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ORIGINAL RESEARCH

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PD-L1 (*CD274*) promoter hypomethylation predicts immunotherapy response in metastatic urothelial carcinoma

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ABSTRACT

PD-L1 status assessed by immunohistochemistry (IHC) has failed to reliably predict outcomes for patients with metastatic urothelial carcinoma (mUC) on immune checkpoint blockade (ICB). *PD-L1* promoter methylation is an epigenetic mechanism that has been shown to regulate *PD-L1* mRNA expression in various malignancies. The aim of our present study was to evaluate the predictive potential of *PD-L1* promoter methylation status (*mPD-L1*) in ICB-treated mUC compared to conventional IHC-based PD-L1 assessment. We quantified *mPD-L1* in formalin-fixed and paraffin-embedded tissue sections using an established quantitative methylation-specific PCR assay (qMSP) in a well-characterized multicenter ICB-treated cohort comprising $N = 107$ patients with mUC. Additionally, PD-L1 protein expression in tumor tissues was assessed using regulatory approved IHC protocols. The effect of pharmacological hypomethylation by the DNA methyltransferase inhibitor decitabine in combination with interferon-γ stimulation in urothelial carcinoma cell lines was investigated by IHC and FACS. *mPD-L1* hypomethylation predicted objective response rate at the first staging on ICB. Patients with tumors categorized as *PD-L1* hypomethylated (lower quartile) showed significantly prolonged progression-free (PFS) and overall survival (OS) after ICB initiation. In contrast, PD-L1 protein expression status neither correlated with response nor survival. In multivariable Cox regression analyses, *PD-L1* promoter hypermethylation remained an independent predictor of unfavorable PFS and OS. In urothelial carcinoma cell lines, pharmacological demethylation led to an upregulation of membranous PD-L1 expression and an enhanced inducibility of PD-L1 expression by interferon γ. Hypomethylation of the *PD-L1* promoter is a promising predictive biomarker for response to ICB in patients with mUC.

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Background

The therapeutic landscape of metastatic urothelial carcinoma (mUC) has undergone substantial changes in recent years. With the broad implementation of immune checkpoint blockade (ICB) and the recent advent of FGFR inhibition and antibody-drug conjugates (ADC), the therapeutic armamentarium in mUC has expanded considerably.[1,2]

In the presence of PD-L1 expression on immune and/or tumor cells that exceeds certain thresholds, ICB can be used as a first-line treatment in cisplatin-ineligible patients.[3,4] Further, ICB presents the second-line therapy of choice in chemotherapy pretreated patients with a more favorable side effect profile compared to the second-line chemotherapy-based regimen.

However, in both, first-line and second-line, only a minority of patients exhibit durable responses to ICB. Due to the emerging new therapeutic approaches in mUC, it is an increasing challenge to find the most promising

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therapy tailored to the patient's specific disease. For this purpose, biomarkers that enable precise individual therapy prediction are of utmost importance. Regarding ICB therapy prediction in mUC, various attempts were conducted to identify therapy responders by biomarkers, including tumor mutational burden and PD-L1 expression.[5,6] A recently published meta-analysis showed that PD-L1 positive tumors respond better to ICB than PD-L1 negative tumors, but the value of PD-L1 in mUC is highly inconsistent.[7] However, application of PD-L1 immunohistochemistry (IHC) as a predictive biomarker is confounded by multiple technical and biological challenges, such as the usage of different antibodies and expression scores, interlaboratory and inter-observer variability, as well as intratumoral heterogeneity and evolution of PD-L1 expression during metastatic progression leading to sampling bias.[5,6,8,9]

Biomarkers based on methylation signatures can overcome some of these shortcomings: DNA methylation is an epigenetic modification that is not subjected to dynamic variations as mRNA or protein expression. Furthermore, it is chemically stable and can be quantified investigator-independently by applying polymerase chain reaction (PCR)-based methods. In addition, quantitative measurement of methylation signatures is also feasible even with small sample quantities (microdissected cells, biopsies), which are frequently found in oncological practice and can be problematic, particularly for morphological assays such as PD-L1 IHC.[10,11]

Research has shown that *PD-L1* expression is epigenetically regulated via DNA promoter methylation across various tumor entities.[12–18] In melanoma, hypomethylation of the *PD-L1* promoter appeared to be associated with an improved response to ICB, although the results did not reach statistical significance ($p = 0.11$) which was probably attributed to a small cohort size of $N = 43$.[16] However, it has already been proven that the methylation status of immune checkpoint genes such as *CTLA4* can predict response to ICB.[19–21]

The aim of our present study was therefore to evaluate the predictive potential of *PD-L1* promoter methylation status (*mPD-L1*) in ICB-treated mUC as valid predictive and prognostic biomarkers are lacking. We performed *mPD-L1* assessment using an established quantitative methylation-specific PCR assay (qMSP)[12,13] in a well-characterized multicenter ICB-treated cohort of $N = 107$ patients with mUC from five academic centers in Germany.[22]

Material and methods

Study cohort and histopathological review

A multicenter ICB-treated mUC cohort was assembled ($N = 107$). The cohort comprised pre-treatment samples from patients who received ICB in the first- or second therapy-line setting. All specimens underwent central histopathological re-evaluation by two experienced uro-pathologists (AH, ME) according to the UICC TNM 2017 system and 2016 WHO classification of genitourinary tumors. Response to ICB was defined according to RECIST v1.1 criteria. Progression-free survival (PFS) was defined as the time from the initiation of ICB to disease progression according to RECIST v1.1 or death from any cause.

This study was conducted according to the declaration of Helsinki and approved by the responsible ethical review board (reference # 187/16; 2018-829 R-MA; 217_18Bc).

Stromal tumor-infiltrating lymphocytes (sTils) assessment

sTILs were analyzed semiquantitatively on hematoxylin and eosin (H&E)-stained tissue sections by experienced uropathologists, according to recommendations of the international working group on sTILs as previously described for urothelial cancer.[23–26]

Immunohistochemistry (IHC)

IHC was performed on 4 µm tissue sections on a Ventana BenchMark ULTRA autostainer (Ventana) according to accredited staining protocols (https://www.dakks.de/en) using the following antibodies: PD-L1 expression on tumor and immune cells was assessed using a laboratory developed PD-L1 assay based on the 28–8 antibody clone (dilution 1:50, Abcam®, United Kingdom). PD-L1 positivity on immune cells (IC) and tumor cells (TC) was scored by experienced uropathologists according to currently clinically applied and approved PD-L1 scoring algorithms including Ventana IC-score, tumor proportion score (TPS/TC), and combined positivity score (CPS) as previously described.[6,23] To identify luminal and basal subtypes, we applied a six-marker panel consisting of CK5 (clone XM26, mouse monoclonal, Diagnostic BioSystems®, USA, dilution 1:50), CK20 (clone Ks 20.8, mouse monoclonal, Dako, Denmark, dilution 1:50), GATA3 (clone L50–823, mouse monoclonal, DCS, Germany, dilution 1:100), FOXA1 (rabbit polyclonal ab23738, Abcam®, dilution 1:400), and CD44 (clone DF1485, mouse monoclonal, Dako, dilution 1:50) according to the recommendations provided by the Bladder Cancer Molecular Taxonomy Group (BCMTG); staining intensities were quantified using the semiquantitative immunoreactive score (IRS; range: 0–12) as described previously.[22,27,28]

PD-L1 IHC 22C3 pharmDx (cat. no. SK006, RRID: AB_2889976; Agilent, CA, USA) was performed on cell pellets according to manufacturer's instruction.

mPD-L1 qMSP assay

The detailed protocol for nucleic acid isolation from the multicenter ICB-treated UC cohort has been described elsewhere[22]. Quantitative methylation-specific real-time PCR (qMSP) for quantification of *mPD-L1* was performed as previously described.[12,13] In brief, qMSP represents a duplex real-time PCR for the sensitive and quantitative detection of *PD-L1* DNA promoter methylation with a reference PCR for the quantification of total DNA using the *ACTB* locus.

Cell culture

The human bladder cancer cell lines TCCSUP (RRID: CVCL_1738), RT-112 (RRID:CVCL_1670), T24 (RRID: CVCL_0554), and RT-4 (RRID:CVCL_0036) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultured in complete RPMI 1640 medium (cat. no. 21875059, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 1 mM 2-mercaptoethanol (cat. no. 21985023, Thermo Fisher Scientific), 1 mM HEPES (1 M stock, cat. no. 15630056, Thermo Fisher Scientific), 10% [v/v] fetal bovine serum (FBS, heat inactivated, cat. no. FBS. S 0615HI, Bio&SELL GmbH, Nuremberg, Germany), 1X MEM (Minimum Essential Medium) Non-Essential Amino Acids Solution (100X stock, cat. no. 11140035, Thermo Fisher Scientific), 1 mM sodium pyruvate (100 mM stock, cat. no. 11360070, Thermo Fisher Scientific), and 100 U/ml penicillin and streptomycin (10,000 U/ml stock, cat. no. 15140122, Thermo Fisher Scientific). The cell lines were treated with 100 µM decitabine (5-aza-2-deoxycytidine; cat. no. ab120842, Abcam, Cambridge, UK) for 240 h and/or treated with recombinant IFN-γ (1,000 U/ml IFN-γ, PeproTech, Rocky Hill, NJ, USA) 24 h prior to cell harvest. Untreated cell lines were used as control. The growth medium was changed every 24 h.

Fluorescence-activated cell sorting (FACS)

Cell line pellets were washed with a flow cytometry buffer (1X Dulbecco's Phosphate-Buffered Saline [cat. no. 14190094, Thermo Fisher Scientific], 4% [v/v] FBS, 2 mM ethylenediaminetetraacetic acid [EDTA]). Cell suspensions were stained with the fluorescein-5-isothiocyanate (FITC)-labeled anti-human PD-L1 (clone 28-8 [cat. no. ab224027, Abcam, Cambridge, UK], 1:100 in flow cytometry buffer) and LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (cat. no. L10119, Thermo Fisher Scientific, 1:1,000 in flow cytometry buffer). Flow cytometry was performed with a FACSCanto™ Flow Cytometer (Becton, Dickinson and Company, NJ, USA) and analyzed with FlowJo software (version 10.8.0, Becton, Dickinson and Company).

Statistics

PFS and overall survival (OS) after ICB initiation were estimated by univariate Kaplan–Meier regression analysis and tested with log-rank tests. Univariate and multivariate Cox regression analyses were performed to compare the prognostic value of each parameter. Variables were included in the multivariate Cox regression models only if survival effects were significant in the univariate analyses. Statistical analyses were performed using R Studio (version 1.4.1106), GraphPad Prism (version 9.0.0), and JMP SAS (version 13.2). The Kruskal–Wallis rank sum test, Mann–Whitney *U* test, Pearson's chi-square test, and Fisher's exact test were used to perform group comparisons. Cluster analysis was performed as previously described.[23] In brief, we performed unsupervised hierarchical clustering based on Ward's method using Euclidean distance as the metric scale. All tests were two-sided, and *P*-values <0.05 were considered significant.

Results

Baseline characteristics

In a well-characterized multicenter cohort of $N = 107$ patients with metastatic urothelial carcinoma (mUC),[22] we aimed to assess *mPD-L1* with an established qMSP assay.[12,13] The mean age of the patients was 67 years (interquartile range, IQR 58–74). Seventy-seven percent of patients were male. Seventy-five percent received an anti-PD-1 antibody, the remaining 25% received an anti-PD-L1 antibody. The ECOG (Eastern Cooperative of Oncology Group) score was ≤2 in 99% (only $N = 1$ ECOG 2).

Histologically, $N = 106$ cases were urothelial carcinomas ($N = 1$ pure neuroendocrine carcinoma of the bladder). Conventional urothelial morphology (not otherwise specified [NOS]) was present in 57% and squamous histology in 23% of patients. The distribution of rare UC variants (e.g., micropapillary, nested, plasmacytoid, etc.) reflected those from other cohorts; hence, from a pathological perspective, this was a balanced real-life UC cohort (Table 1; Suppl. Table S1). Due to the overall low numbers of specific variants, urothelial carcinoma with variant histology was summarized as "other". Molecular UC subtypes (luminal vs. basal) were defined using an established marker panel (CK5, CK20, FOXA1, GATA3, CD44).[29,30] Seventy percent ($N = 75$) of tumor samples were defined as luminal and the other 30% as basal ($N = 32$). Detailed baseline characteristics are summarized in Table 1.

PD-L1 promoter hypomethylation predicts immunotherapy response and outcome

First, we investigated the predictive power of tissue-based *mPD-L1* for immunotherapy response. *mPD-L1* was significantly associated with objective response (Figure 1a). Patients with complete response (CR) exhibited significantly lower *mPD-L1* (IQR: 2.12–9.20%) as compared to PD (IQR: 3.76–17.49%, $P = 0.003$; Figure 1a). Next, we wanted to assess whether the predictive value of *mPD-L1* also translates into prolonged PFS and OS. In univariable Cox, *mPD-L1* methylation as a continuous variable showed a trend toward improved PFS and OS (PFS: HR = 1.01 [95%-CI 1.00–1.03], $P = 0.079$; OS; HR = 1.01 [95%-CI 1.00–1.03], $P = 0.072$). Next, we divided the cohort into quartiles based on *mPD-L1* and found that the quartile with the lowest *mPD-L1* methylation was associated with exceptional PFS and OS following immunotherapy initiation (Figure 1b,c). Baseline patient and histological characteristics were balanced between *mPD-L1* quartile groups (Suppl. Table S1).

We next comprehensively examined prognostically relevant patient and histologic parameters in the multicenter ICB-treated UC cohort using univariate Cox regression models. Results for univariate Cox regression are summarized in Table 2. Of note, higher CPS (cutoff: ≥10) showed a trend ($P = 0.057$) toward a lower response rate

Table 1. Patients characteristics at baseline.

Characteristic	Patients (*N* [%], Median [IQR])
Total	107 (100%)
Age	67 (58–74)
Sex	
Female	25 (23%)
Male	82 (77%)
ECOG	
0	86 (80%)
≥1	21 (20%)
Checkpoint Inhibitor	
PD-1 Inhibitor (Nivolumab, Pembrolizumab)	79 (75%)
PD-L1 Inhibitor (Atezolizumab, Durvalumab)	26 (25%)
Unknown	2
Line of Therapy	
1st	34 (32%)
2nd	73 (68%)
Smoking Status	
Non-smoker	44 (54%)
Smoker	37 (46%)
Unknown	26
Histology	
Neuroendocrine	1 (0.9%)
Urothelial	106 (99%)
Histological Variant	
Squamous	25 (23%)
NOS	61 (57%)
Other	21 (20%)
Protein Subtype	
Basal	32 (30%)
Luminal	75 (70%)
***FGFR3* Alteration**	
Alterated	16 (15%)
Wild type	91 (85%)
PD-L1 IC	1 (0–5)
PD-L1 TPS	0 (0–12)
PD-L1 CPS	5 (0–40)
PD-L1 CPS	
CPS <10%	64 (60%)
CPS ≥10%	43 (40%)
sTILs	5 (2–20)
mPD-L1	9 (4–17)

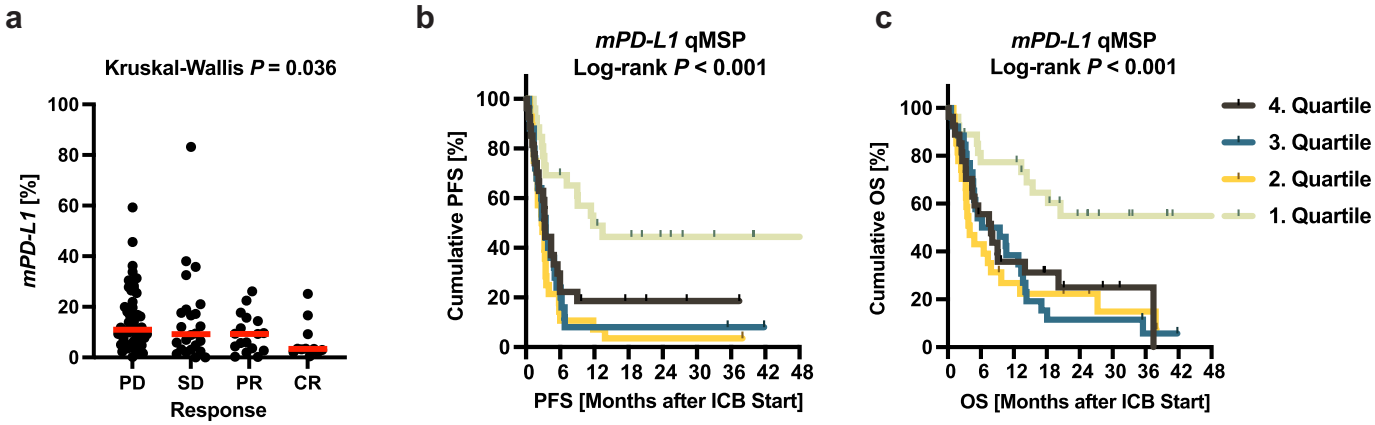

Figure 1. (a) Distribution of *mPD-L1* stratified by response at first staging according to RECIST (CR=complete response, PR=partial response, SD=stable disease, PD=progressive disease; Mann–Whitney *U* tests: PD vs. SD $P = 0.49$, PD vs. PR $P = 0.13$, PD vs. CR $P = 0.003$, SD vs. PR $P = 0.56$, SD vs. CR $P = 0.12$, PR vs. CR $P = 0.20$). Kaplan–Meier survival curves showing the progression-free (PFS, b) and overall survival (OS, c) after ICB initiation stratified according to *mPD-L1* status (quartiles).

(Supplemental Figure S1) and was negatively associated with ICB outcome, highlighting the limited robustness and the inconsistent value of this biomarker. In addition to the prognostic impact of *PD-L1* methylation status on PFS and OS, only ECOG ≥ 1 and the use of PD-L1 inhibitors (vs. PD-1 inhibitors) were significantly associated with unfavorable outcomes. These influencing factors were further examined using multivariable Cox regression analyses. Only ECOG ≥ 1 and *mPD-L1* ≥2nd quartile (second/third and fourth quartiles) remained as independent predictors of unfavorable OS, whereas for PFS only *mPD-L1* ≥2nd quartile remained an independent risk factor (Table 3).

We next analyzed whether immune infiltration (measured as sTILs) and expression of PD-L1 on immune and tumor cells

Table 2. Univariate Cox proportional hazards analyses of progression-free and overall survival. HR: hazard ratio, 95% CI: 95% confidence interval.

		PFS		OS	
Characteristics	*N*	HR [95%CI]	*P*-Value	HR [95%CI]	*P*-Value
Age	107				
Age ≤75 Years		—		—	
Age >75 Years		0.97 [0.58–1.61]	0.9	0.78 [0.43–1.39]	0.4
Sex	107				
Female		—		—	
Male		1.18 [0.72–1.95]	0.5	0.96 [0.58–1.60]	0.9
ECOG	107				
0		—		—	
≥1		1.35 [0.80–2.27]	0.3	1.80 [1.05–3.11]	0.034
Checkpoint Inhibitor	105				
PD-1 Inhibitor (Nivolumab, Pembrolizumab)		—		—	
PD-L1 Inhibitor (Atezolizumab, Durvalumab)		2.02 [1.26–3.23]	0.004	1.47 [0.90–2.40]	0.13
Line of Therapy	107				
1st		—		—	
2nd		1.41 [0.88–2.24]	0.15	1.32 [0.81–2.16]	0.3
Smoking Status	81				
Non-smoker		—		—	
Smoker		0.78 [0.48–1.27]	0.3	0.79 [0.48–1.31]	0.4
Histology Variant	107				
Squamous		—		—	
NOS		0.75 [0.45–1.25]	0.3	0.62 [0.36–1.06]	0.081
Other		0.92 [0.49–1.74]	0.8	1.05 [0.56–1.98]	0.9
Protein Subtype	107				
Basal		—		—	
Luminal		0.97 [0.62–1.53]	>0.9	0.73 [0.45–1.17]	0.2
***FGFR3* Alteration**	107				
Altered		—		—	
Wild Type		0.84 [0.47–1.52]	0.6	0.88 [0.47–1.62]	0.7
PD-L1 IC	107	1.00 [0.98–1.02]	0.8	1.00 [0.97–1.02]	>0.9
PD-L1 CPS	107				
CPS <10%		—		—	
CPS ≥10%		1.73 [1.13–2.65]	0.012	1.74 [1.10–2.73]	0.017
PD-L1 CPS	107	1.01 [1.00–1.02]	0.009	1.01 [1.00–1.02]	0.017
PD-L1 TPS	107	1.01 [1.00–1.02]	0.003	1.01 [1.00–1.02]	0.012
mPD-L1	107	1.01 [1.00–1.03]	0.079	1.01 [1.00–1.03]	0.072
***mPD-L1* Quartile**	107				
1st Quartile		—		—	
2nd–4th Quartile		2.93 [1.66–5.16]	<0.001	3.39 [1.78–6.45]	<0.001
sTILs	107	1.00 [0.99–1.01]	>0.9	1.00 [0.99–1.01]	0.4

Table 3. Multivariate Cox proportional hazards analyses of progression-free and overall survival. Included are *N* = 105 patients with complete data records. HR: hazard ratio, 95% CI: 95% confidence interval.

	PFS		OS	
Characteristic	HR [95%CI]	*P*-Value	HR [95%CI]	*P*-Value
ECOG				
0	—		—	
≥1	1.54 [0.90–2.63]	0.11	2.19 [1.24–3.87]	0.007
Checkpoint Inhibitor				
PD-1 Inhibitor (Nivolumab, Pembrolizumab)	—		—	
PD-L1 Inhibitor (Atezolizumab, Durvalumab)	1.56 [0.95–2.57]	0.080	1.14 [0.68–1.91]	0.6
PD-L1 CPS	0.99 [0.96–1.02]	0.6	0.99 [0.96–1.03]	0.7
PD-L1 CPS				
CPS <10%	—		—	
CPS ≥10%	1.47 [0.66–3.27]	0.3	1.59 [0.67–3.78]	0.3
PD-L1 TPS	1.02 [0.99–1.05]	0.2	1.01 [0.98–1.04]	0.5
***mPD-L1* Quartile**				
1st Quartile	—		—	
2nd–4th Quartile	2.86 [1.55–5.27]	<0.001	3.29 [1.68–6.44]	<0.001

associated with *mPD-L1*. By applying an unsupervised hierarchical cluster analysis of sTILs, PD-L1 expression on tumor cells (TPS/TC, %) and immune cells (IC, %), PD-L1 CPS, and continuous methylation of the *PD-L1* promoter, we identified four different tumor clusters (Figure 2a,b): "Cluster 1 (*mPD-L1* Intermediate, Inflamed, PD-L1 Tumor Cell (TC) Low)" with intermediate *mPD-L1* (IQR: 5.32–25.13%), high sTILs (IQR: 30–90%), low PD-L1 TC expression (IQR: 0–40%), and high PD-L1 immune cell expression (IQR: 20–35%); "Cluster 2 (*mPD-L1* Intermediate, PD-L1 TC High)" with intermediate *mPD-L1* (IQR: 4.19–16.34%), low to intermediate sTILs (IQR: 2–20%), high PD-L1 TC expression (IQR: 40–80%), and low PD-L1 immune cell expression (IQR: 0–10%); "Cluster 3 (*mPD-L1* Low, Uninflamed)" with low *mPD-L1* (IQR: 2.79–9.29%), low sTILs (IQR: 2–11%), mostly absent PD-

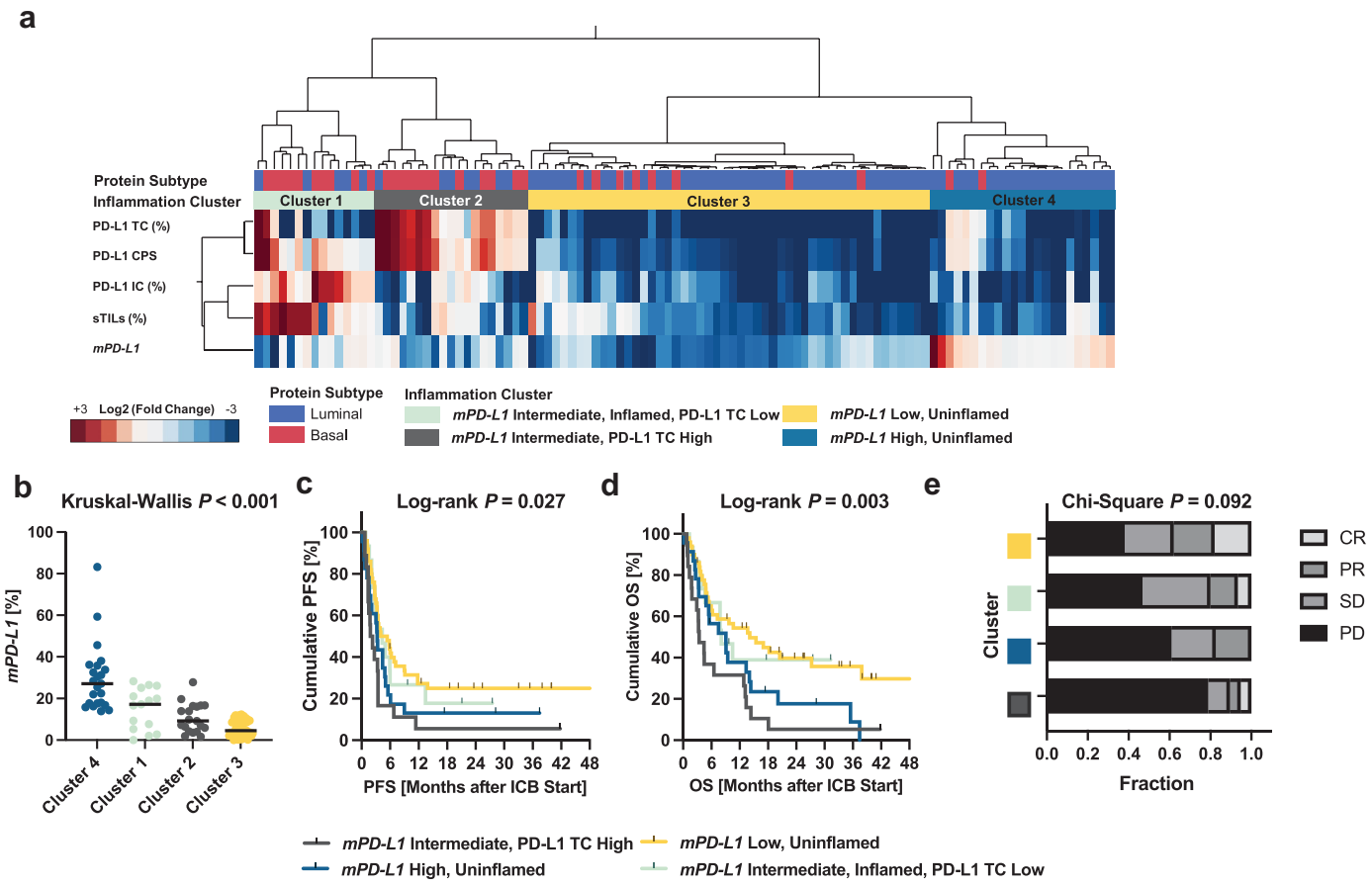

Figure 2. (a) Unsupervised hierarchical cluster analysis of PD-L1 expression on tumor cells (TPS, %) and immune cells (IC, %), PD-L1 combined positive score (CPS), overall immune infiltration (stromal tumor infiltrating lymphocytes, sTils, %), and *PD-L1* promoter methylation. (b) Distribution of continuous *mPD-L1* across different cluster groups (Mann–Whitney *U* tests: Cluster 4 vs. Cluster 1 $P = 0.004$, Cluster 4 vs. Cluster 2 $P < 0.001$, Cluster 4 vs. Cluster 3 $P < 0.001$ Cluster 1 vs. Cluster 2 $P = 0.14$, Cluster 1 vs. Cluster 3 $P = 0.002$, Cluster 2 vs. Cluster 3 $P = 0.007$). (c) Progression-free (PFS) and (d) overall (OS) survival analyses based on cluster group assignments. (e) Objective response rates (according to RECIST v1.1) based on cluster group assignments.

L1 TC expression (IQR: 0–0%), and low PD-L1 immune cell expression (IQR: 0–3%); "Cluster 4 (*mPD-L1* High, Uninflamed)" with high *mPD-L1* (IQR: 17.59–35.80%), low sTILs (IQR: 1–10%), low PD-L1 TC expression (IQR: 0–10%), and mostly absent PD-L1 immune cell expression (IQR: 0–1%).

Tumors with an uninflamed microenvironment and *mPD-L1* hypermethylation ("Cluster 4") showed worse outcomes (Figure 2c,d) and objective response rates (Figure 2e).

Interestingly, tumors within "Cluster 3 (*mPD-L1* Low, Uninflamed)" with a remarkable *mPD-L1* hypomethylation not only showed the most favorable outcome in terms of PFS and OS (Figure 2c,d) and highest objective response rates (Figure 2e), but also a mostly uninflamed immune phenotype indicating that *mPD-L1* assessment might be a suitable tool to identify ICB responders with low or absent immune infiltration and PD-L1 expression (uninflamed but ignitable tumors). On the other hand, low to intermediate *mPD-L1* levels together with substantial correlates of a preexisting antitumoral immune responses (high sTILs, high expression of PD-L1 on immune cells) found in "Cluster 1" also predicted favorable outcomes and objective response rates to immune checkpoint inhibition, while tumors with moderate–high *mPD-L1* levels and PD-L1 TC expression ("Cluster 2") showed poor outcomes and objective response rates toward ICB regardless of *mPD-L1* status (Figure 2c–e).

Pharmacological demethylation induces PD-L1 expression in urothelial cancer cells

To functionally substantiate our findings, we analyzed whether *PD-L1* promoter hypomethylation associates with functional susceptibility to immune responses. In cell cultures, pharmacological demethylation using the DNA methyltransferase (DNMT) inhibitor decitabine, particularly in combination with IFN-γ stimulation, resulted in profound membranous PD-L1 protein upregulation (Figure 3), especially being prominent in the lines RT4 and RT112, that showed no or very weak PD-L1 membranous expression at baseline. This finding indicates that *PD-L1* promoter hypomethylation associates with susceptibility to IFN-γ induced immune responses.

Discussion

The overriding goal in modern oncology is to tailor a therapy regimen adapted to the patient's individual tumor biology, independent of rigidly defined therapy lines.[31,32] This, however, can only be achieved with the integration of robust biomarkers that enable precise response prediction. In the present study, we comprehensively examined *PD-L1* promoter methylation status with regard to ICB response and clinical outcomes in a multicenter ICB-treated mUC cohort. *PD-L1* promoter

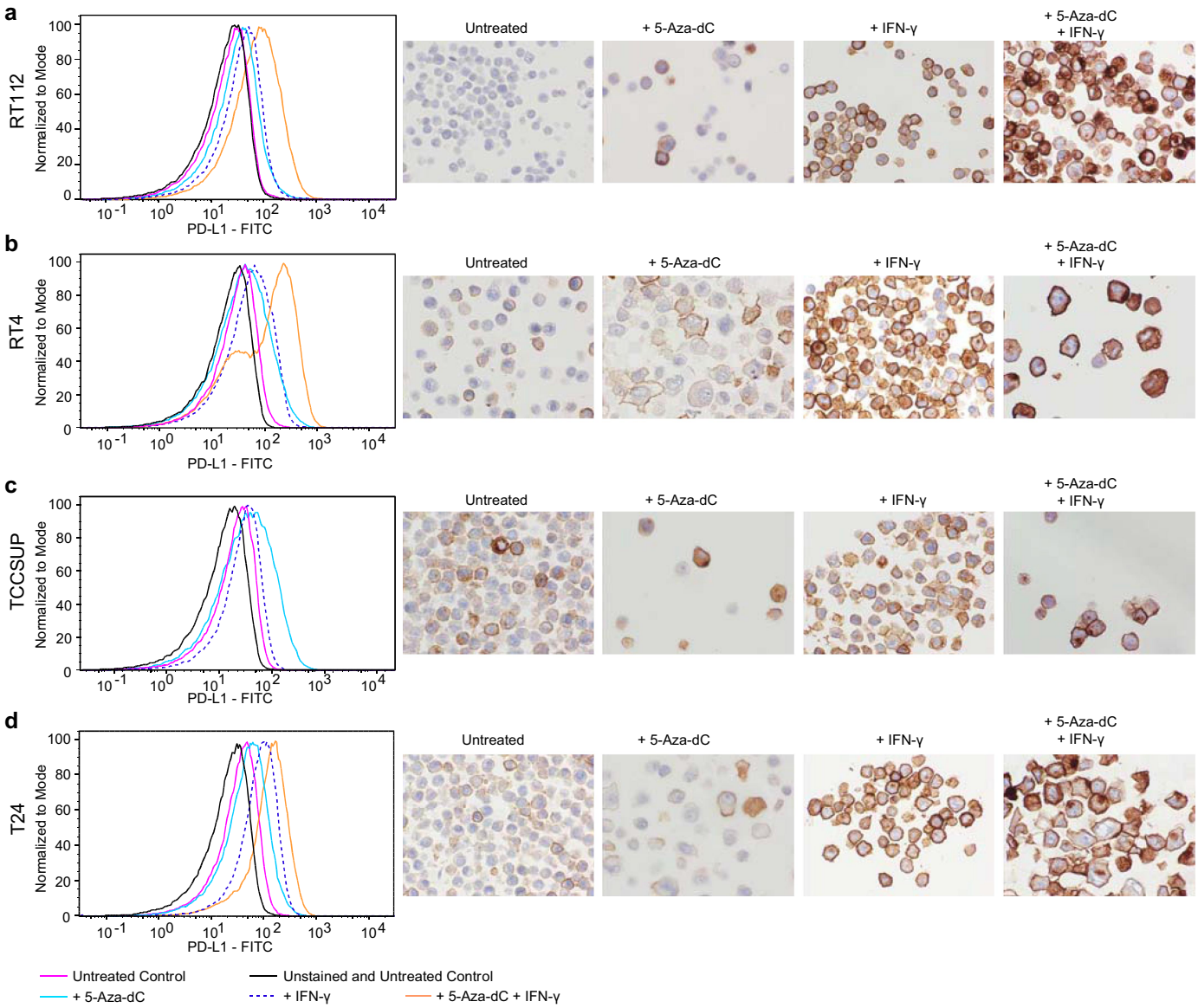

Figure 3. Pharmacological demethylation induces PD-L1 expression and enhances IFN-γ inducibility in urothelial cancer cells. Normalized histograms and PD-L1 IHC illustrate the induction of membranous PD-L1 expression in pharmacologically demethylated (5-aza-dC treated) urothelial cancer cell lines (a: RT112; b: RT4; c: TCCSUP; d: T24), with and without IFN-γ stimulation, compared to untreated.

methylation status was assessed in pre-treatment tissue samples using an established qMSP assay.[12,13] Patients with objective response and especially those with complete response to ICB showed significantly reduced *PD-L1* promoter methylation. Promising data for the methylation patterns of immune checkpoint genes have also been obtained for renal cancer and melanoma, where we recently demonstrated the predictive value of *CTLA4* promoter methylation in relation to ICB response.[19–21] When considering the clinical outcome on immunotherapy, a correlation between *mPD-L1* and survival was observed, and in particular, the quartile with the lowest *mPD-L1* methylation showed exceptional survival under immunotherapy. If these data were validated prospectively, it is reasonable to envision that this group of patients would benefit from ICB-based therapy in the first-line setting, regardless of the less robust and inconsistent PD-L1 protein expression status.

Early companion biomarker analyses from clinical trials indicated a predictive value of PD-L1 expression, ultimately leading to a restricted FDA approval in specific indications, i.e. pembrolizumab and atezolizumab treatment of cisplatin-ineligible patients with a CPS ≥ 10 and an IC of ≥5%, respectively.[1,33] However, responses were observed in all PD-L1 CPS or IC class categories and further biomarker analyses produced inconclusive results,[3,34] which is in line with our presented data. Moreover, our data from a heterogeneous cohort comprised of first-line and second-line atezolizumab and pembrolizumab treated patients with mUC rather point to a remarkably high response in a subset of patients with low PD-L1 expression. We recently described that a certain subset of urothelial cancers with constitutive PD-L1 TC associated with poor outcomes and aggressive disease behavior as well as immunotherapy resistance likely caused by their strong myeloid microenvironment.[23,35] An interesting finding in our present study is that we see that those tumors exhibiting strong PD-L1 TC expression and higher levels of *PD-L1* promoter methylation

seem to be resistant to immunotherapy, which is in line with our previous findings, while tumors with low *PD-L1* methylation levels and low expression of PD-L1 on TCs or/and substantial inflammation respond very well. Concordantly, we showed that our *mPD-L1* assay identifies a high number of therapy responders with the absence of inflammatory biomarkers such as PD-L1 or immune infiltration (ignitable cold tumors), but also tumors with marked inflammation and PD-L1 expression that were not susceptible to immunotherapy. Therefore, we hypothesized that *mPD-L1* is a correlate of susceptibility to immunotherapy, but independent of the current inflammatory tissue context. In line, we were able to demonstrate that the treatment of various urothelial cancer cell lines with a demethylating agent (decitabine) led to a marked sensitization of IFN-γ-induced PD-L1 upregulation on tumor cell membrane, especially in cell lines which showed negativity for PD-L1 baseline expression.

DNA methylation is cell type-specific and changes in DNA methylation are hallmarks of hematopoietic T cell maturation, frequently associated with open chromatin marks and enhancer elements.[36,37] Genome-wide analyses revealed significant dynamic methylation changes in the course of T cell activation and differentiation.[38] A comprehensive genome-wide analysis of the epigenetic landscape during $CD4^+$ T memory cell differentiation has been reported by Durek and colleagues.[39] Moreover, T cell exhaustion, a key factor governing response to ICB, is accompanied by profound epigenetic changes.[40–42] In our present study, we analyzed bulk tumor tissue which does not allow to attribute *mPD-L1* to specific cell types and particularly to specific immune cell subsets. Finally, we did not analyze *PD-L1* copy number variations which are prevalent in urothelial carcinomas[43] and might affect *PD-L1* promoter methylation. These limitations of our study warrant further investigation, e.g., employing methylation analyses of FACS (fluorescence-activated cell sorting) sorted cells and copy number analyses.

Overall, previously established predictive biomarkers, in particular PD-L1 IHC, failed to predict ICB response and were outperformed by *PD-L1* promoter hypomethylation in our mUC cohort.

Our study points toward *mPD-L1* as a promising biomarker for ICB response prediction in mUC. However, further, prospective elucidations are needed to evaluate the predictive potential of *mPD-L1* (alone or in conjunction with PD-L1 IHC) for rational treatment decisions in mUC.

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Disclosure statement

DD owns patents and patent applications on biomarker technologies and methylation of immune checkpoint genes as predictive and prognostic biomarkers (DE 10 2016 005 947.8, DE 10 2015 009 187.5, DE 10 2017 125 780.2, PCT/EP2016/001237). The patents are licensed to Qiagen GmbH (Hilden, Germany). DD is a consultant of Qiagen. The University Hospital Bonn (PI DD) received research funding from Qiagen. RMW is founder and CEO of STRATIFYER Molecular pathology. All other authors declare no conflicts of interest regarding the present study.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. https://www.sciencedirect.com/science/article/pii/S030228382102056X?via%3Dihub

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