, MSTS: Result obtained in multisite tumor sampling (annotated is the highest obtained in any region, not the sum). RS: Result obtained in routine sampling (one sample), Result: Reflects the respective result of multisite and routine tumor samplings considering the positive cut-off is ≥ 1. Bold rows highlight the 5 cases in which multisite tumor sampling outperforms routine sampling in PD-L1 detection.

demonstrated a significant loss of PD-L1 expression in the renal vein tumor thrombus microenvironment in a series of advanced CCRCC [20], and others have also shown differences in PD-L1 expression between primary CCRCC and their metastases [21]. Jilaveanu et al [21] have shown a greater PD-L1 expression in metastases when comparing 34 matched pairs of CCRCC and argue that a single core biopsy may not be enough to determine PD-L1 expression. Same as happens in others neoplasms [22,23], PD-L1 ITH is high in CCRCC, as reflected in several recent studies [21,24] and meta-analyses [25,26]. Although PD-1/PD-L1 expression is being been tested mainly in CCRCC, papillary [27], chromophobe [28], translocation [29] and fumarate-hydratase [30] RCCs have also been analyzed.

Most studies of ITH generally focus on mutational heterogeneity, and therefore only consider tumour cells, it is important to note that heterogeneity also extends to the tumour microenvironment as this is dependent upon extensive cross-talk with tumor cells [31].

The issue of under-estimating the extent of ITH in large tumors is not restricted to PD-L1 expression but remains a crucial problem for pathologists and by extension the oncologists that treat patients on the basis of the information provided by the pathologist, which could be incomplete. Several examples of the inadequacy of current sampling protocols have been published including Zaldumbide et al. [32] and Guarch et al. [33], in CCRCC, and Nassar et al. in breast carcinoma [34]. MSTS has shown in the present study a higher performance compared with RS in detecting PD-L1 (SP-142) expression at no extra cost to the health service, with the potential to identify CCRCC patients that might benefit from immunotherapy treatment that otherwise could have been missed using current sampling protocols.

Based on the results of the present study, and previous studies [35–37], we urge pathologists to consider MSTS as an alternative to current sampling protocols and call for independent assessment of this technique within routine pathology laboratories.

## Compliance with ethical standards

The study has been approved by local ethical committee (CEIC-Euskadi PI2016096)

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## Conflict of interest

The authors declare no conflict of interest

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