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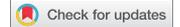
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ORIGINAL RESEARCH



## **PDL1 expression is associated with longer postoperative, survival in adrenocortical carcinoma**

Emilien Billon<sup>b</sup>, Pascal Finetti<sup>a</sup>, Alexandre Bertucci<sup>b</sup>, Patricia Niccoli<sup>b</sup>, Daniel Birnbaum<sup>a</sup>, Emilie Mamessier<sup>a</sup>, and François Bertucci<sup>a,b,c</sup>

<sup>a</sup>Laboratoire Oncologie Prédictive, Centre de Recherche en Cancérologie de Marseille (CRCM), Institut Paoli-Calmettes, INSERM UMR1068, CNRS UMR725, Aix-Marseille Université, Marseille, France; <sup>b</sup>Département d'Oncologie Médicale, Centre de Recherche en Cancérologie de Marseille (CRCM), Institut Paoli-Calmettes, Marseille, France; <sup>c</sup>Faculté de Médecine, Aix-Marseille Université, Marseille, France

### **ABSTRACT**

Adrenocortical carcinomas (ACCs) are heterogeneous cancers associated with a very poor prognosis. The improvement of prognostic tools and systemic therapy are urgently needed. Targeting the immune system using checkpoint inhibitors such as PD1/PDL1 inhibitors is an attractive novel therapeutic strategy for poor-prognosis tumors. Multiple clinical trials are ongoing, including in advanced ACC. However, PDL1 expression has been studied in ACC in only one heterogeneous series of 28 clinical samples. Here, we have retrospectively analyzed *PDL1* mRNA expression in 146 clinical ACC samples and searched for correlations between expression and biological and clinicopathological data, including post-operative disease-free survival (DFS). *PDL1* mRNA expression was heterogeneous across samples. "PDL1-high" tumors were not associated with the classical prognostic variables but were associated with longer DFS in both uni- and multivariate analyses. High PDL1 mRNA expression was associated with biological signs of the cytotoxic local immune response. Supervised analysis between "PDL1-high" and "PDL1-low" tumors identified a robust 370-gene signature whose ontology analysis suggested the existence in "PDL1-high" tumors of a cytotoxic T-cell response, however, associated with some degree of T-cell exhaustion. In conclusion, *PDL1* mRNA expression refines the prognostication in ACC and high expression is associated with longer DFS. Clinical validation at the protein level and functional validation are required to fully understand the role of PDL1 in ACC. Reactivation of dormant tumor-infiltrating lymphocytes by PDL1-inhibitors could represent a promising strategy in "PDL1-high" ACCs, supporting the ongoing clinical trials.

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## **Introduction**

Adrenocortical carcinomas (ACCs) are rare cancers with 0.5–2 new cases per million diagnosed every year in the United States.<sup>1,2</sup> ACC is associated with a very poor prognosis. Complete surgical resection is the only curative therapeutic option but is possible in less than half of patients.<sup>3–5</sup> ACC is a heterogeneous disease. Currently, the most validated prognostic factor is initial tumor stage and nodal or distant metastases; other unfavorable factors include increasing age, poorly differentiated or high-grade tumors, and involved surgical margins. But the current prognostic clinicopathological classifications are imperfect and need to be redefined. Despite treatment, nearly 80% of operated patients relapse. The 5-year survival rate is lower than 35% in patients with non-operable tumors. The mitotane adrenolytic drug, a 55-year-old therapy, remains the cornerstone of medical treatment and the currently used cytotoxic chemotherapy agents include old drugs such as etoposide, doxorubicin, cisplatin, and streptozotocin, which display limited efficacy and high toxicity. Clearly, the improvement of prognostic tools and the development of more efficient systemic therapy are crucial.<sup>6</sup>

Development of ACC is not well characterized at the biological level. Transcriptional profiling identified prognostic classes,<sup>7</sup> and expression signatures associated with malignancy,<sup>8,9</sup> with hormonal activities<sup>8</sup> and with survival in adrenocortical tumors.<sup>8,10</sup> Integrated genomic characterization recently identified recurrent alterations in known driver genes and in genes not previously reported in ACC, and distinct molecular subtypes with distinct clinical outcome.<sup>11,12</sup> If genomic profiling is expected to become clinically relevant in the future, the KI67 proliferation index seems the best prognostic tool currently available.<sup>13</sup> Regarding the targeted therapies, no study has demonstrated any benefit with agents blocking molecular pathways such as EGFR, VEGF, mTOR, IGF-1R or FGFR in advanced disease.<sup>14–18</sup>

The concept that cancer cells can exploit the immune checkpoint pathway to evade the immune system and allow tumor growth has been known for decades.<sup>19</sup> Immune response is a complex phenomenon based on a balance between activator and inhibitor pathways that regulate tumor-infiltrating lymphocytes (TILs) activity. One key inhibitor is the PD1/PDL1 pathway. PD1 (Programmed cell Death 1) is expressed at the cell surface of several immune cell

populations including T-cells. PD1 is activated by its ligands PDL1 and PDL2, and expressed by antigen-presenting cells but also by tumor cells from different locations. The PD1/PDL1 pathway is a major actor in cancer progression through inhibition of anti-cancer immune response.<sup>20–22</sup> Recently, immune checkpoint inhibitors have revolutionized cancer treatment and represent the most successful class of immunotherapies. Remarkably, durable responses have been reported in the clinical trials testing monoclonal antibodies (mAbs) directed against PD1 or PDL1, notably in melanoma, lung, renal cell, head and neck, and bladder carcinomas.<sup>23–25</sup> Today, several anti-PD1 and anti-PDL1 mAbs are approved for the treatment of different cancers or are at an advanced stage of clinical development. Furthermore, the relationship between PDL1 expression on tumor and/or immune cells and objective response has been reported.<sup>23,24,26,27</sup>

Few data are available regarding the clinical activity of anti-PDL1/PD1 mAbs in ACC. Preliminary data from two small phase 2 studies have been reported.<sup>28,29</sup> Of seven patients who received nivolumab, five had disease progression as best response and two were awaiting evaluation.<sup>28</sup> Of 11 patients who received pembrolizumab, two had a partial response, one achieved stable disease, and the 6-month progression-free survival rate was 27%.<sup>29</sup> The JAVELIN Solid Tumor trial (NCT01772004) was a phase 1a, multi-cohort, dose-escalation trial assessing avelumab, a mAb directed against PDL1, in patients with metastatic or locally advanced previously treated solid tumors.<sup>30</sup> A phase 1b expansion cohort enrolled 50 patients with platinum-pretreated metastatic ACC.<sup>31</sup> To our knowledge, this is the largest prospective trial for a checkpoint inhibitor in this disease. The objective response rate (ORR) was 6%, and 21 patients (42%) had the stable disease as a best response (disease control rate: 48%). In evaluable patients with PDL1-positive (N = 12) or PDL1-negative (N = 30) tumors ( $\geq 5\%$  tumor cell as IHC cutoff), the ORR was 16.7% versus 3.3% ( $p = .192$ ) respectively. Possible mechanisms of ACC resistance to PD1/PDL1 inhibitors have been suggested, such as WNT- $\beta$ -catenin amplification, TP53 mutation, abnormal PD-L1 expression and increased production of steroids.<sup>32,33</sup>

PDL1 expression has been studied in many cancers, with evidence of correlations with clinicopathological features, including survival, in several studies.<sup>34–48</sup> In ACC, only one study analyzed the prevalence and prognostic value of PDL1 expression<sup>49</sup> in a small series of 28 samples and at the protein level using immunohistochemistry (IHC). Here, we have analyzed PDL1 mRNA expression in 146 clinical ACC samples. We searched for correlations between PDL1 mRNA expression and biological and clinicopathological data, including survival.

## Results

### Patients' population and PDL1 expression and copy number alteration

PDL1 mRNA expression was analyzed in the 79 TCGA ACC samples, whose clinicopathological characteristics are summarized in Table 1. The median patients' age was 49 (range, 14 to 77) years. There were more females (61%) than males

**Table 1.** Clinicopathological characteristics of ACC patients.

Characteristics		N (%)
Age, median	years (range)	49 (14–77)
Sex	female	48 (61%)
	male	31 (39%)
ENSAT stage	1	9 (12%)
	2	37 (48%)
	3	16 (21%)
	4	15 (19%)
Pathological tumor size (pT)	pT1	9 (12%)
	pT2	42 (55%)
	pT3	8 (10%)
	pT4	18 (23%)
Pathological lymph node (pN)	pN0	68 (88%)
	pN1	9 (12%)
Surgical margins status	R0	55 (79%)
	R1	6 (9%)
	R2	9 (13%)
Weiss classification	low (1–3)	14 (22%)
	high (4–9)	50 (78%)
Steroid hormone secretion excess	no	26 (35%)
	yes	48 (65%)
Adjuvant mitotane	no	4 (9%)
	yes	39 (91%)
Adjuvant radiotherapy	no	59 (79%)
	yes	16 (21%)
TP53 mutation status	wild type	64 (81%)
	mutated	15 (19%)
MKI67 mRNA status	low	40 (51%)
	high	39 (49%)
DFS event*		27 (42%)
5-year DFS*		52% [39–70]

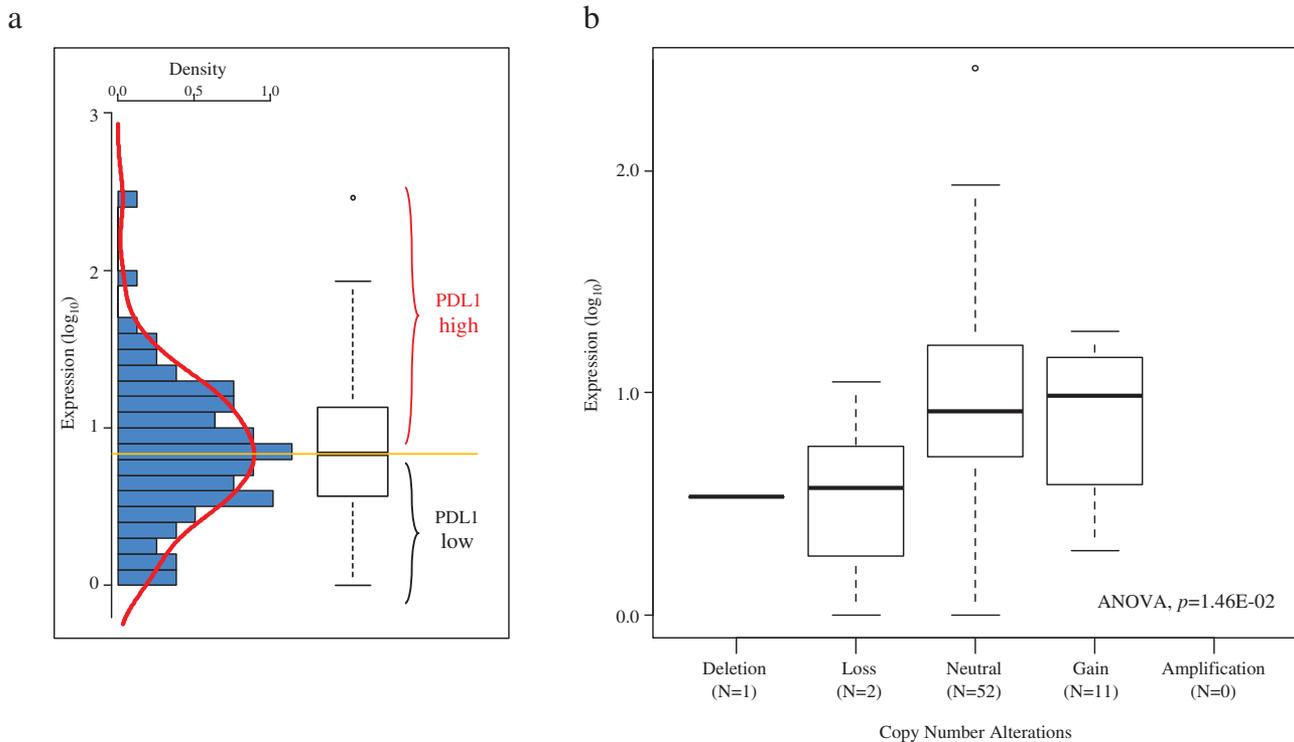
\*, M0 patients only (N = 64)

(39%). The most frequent ENSAT stage was stage 2 (48%). Most of the operated tumors were pathologically classified as pT2 (55%), pN0 (88%), and with negative surgical margins (79%). In the Weiss classification, 78% of cases displayed a high score. The majority of tumors (65%) were functional with an excess of steroid hormone secretion. Ninety-one percent of informative patients received adjuvant mitotane therapy and 21% received adjuvant radiotherapy.

PDL1 mRNA expression level was heterogeneous across all samples with a range of intensities over nearly three decades in a log<sub>10</sub> scale (Figure 1A). SNP-array data were available for 76 out of 79 tumors. PDL1 copy number alterations were rare, observed in 24 samples (32%): one tumor displayed deletion (homozygous loss), 12 displayed loss (1 copy), and 11 displayed gain (3 copies). A small positive correlation existed between DNA copy number and mRNA expression level ( $p = 1.46E-02$ , ANOVA; Figure 1B).

### PDL1 expression and clinicopathological features

We searched for correlations between PDL1 mRNA expression assessed as a binary variable (high versus low using the median expression level as cutoff) and clinicopathological features. As shown in Table 2, no correlation was found with all tested variables: patients' age, sex, ENSAT stage, pathological tumor size, pathological lymph node status, surgical margins status, Weiss classification, steroid secretion, adjuvant mitotane therapy, adjuvant radiotherapy, MKI67 mRNA expression, and TP53 mutation status. The “PDL1-high” class tended to be associated with younger patients' age ( $p = 6.45E-02$ , Student t-test), and with higher MKI67 expression ( $p = 7.29E-02$ , Fisher's exact test).



**Figure 1.** PD-L1 mRNA expression across the 79 ACC samples.

A/Left: histogram of distribution of *PDL1* mRNA expression levels ( $\log_{10}$ ) across the 79 TCGA samples. The red line represents the density curve of distribution. Right: *PDL1* expression level reported as a box plot. The median expression level (orange horizontal line) defines the “PDL1-low” and “PDL1-high” classes. B/Similar to A-Right, but according to *PDL1* DNA copy number alteration (SNP-array).

### **PDL1 expression and disease-free survival**

We assessed the prognostic value of *PDL1* mRNA expression for post-operative DFS in the 64 patients non-metastatic at diagnosis (M0). Fifty-seven patients remained event-free during a median follow-up of 22 months (range, 1 to 154) and 27 (42%) displayed a DFS event. The 5-year DFS rate was 52% (95%CI, 39–70) (Figure 2A). DFS was correlated with *PDL1* expression: in the “PDL1-high” class, the number of patients with DFS event was 6 out of 39 (18%) and the 5-year DFS rate was 76% (95%CI, 58–100); whereas in the “PDL1-low” class, the number of patients with DFS event was 21 out of 40 (70%;  $p = 3.47E-05$ , Fisher’s exact test) and the 5-year DFS rate was 29% (95%CI, 16–53;  $p = 5.11E-05$ , log-rank test; Figure 2B).

In univariate analysis (Table 3), pathological tumor size, surgical margins, Weiss classification, *MKI67* mRNA expression, *TP53* mutation status, and *PDL1* class were ( $p < .05$ ) or tended to be ( $0.05 < p < .10$ ) associated with DFS (Wald test). The hazard ratio (HR) for DFS event was 0.19 (95%CI 0.08–0.46) in the “PDL1-high” class when compared with the “PDL1-low” class ( $p = 3.00E-04$ , Wald test). In multivariate analysis incorporating all these variables, two variables remained significantly associated with shorter DFS: higher pathological tumor size, and the “PDL1-low” class, suggesting independent prognostic value. Of note, the same independent prognostic value was observed for *PDL1* mRNA expression when analyzed in continuous value ( $p = 2.99E-03$  in univariate analysis, and  $p = 9.01E-03$  in multivariate analysis; Wald test).

Since the adjuvant mitotane and/or radiotherapy can impact on DFS, we reassessed the prognostic value of *PDL1* expression according to the delivery or not of adjuvant therapy. Among the 35 patients treated with adjuvant mitotane, the 5-year DFS was 87% (95%CI, 72–100) in the “PDL1-high” class versus 23% (95%CI, 08–66) in the “PDL1-low” class ( $p = 9.09E-04$ , log-rank test; Figure 2C). Among the 50 patients untreated with adjuvant radiotherapy, the 5-year DFS was 90% (95%CI, 77–100) in the “PDL1-high” class versus 34% (95%CI, 19–60) in the “PDL1-low” class ( $p = 4.54E-04$ , log-rank test; Figure 2D). Among the 12 patients treated with adjuvant radiotherapy, the 5-year DFS was 64% (95%CI, 34–100) in the “PDL1-high” class versus 0% (95%CI, NA-NA) in the “PDL1-low” class ( $p = 1.43E-02$ , log-rank test; Figure 2D).

### **PDL1 expression and immune features**

We studied if *PDL1* mRNA expression was associated (Student t-test) with immune variables in clinical samples of the whole TCGA data set. As shown in Table 4, we found higher expression of the three Palmer’s metagenes representing B-cells, T-cells, and CD8 + T-cells<sup>50</sup> in the “PDL1-high” class. Similarly, the probability of activation<sup>51</sup> of the four immune-related pathways, IFN $\alpha$ , IFN $\gamma$ , STAT3 and TNF $\alpha$ , was higher in the “PDL1-high” class, as was the Rooney’ cytolytic activity score.<sup>52</sup> This immune pattern was confirmed and refined using the 24 Bindea signatures for

**Table 2.** Correlations of *PDL1* expression with clinicopathological variables.

Characteristics	N	PDL1		p-value
		Low (N = 40)	High (N = 39)	
Age, median	79	52	44	<b>0.065</b>
years (range)		(23–77)	(14–71)	
Sex				0.254
female	48	27 (68%)	21 (54%)	
male	31	13 (32%)	18 (46%)	
ENSAT stage				0.160
1	9	4 (10%)	5 (14%)	
2	37	15 (38%)	22 (59%)	
3	16	11 (28%)	5 (14%)	
4	15	10 (25%)	5 (14%)	
Pathological tumor size (pT)				0.429
pT1	9	4 (10%)	5 (14%)	
pT2	42	19 (48%)	23 (62%)	
pT3	8	5 (12%)	3 (8%)	
pT4	18	12 (30%)	6 (16%)	
Pathological lymph node (pN)				0.156
pN0	68	33 (82%)	35 (95%)	
pN1	9	7 (18%)	2 (5%)	
Surgical margins status				0.576
R0	55	25 (74%)	30 (83%)	
R1	6	4 (12%)	2 (6%)	
R2	9	5 (15%)	4 (11%)	
Weiss classification				0.235
low (1–3)	14	10 (28%)	4 (14%)	
high (4–9)	50	26 (72%)	24 (86%)	
Steroid hormone secretion excess				0.223
no	26	16 (43%)	10 (27%)	
yes	48	21 (57%)	27 (73%)	
Adjuvant mitotane				0.610
no	26	3 (13%)	1 (5%)	
yes	49	20 (87%)	19 (59%)	
Adjuvant radiotherapy				0.261
no	59	26 (72%)	33 (85%)	
yes	16	10 (28%)	6 (15%)	
<i>TP53</i> mutation status				0.252
mt	15	5 (13%)	10 (25%)	
wt	64	34 (87%)	30 (75%)	
<i>MKI67</i> mRNA status				<b>0.073</b>
low	40	24 (62%)	16 (40%)	
high	39	15 (38%)	24 (60%)	
DFS event*		21 (70%)	6 (18%)	<b>3.47E-05</b>
5-year DFS*		29%	76%	
		[16–53]	[58–100]	<b>5.11E-05</b>

\*, M0 patients only (N = 64)

immune cell subsets<sup>53</sup> showing a strong enrichment in the “PDL1-high” class versus “PDL1-low” class for B-cells, T-cells, cytotoxic T-cells, Th1 cells, and CD8 + T-cells, TFH cells and neutrophils. Among T-helper cells, the Th1/Th2 ratio was higher in the “PDL1-high” class. This anti-tumor activation was also correlated to subsets involved in antigen presentation, such as activated dendritic cells (aDC), DC, B-cells, and macrophages, which were higher in the “PDL1-high” class. The “PDL1-high” class was also enriched in samples with ICR4 signature, a reflect of an anti-tumor immune response. Altogether, these results suggested an association of *PDL1* mRNA expression with an anti-tumor T-cell response in ACC samples.

### **PDL1 expression and associated biological processes**

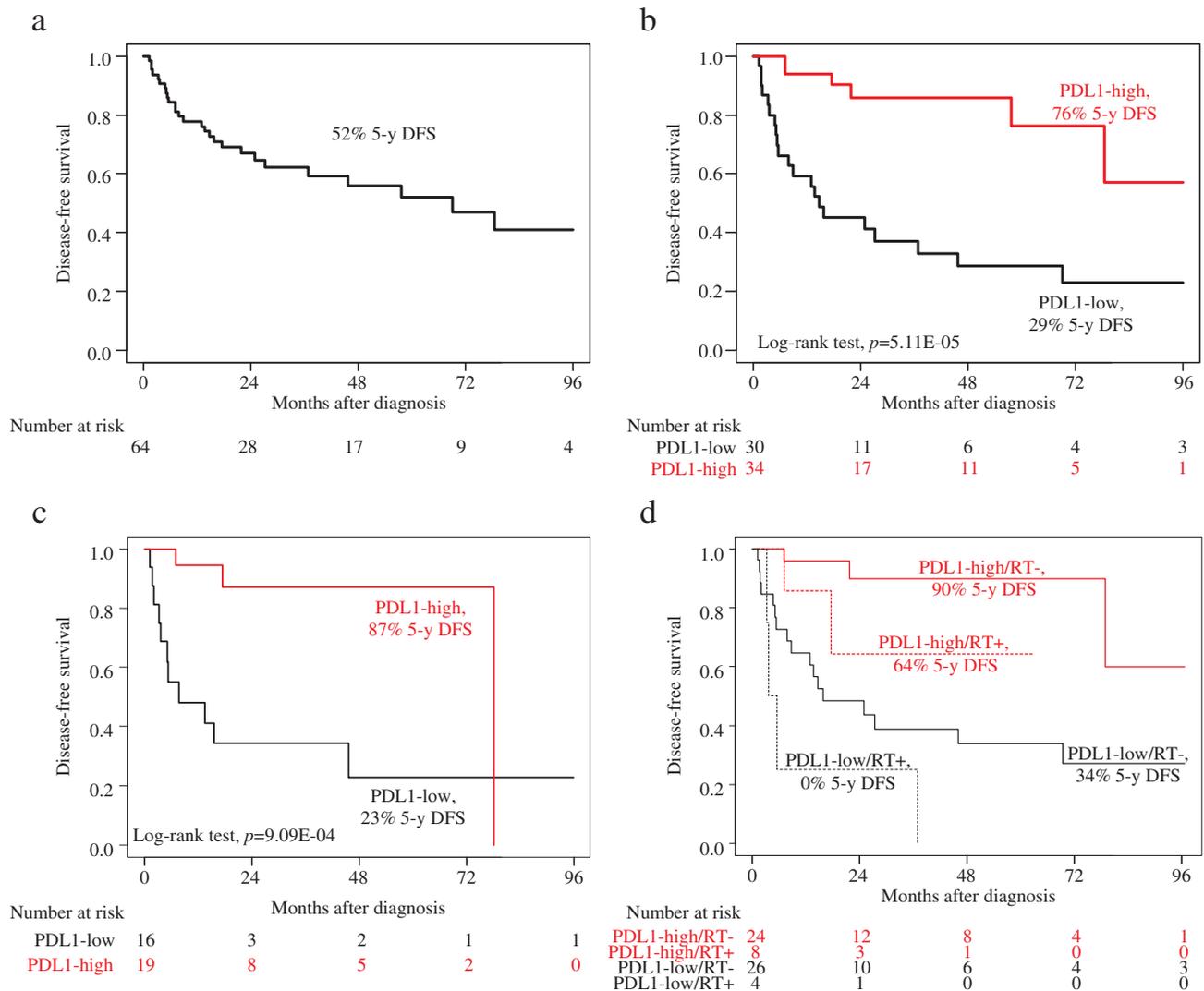
Finally, to further explore the biological pathways associated to *PDL1* mRNA expression in ACC, we compared within the whole TCGA data set the expression profiles of all genes between

“PDL1-high” tumors and “PDL1-low” tumors. We identified 370 genes differentially expressed, including 233 genes upregulated and 137 genes downregulated in the “PDL1-high” samples (Supplementary Table 1, Figure 3A). Ontology analysis of these 370 genes (Supplementary Table 2, Figure 3B) revealed that the “PDL1-high” samples overexpressed genes mainly involved in the regulation of the local immune response. More specifically, we found evidence for macrophages (*CD68*, *CD14*) and T-cells (*CD2*, *CD4*, *CD8B*, *CD244*, *CD247*, *KLRB1*, *KLRD1*, *PTPRC*) infiltrates. Numerous genes from the PDL1-high signature were related to antigen processing and presentation of peptides via MHC class II (*B2M*, *CIITA*, *CD74*, *HLA-DPA1*, *HLA-DRB1*, *HLA-DPB1*, *HLA-DQA1*, *HLA-DRA*, *HLA-DMA* ...). This immune function, responsible for T-cells activation, is performed by antigen-presenting cells only, including macrophages, which were found more abundant in the PDL1-high tumors. Inflammatory response (*GBP5*, *LY96*, *CYSLTR1*, *CYSLTR2*, *CYBB*, *CSF1*, *DAGLA*, *TLR5*, *CSF1R*, *P2RX7*, *CLEC7A*, *NLRP3*, *IRF1*, *INPP5D*, *TRIM22*, *TLR4*, *TLR5*), chemokines and cytokines family members involved in immune cells recruitment and activation were also upregulated in the “PDL1-high” samples (*CCR5*, *ITGAL*, *CX3CR1*, *CXCR6*, *CCL8*, *IL15*, *IL16*, *CXCL10*, *CCR2*, *CSF1*, *CCR5*, *IL2RA*, *CCL5*, *CCR1*, *IL15*, *XCL1*). Some of these chemokines are essential for T-cell homing to the tumor site. In this line, some transcripts upregulated in the “PDL1-high” group suggested local anti-tumor activation of T-cells (*ITGAL*, *IL2RA*, *IL2RB*, *IL12RB1*, *ITK*, *CRTAM*, *CD69*, *PRF1*, *STAT4*, *EOMES*, *GZMA*, *GZMK*, *LCK*, *SYK*, ...). However, additional candidates indicated an exhausted/ anergic status of T-cells (*IL10RA*, *PDCD1LG2*, *PTPRJ*, *HLA-E*, *HAVCR2*, *TIGIT*, *EOMES*, *IKZF1*, ...). Altogether, this transcriptional profile of the “PDL1-high” group showed an anti-tumor response being under the control of tumor cells at the time of analysis. Many genes overexpressed in the “PDL1-low” class were involved in the steroid and lipid metabolism, the gland development, and differentiation. The robustness of this gene list was confirmed in the independent validation set including 33 “PDL1-high” tumors and 34 “PDL1-low” tumors ( $p = 31E-03$ , Fisher’s exact test; Figure 3C).

### **Discussion**

The need for new therapeutic and/or prognostic targets are crucial in ACC. Blockade of the PD1-PDL1 pathway in tumors is a recent promising therapeutic strategy. To our knowledge, only one study (JAVELIN Solid Tumor trial) with an immune checkpoint inhibitor (avelumab) has included more than 15 patients with advanced ACC treated.<sup>29</sup> The ORR was 6% in the whole 50-patient series, higher in the PDL1-positive tumors (16.7%) than in the PDL1-negative tumors (3.3%). Our objective was to document the expression of *PDL1* in a large series of ACC clinical samples and to search for correlations with tumor features. We found that *PDL1* mRNA expression was heterogeneous and associated with better DFS independently from classical prognostic variables. To our knowledge, with 146 cases analyzed this study is the largest series reported in the literature.

During the last years, PDL1 expression in cancer has been mainly studied at the protein level using IHC. However,



**Figure 2.** Disease-free survival according to *PDL1* mRNA expression in the M0 patients.

A/Kaplan-Meier DFS curves in the whole population (N = 64). B/Kaplan-Meier DFS curves in the “PDL1-low” and “PDL1-high” classes in the whole population (N = 64). C/Kaplan-Meier DFS curves in the “PDL1-low” and “PDL1-high” classes in the patients treated with adjuvant mitotane (N = 35). D/Kaplan-Meier DFS curves in the “PDL1-low” and “PDL1-high” classes in the patients treated with adjuvant radiotherapy (RT+; dashed curves; N = 12) and in the patients untreated with adjuvant radiotherapy (RT-; solid curves; N = 50).

**Table 3.** Univariate and multivariate prognostic analyses for DFS.

Characteristics*	Univariate			Multivariate			Multivariate		
	N	HR [95%CI]	p-value	N	HR [95%CI]	p-value	N	HR [95%CI]	p-value
Age, median	64	1.00 [0.96–1.03]	.834						
Sex	64	1.45 [0.51–4.15]	.485						
ENSAT stage	62	2.86 [0.67–12.18]	.155						
Pathological tumor size	pT2 vs. pT1	1.85 [0.22–15.5]	<b>5.56E-03</b>	43	1.33 [0.26–6.83]	.730	43	1.89 [0.37–9.64]	.443
	pT3 vs. pT1	6.83 [0.57–81.8]		43	27.70 [2.78–276]	<b>4.66E-03</b>	43	31.04 [2.89–333.53]	<b>4.57E-03</b>
	pT4 vs. pT1	21.6 [2.08–224]		43	48.1 [1.78–1296]	<b>2.12E-02</b>	43	22.86 [0.75–695.62]	.073
Pathological lymph node	62	0.00 [0.00–Inf]	.998						
Surgical margins status	R1 vs. R0	22.8 [1.43–366]	<b>8.77E-03</b>	43	0.42 [0.03–5.82]	.519	43	1.07 [0.09–12.42]	.956
	R2 vs. R0	94.2 [4.32–2051]		43	<NA> [NA-NA]	<NA>	43	<NA> [NA-NA]	<NA>
Weiss classification	49	4.79 [0.92–24.8]	.062	43	3.24 [0.68–15.46]	.141	43	2.13 [0.47–9.6]	.324
Steroid hormone secretion excess	59	3.17 [0.7–14.30]	.134						
Adjuvant mitotane	37	7.24E07 [0–Inf]	.999						
Adjuvant radiotherapy	62	1.06 [0.23–4.84]	.938						
<i>MKI67</i> mRNA status	64	8.87 [2.45–32.1]	<b>8.99E-04</b>	43	0.68 [0.18–2.58]	.569	43	1 [0.26–3.83]	.995
<i>TP53</i> mutation status	64	0.15 [0.04–0.60]	<b>7.79E-03</b>	43	0.32 [0.03–3.35]	.340	43	0.28 [0.03–2.87]	.285
PDL1 class	64	0.19 [0.08–0.46]	<b>3.00E-04</b>	43	0.09 [0.02–0.39]	<b>1.58E-03</b>			
PDL1, continuous value	64	0.66 [0.51–0.87]	<b>2.99E-03</b>				43	0.56 [0.36–0.86]	<b>9.01E-03</b>

\*, N = 64 M0 patients

**Table 4.** Correlations of *PDL1* expression with immune features.

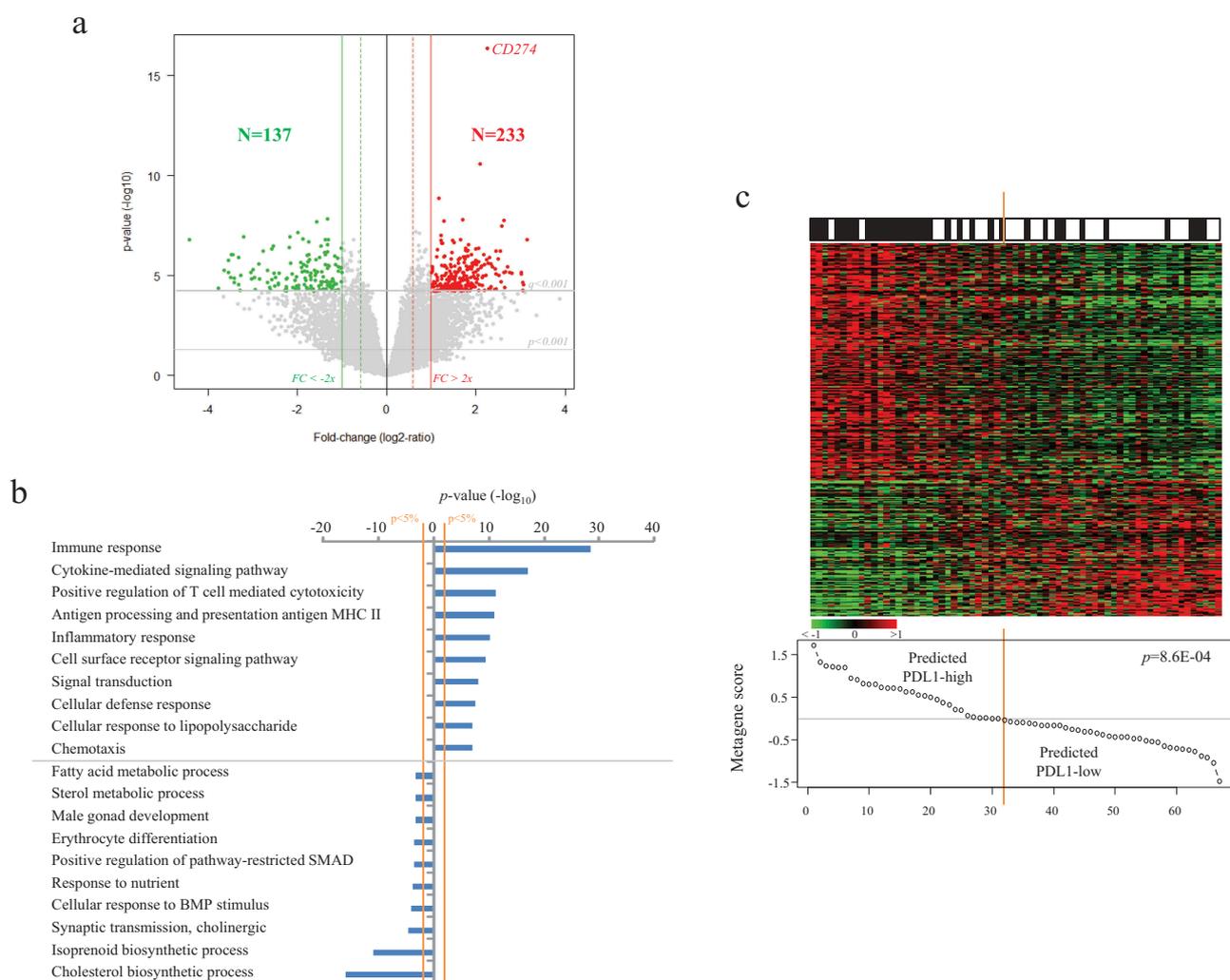
Characteristics		N	PDL1		<i>p</i> -value	
			Low (N = 40)	High (N = 39)		
Palmer et al.	B module	79	-0.18 (-0.67-0.78)	0.18 (-0.48-1.04)	<b>4.81E-05</b>	
	CD8 module	79	-0.31 (-1.84-1.72)	0.49 (-1.13-2.91)	<b>7.94E-05</b>	
	T module	79	-0.22 (-0.81-0.64)	0.17 (-0.82-1.13)	<b>8.30E-05</b>	
Gatza et al.	IFN $\alpha$ biological pathway	79	0.25 (0.02-0.86)	0.69 (0.11-0.98)	<b>3.58E-05</b>	
	IFN $\gamma$ biological pathway	79	0.37 (0.04-0.96)	0.71 (0.04-0.99)	<b>1.88E-03</b>	
	STAT3 biological pathway	79	0.37 (0.02-0.92)	0.54 (0.14-1.00)	<b>7.98E-04</b>	
	TNF $\alpha$ biological pathway	79	0.45 (0.01-0.86)	0.58 (0.09-1.00)	<b>8.21E-03</b>	
Bindea et al.	B cells	79	-0.22 (-0.57-1.22)	0.07 (-0.41-1.42)	<b>2.99E-04</b>	
	T cells	79	-0.39 (-1.18-0.97)	0.25 (-1.01-1.56)	<b>1.78E-03</b>	
	T helper cells	79	-0.12 (-0.43-0.87)	-0.01 (-0.42-0.69)	.259	
	Tcm	79	-0.08 (-0.69-0.47)	0.09 (-0.57-0.66)	<b>3.32E-02</b>	
	Tem	79	-0.07 (-0.58-0.45)	0.07 (-0.47-0.75)	.101	
	Th1 cells	79	-0.19 (-0.67-0.64)	0.05 (-0.48-1.33)	<b>1.16E-03</b>	
	Th2 cells	79	0.03 (-0.78-0.57)	-0.11 (-0.68-0.86)	<b>2.15E-02</b>	
	Th1/Th2 log <sub>2</sub> -ratio	79	-0.21 (-1.05-0.66)	0.25 (-0.88-0.9)	<b>1.18E-05</b>	
	TFH	79	-0.12 (-0.62-0.48)	0.15 (-0.47-0.77)	<b>2.15E-04</b>	
	Th17 cells	79	-0.18 (-1.1-0.78)	0.02 (-1.19-2.45)	<b>3.67E-02</b>	
	TReg	79	-0.07 (-1.74-3.7)	-0.26 (-1.52-2.05)	.372	
	CD8 T cells	79	-0.05 (-0.59-0.37)	0.08 (-0.43-0.42)	<b>1.17E-02</b>	
	Tgd	79	-0.19 (-1.04-0.97)	0.03 (-0.77-1.34)	.097	
	Cytotoxic cells	79	-0.35 (-1.39-0.88)	0.32 (-0.85-1.62)	<b>1.13E-04</b>	
	NK cells	79	-0.02 (-0.5-0.47)	0.02 (-0.49-0.57)	.205	
	NK CD56dim cells	79	-0.14 (-0.7-1.06)	0.08 (-0.8-1.2)	.082	
	NK CD56bright cells	79	-0.02 (-0.65-1.09)	0.11 (-0.8-1.01)	.562	
	DC	79	-0.23 (-1.08-1.64)	0.02 (-0.75-1.9)	<b>3.01E-02</b>	
	iDC	79	-0.07 (-0.61-0.82)	0.08 (-0.43-0.98)	.059	
	aDC	79	-0.12 (-1.11-0.69)	0.16 (-1-2.06)	<b>1.23E-04</b>	
	pDC	79	-0.08 (-2.45-1.85)	0.09 (-2.3-2.14)	.595	
	Eosinophils	79	0.03 (-0.43-0.38)	0 (-0.42-0.64)	.664	
	Macrophages	79	-0.15 (-0.88-0.62)	0.08 (-0.56-1.65)	<b>5.65E-04</b>	
	Mast cells	79	-0.24 (-0.59-1.13)	0.04 (-0.53-1.76)	<b>1.20E-03</b>	
	Neutrophils	79	-0.19 (-0.88-1.22)	0.11 (-0.75-2.08)	<b>1.67E-04</b>	
	Rooney et al.	Cytolytic activity	79	-0.61 (-2.09-1.35)	0.49 (-1.12-2.28)	<b>2.48E-05</b>
	Bertucci et al.	ICR1-3 classes	74	40 (100%)	34 (87%)	<b>2.55E-02</b>
ICR4 class		5	0 (0%)	5 (13%)		

divergent results have been reported, notably regarding its prognostic value.<sup>54</sup> Such divergence has often been related to the usual limitations of IHC such as the absence of standardization for PDL1, notably in terms of specificity and reproducibility of available antibodies,<sup>55,56</sup> the definition of positivity cutoff, and interpretative subjectivity. Our analysis at the mRNA level allowed us to avoid these limitations, to work on a large series of samples, and to search for associations with expression of other genes on a whole-genome scale.

*PDL1* mRNA expression was heterogeneous between samples with a relatively wide range of values (~3 decades) and showed a small positive correlation with *PDL1* DNA copy number in the TCGA data set. However, DNA copy number alterations were rare (32% of samples), confirming the existence of many other levels of regulation for PDL1 expression in tumors.<sup>57</sup> Such heterogeneity allowed the search for correlations between *PDL1* mRNA expression and clinically and biologically relevant features. No correlation was found with patients' age, sex, ENSAT stage, pathological tumor size or lymph node status, surgical margins status, Weiss classification, steroid secretion. There was a trend toward association with a younger patients' age reported as a good-prognosis feature<sup>58</sup> and with higher *MKI67* expression known as a poor-prognosis feature.<sup>59</sup> To our knowledge, only one study in literature characterized PDL1 expression in ACC and searched for clinicopathological correlations.<sup>49</sup> The analysis was limited to a series of 28 samples, including 20 primary tumors and 8 metastases, and was based on IHC and an

antibody developed in an academic laboratory. PDL1 staining was assessed on tumor cell membrane with a positivity cutoff equal to 5% of tumor cells (observed in 10.7% of samples) and in tumor-infiltrating mononuclear cells (TIMC) with a positivity cutoff equal to 1% of cells (observed in 70.4% of samples). PDL1 positivity at the cell surface of either tumor cells or TIMC was not associated with stage at diagnosis, tumor grade, excessive hormone secretion, or overall survival. Our results observed in a larger (N = 79) and more homogeneous (only primary tumors) series confirms the absence of association between *PDL1* mRNA expression and the classical prognostic variables. However, we show for the first time the prognostic value of *PDL1* mRNA expression in ACC. In uni- and multivariate analyses for DFS, high expression was associated with longer DFS, suggesting independent prognostic value, and this result was observed both as discrete value and as a continuous value. Possible explanations for this discordance with the IHC study may be related to the IHC limitations described above, the small size and the heterogeneity of the IHC series, and the non-perfect correlation between mRNA and protein expression.

To date, the prognostic role of immunity in ACC has been little documented. Two studies suggest that the immune response may impact the clinical outcome of patients. Elevated neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) were associated with shorter post-operative relapse-free survival and disease-specific survival in a retrospective series of 84 patients.<sup>60</sup> In a comprehensive



**Figure 3.** Identification and validation of the *PDL1* gene expression signature.

A/Identification of the signature in the TCGA data set ( $N = 79$ ). Volcano-plot showing the 370 genes differentially expressed between the “PDL1-high” versus “PDL1-low” ACC samples. B/Top 20 ontologies associated with the 370 genes differentially expressed based on GO biological processes of the Database for Annotation, Visualization and Integrated Discovery (DAVID). C/Validation of the signature in the pooled Giordano’s and De Reynies’s sets ( $N = 67$ ). Up, observed *PDL1* expression status (black: positive; white: negative); Middle, expression of the 370 differential genes. Each row represents a gene and each column represents a sample. Expression levels are depicted according to the color scale. Genes are ordered from top to bottom by their decreasing t-test statistics, and samples are ordered from left to right according to the decreasing “PDL1 metagene” score (Bottom). The solid orange line indicates the threshold 0 that separates the two predicted classes of samples, “PDL1-high” (at the left of the line) and “PDL1-low” (right to the line). The  $p$ -value is for the Student t-test comparing the “PDL1 metagene” score in the two observed *PDL1* classes.

pan-cancer meta-analysis of mRNA expression in TCGA data,<sup>61</sup> the intra-tumoural immune cytolytic activity (CYT), calculated as the geometric mean of *GZMA* and *PRF1* expression levels, was associated with improved overall survival in ACC. In fact, the favorable prognostic value of high *PDL1* expression may seem paradoxical given the known immunosuppressive role of *PDL1*. Similar favorable prognostic value was previously reported in other cancers such as lung cancer,<sup>54</sup> breast cancer,<sup>47,62</sup> colorectal cancer,<sup>41</sup> Merkel cell carcinoma,<sup>63</sup> and GIST.<sup>44</sup> It is even more puzzling that there was a positive correlation between *PDL1* mRNA expression and *MKI67* mRNA expression, which makes these tumors more likely to have a worse prognosis. The results of our supervised analysis provide a few elements to help in understanding these paradoxes. The list of genes that were correlated with *PDL1* mRNA expression identified a coherent

immunological signature. This signature was related to chemo-attractive factors for leukocytes, and macrophages and T-cells infiltrates. Because of the very good antigen presentation capabilities (CMH-II-related molecules were strongly upregulated), the T-cells probably received convincing positive anti-tumor activation signals. In this line, the co-expression of *STAT4* and *EOMES* was indeed reported in effector T-cells.<sup>64</sup> Some molecules involved in T-cell cytotoxicity against tumor cells were upregulated (*PRF1*, *GZM* ...). Considering this, one hypothesis would be that *PDL1* expression was the consequence of IFN $\gamma$  activation as already demonstrated in this context.<sup>26,65</sup> The correlation between high *PDL1* expression and elevated tumor cell proliferation (Ki67) might be explained by the higher mutation rate of hyperproliferative tumor cells, potentially responsible for higher immunogenicity due to the rapid appearance of

neoantigens. Similar positive correlations between PDL1 expression and cell proliferation and longer survival were previously reported in breast cancer,<sup>47,62</sup> and GIST.<sup>44</sup> However, some very well-known markers of exhaustion (*TIGIT*, *HAVCR2*, the simultaneous expression of *EOMES* and *IKAROS* ...) were also found upregulated in the “PDL1-high” samples, which is certainly the consequence of the tumor overcoming the anti-tumor T-cell response. Altogether, this is laying the ground for the use of immune-checkpoint inhibitors in patients with “PDL1-high” ACC. In those patients, there is a real potential to re-activate the host immune response as reported for melanoma or lung cancers.

**In conclusion**, we showed that *PDL1* mRNA expression is heterogeneous in ACC and represents an independent prognostic feature. Samples with high expression are associated with longer DFS independently from the other prognostic features, suggesting that *PDL1* expression cooperates with tumor cell-intrinsic features to influence survival. The strength of our study lies in its originality, the number of samples analyzed ( $N = 146$ ), important when considering the low incidence of the disease, the homogeneity of the series (primary tumors only), the independent prognostic value of *PDL1* mRNA expression as discrete or continuous value, and its biological relevance. Limitations include its retrospective nature and associated biases, such as the absence of information with respect to overall survival, the analysis at the mRNA rather protein level, the use of DNA microarrays and RNA-sequencing that quantify expression levels of both epithelial and stromal cells, and the absence of metastatic samples tested. Even if the biological relevance of our *PDL1* signature provides an indirect indication that increased *PDL1* mRNA expression in ACC is likely associated with an increase in its activity, and thus its protein expression, it is warranted to validate these preliminary results at the protein level. Analysis of larger patients' series, retrospective, then prospective, is warranted to confirm our results and to fully understand the role of PDL1 in ACC. If confirmed, *PDL1* expression might refine the prediction of postoperative clinical outcome in ACC and improve our ability to better tailor adjuvant therapy. Given the suspected link between PDL1 expression and tumor response to PDL1-inhibitors, our study also suggests that the therapeutic targeting of PDL1 in ACC could enhance the local immune response, thus providing an antitumor effect. Functional and clinical validation of this hypothesis is urgently warranted in front of this devastating disease. Phase 2 clinical trials are ongoing with nivolumab combined with ipilimumab (NCT03333616), and with pembrolizumab (NCT02721732; NCT02673333). In this setting, analysis of metastatic and/or pre-treated samples is warranted to assess PDL1 expression and to test if it can predict the response to immune checkpoint inhibitors.

## Materials and methods

### Adrenocortical carcinoma samples

We collected clinicopathological and gene expression data of clinical ACC samples from three publicly available data sets<sup>7,10,11</sup> comprising at least one probe set representing *CD274/PDL1*. Data were collected from the National Center for Biotechnology Information (NCBI)/Genbank GEO, and TCGA databases (Supplementary Table 3). All samples were

pre-treatment primary tumor samples collected on the surgical resection specimen. The TCGA set included 79 cases profiled using RNA-seq (Illumina) and clinically annotated for many variables including classical prognostic variables, adjuvant treatment, and clinical outcome. The Giordano's and De Reynies's sets included 33 and 34 cases, respectively; all were profiled using whole-genome DNA microarrays (Affymetrix U133 Plus 2.0). Only the TCGA set could be used for the prognostic analysis since the other datasets<sup>6-9</sup> were not annotated for both expression, prognostic and survival data.

### Gene expression data analysis

Data analysis required pre-analytic processing. The first step was to normalize each data set separately: we used the Robust Multichip Average (RMA)<sup>66</sup> with the non-parametric quantile algorithm for the raw data from the Affymetrix data sets. Normalization was done in R using Bioconductor and associated packages. Then, hybridization probes were mapped across the different technological platforms represented. We used NetAffx Annotation files ([www.affymetrix.com](http://www.affymetrix.com); release from 01/12/2008) to update the Affymetrix annotations. The probes were then mapped based on their EntrezGeneID. When multiple probes mapped to the same GeneID, we retained the one with the highest variance in a particular dataset. *PDL1* (*CD274*) tumor expression was measured by analyzing different probe sets whose identity and specificity were verified using the NCBI program BLASTN 2.2.31+ (Supplementary Table 3). For the TCGA data, we used the available normalized RNA-seq data that we log<sub>2</sub>-transformed.

Because of the role of PDL1 in immunity, we also analyzed gene expression signatures related to the immune response in cancers. Each of the following signatures was applied to the TCGA data set: three Palmer's B-cell, T-cell, and CD8 + T-cell signatures,<sup>50</sup> the Rooney' cytolytic activity score,<sup>52</sup> four Gatz's signatures of IFN $\alpha$ , IFN $\gamma$ , STAT3, and TNF $\alpha$  biological pathway activity,<sup>51</sup> the signatures of 24 different innate and adaptative immune cell subpopulations defined by Bindea *et al.*,<sup>53</sup> and the Immunologic Constant of Rejection (ICR) signature of Th-1 immunity-mediated response.<sup>67</sup>

Finally, to explore more-in-depth the biological pathways associated to *PDL1* mRNA expression in ACC, we applied a supervised analysis to the whole TCGA data set as a learning set ( $N = 79$ ) and compared the expression profiles of all genes between tumors with low *versus* high *PDL1* mRNA expression using a moderated t-test with the following significance thresholds:  $p < .001$ ,  $q < 0.001$  and fold change (FC) superior to  $|2x|$ . Ontology analysis applied to the resulting gene list was based on GO biological processes of the Database for Annotation, Visualization and Integrated Discovery (DAVID; [david.abcc.ncifcrf.gov/](http://david.abcc.ncifcrf.gov/)). The robustness of this gene list was tested in the 67 pooled Affymetrix-profiled ACC samples<sup>7,10</sup> used as an independent validation set. We computed for each sample a “PDL1 meta-gene” score as the difference between the mean expression of genes upregulated and the mean expression of genes downregulated in the “PDL1-high” group. The natural score of 0 was used as a threshold to define a sample as “PDL1-up” or “PDL1 no up”. Once defined, we analyzed the concordance of the predicted and observed statuses of all 67 samples using Fisher's exact test.

## Statistical analysis

Correlations between tumor groups and clinicopathological features were analyzed using the t-test or Fisher's exact test when appropriate. Disease-free survival (DFS) was calculated from the date of diagnosis until the date of distant relapse or death from any cause. Follow-up was measured from the date of diagnosis to the date of last news for event-free patients. Survivals were calculated using Kaplan–Meier method and curves were compared with the log-rank test. Univariate and multivariate survival analyses were done using Cox regression analysis (Wald test). Variables tested in univariate analyses included patients' age at time of diagnosis, sex, pathological tumor size (pT: pT1 vs pT2, vs pT3, vs pT4), pathological lymph node status (pN: negative vs positive), surgical margins status (R0 vs R1, vs R2), ENSAT stage (1 vs 2–3), Weiss classification (low vs high),<sup>68</sup> excess of steroid hormonal secretion (no vs yes), adjuvant mitotane therapy (no vs yes), adjuvant radiotherapy (no vs yes), *MKI67* mRNA expression (low vs high), and *TP53* mutation status (wild type WT vs mutated). Variables with a *p*-value <0.10 in univariate analysis were tested in multivariate analysis. All statistical tests were two-sided at the 5% level of significance. Statistical analysis was done using the survival package (version 2.30) in the R software (version 2.15.2; <http://www.cran.r-project.org/>). We followed the reporting REcommendations for tumor MARKer prognostic studies (REMARK criteria).<sup>69</sup>

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## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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