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BRIEF REPORT

PD-L1 (*CD274*) promoter methylation predicts survival in colorectal cancer patients

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ABSTRACT

This study evaluates promoter methylation of the programmed cell death ligand 1 (PD-L1) as a biomarker in a cohort of 383 colorectal cancer patients. *PD-L1* methylation (m*PD-L1*) was inversely correlated with PD-L1 mRNA expression ($p = 0.001$) and was associated with significantly shorter overall survival (OS, $p = 0.003$) and recurrence-free survival (RFS, $p < 0.001$). In age-stratified multivariate Cox proportional hazards analyses including sex, tumor, nodal, distant metastasis categories, microsatellite instability (MSI)-status, and PD-L1 mRNA, m*PD-L1* is classified as an independent prognostic factor (OS: $p = 0.030$; RFS: $p < 0.001$). Further studies are needed to evaluate *PD-L1* methylation as a biomarker for response prediction of immunotherapies targeting the PD-1/PD-L1 axis.

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Colorectal cancer (CRC) is one of the leading causes of death in both men and women in the Western hemisphere. On the molecular level, CRC is a highly heterogeneous disease accumulating multiple driver mutations such as mutations within *KRAS, BRAF*, and mismatch repair (MMR) genes.[1] A subset of CRC is characterized by MMR-deficiency, leading to a phenotype with high microsatellite instability (MSI-H). Sessile serrated adenomas with intraepithelial neoplasia are considered precursors of sporadic MMR-deficient CRC. They are unique in that they present with *BRAF* mutations (c.1799T>A) and generalized CpG island methylation, which affect the mismatch repair gene *MLH1*, and thus result in MSI-H.[2] As a consequence, MSI-H CRCs exhibit an extraordinary mutational burden, harboring hundreds to thousands of mutations. Additionally, these tumors typically present with prominent lymphocytic infiltrates.

Various tumor entities with elevated immune response, including MSI-H CRC, have dense $CD8^{pos}$ T-cell infiltrates in common, which are responsible for a local production of interferon gamma (IFNγ).[3,4] IFNγ, in turn, provokes the adaptive upregulation of the programmed death ligand 1 (PD-L1, also known as B7-H1) on nearby tumor cells via NFκB,[5] thereby mediating a negative feedback mechanism that ultimately leads to T-cell exhaustion in tumor-infiltrating lymphocytes. Upon binding of PD-L1 to its programmed death receptor (PD-1) on T lymphocytes, it limits the activity of the T-cell receptor (TCR) and thereby abolishes cytolytic activity. So far, PD-L1 expression in CRC has not been fully addressed, and the function of PD-L1 in CRC remains largely unknown. However, a strong correlation between PD-L1 expression on tumor cells and discrepant clinical outcomes has been observed, and recent reports have shown that PD-L1 may correlate with prognosis in CRC patients.[6,7] Droeser *et al.*, for instance, detected strong PD-L1 expression in 36% (433/1,197) of MMR-proficient and 28% (62/223) of MMR-deficient CRC,[8] which was associated with improved survival in MMR-proficient CRC, possibly due to concomitant increase of $CD8^{pos}$ T-cells infiltration. In contrast, two previous studies reported PD-L1 expression in tumor cells to be an independent predictor of poor prognosis in CRC.[9,10]

Data on the epigenetic regulation of the PD-L1 encoding gene *CD274* are sparse. Pharmacologically induced gene methylation, however, has been shown to adjust PD-L1 expression in various malignancies.[11] In prostate cancer and acute myeloid leukemia cohorts analyzed by The Cancer Genome Atlas (TCGA), *CD274* promoter methylation (m*PD-L1*) correlates with gene expression and is associated with survival.[12,13] We therefore hypothesized that PD-L1 expression might be under direct epigenetic control in CRC as well and consequently might be of major importance for the stratification of patients potentially benefitting from immunotherapeutic PD-1/PD-L1 checkpoint inhibition.

The results shown here are entirely based upon gene methylation data and mRNA expression data generated by the TCGA Research Network (http://cancergenome.nih.gov/) using the Infinium HumanMethylation450 BeadChip (Illumina, Inc., San Diego, CA, USA) and gene expression RNAseq (polyA+ IlluminaHiSeq), respectively. Data from the TCGA Colon and Rectal Cancer (COADREAD) cohort were downloaded from the UCSC Xena browser (http://xena.ucsc.edu) and analyzed. The generation of data is described in detail on UCSC Xena webpage (https://genome-cancer.ucsc.edu/proj/site/composite/datapages/?cohort=TCGA%20Colon%20and%20Rectal%20Cancer%20

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(COADREAD)). For further analysis, two bead pairs (cg15837913 and cg19724470) targeting CpGs within the upstream CpG-island located in the *CD274* promoter were analyzed and mean averaged in order to achieve a stable signal.

Statistical analyses were performed using SPSS, version 23.0 (SPSS Inc., Chicago, IL). Statements regarding potential correlations of characteristics were made using the Spearman's correlation coefficient. Comparisons were performed using the Wilcoxon–Mann–Whitney *U* test and the Kruskal–Wallis test. Survival was defined as time to death (overall survival, OS) and time to recurrence (recurrence-free survival, RFS). Hazard ratios (HRs) were calculated using univariate and multivariate Cox proportional hazards models. These models incorporated age as a stratifying variable rather than as a covariate for two reasons: (1) age is known to affect the outcome of CRC patients. Our primary aim, however, was to obtain estimates of the effects of the other variables given in the equation. (2) Given the very large range of age (31–90 y) in this survival study and the fact that there is an inverse correlation between *CD274* methylation and age, it would seem a reasonable assumption that baseline hazard will vary with age. Specifying age strata allowed for different baseline hazards for each age

Table 1. *PD-L1* promotor methylation (m*PD-L1*, beta-values) and PD-L1 mRNA expression (normalized counts) in a clinico-pathological context. Bold numbers indicate statistically significant correlations with p-values less than 0.05.

	Patients	%	Median m*PD-L1*	*p*-value	Median PD-L1 mRNA	*p*-value
All patients	383	100	−0.371		4.109	
Missing data (methylation/mRNA expression)	11/11	2.9/2.9				
Sex						
Female	168	43.9	−0.372	0.67*	4.089	0.88*
Male	206	53.8	−0.370		4.135	
Unknown	9	2.3				
Follow-up						
Patients with follow-up: OS	364	95				
Events/deaths	70	18.3				
Patients with follow-up: RFS	313	82				
Events/recurrences	60	15.7				
Mean follow-up [mo]	24.5 [21.6–27.5]					
Median follow-up [mo]	13.7					
Range follow-up [mo]	0.1–142.3					
Age [y]						
Mean	64.4 [63.0–65.7]					
Median	66					
≤ 66 y	193	50.4	−0.365	**0.023***	3.924	0.066*
> 66 y	181	47.3	−0.380		4.544	
Unknown	9	2.3				
pT category						
1	10	2.6	−0.369	0.41**	3.722	0.81**
2	55	14.4	−0.389		4.358	
3	250	65.3	−0.371		4.135	
4	46	12	−0.354		4.043	
Unknown	22	5.8				
pN category						
0	197	51.4	−0.381	**<0.001***	4.367	0.15*
1	96	25.1	−0.357		4.022	
2	66	17.2	−0.350		3.983	
Unknown	24	6.3				
M category						
0	242	63.2	−0.375	**0.002***	4.313	**0.045***
1	48	12.5	−0.351		3.734	
Unknown	93	24.3				
Microsatellite instability						
MSS	258	67.4	−0.372	0.54ε	3.837	**<0.001**ε
MSI-L	65	17.0	−0.358		4.136	
MSI-H	55	14.4	−0.371		5.545	
Intermediate	3	0.8	−0.410		3.055	
Unknown	2	0.5				
BRAF						
Wildtype	32	8.4	−0.374	0.80*	2.918	**0.025***
Mutation	3	0.8	−0.383		4.347	
Unknown	348	90.9				
KRAS						
Wildtype	32	8.4	−0.353	0.38*	2.996	0.86*
Mutation	27	7.0	−0.357		3.261	
Unknown	324	84.6				
MLH1 expression by IHC						
Absent	58	15.1	−0.368	0.94*	3.810	**0.014***
Present	176	46.0	−0.370		4.482	
Unknown	149	38.9				

*Wilcoxon–Mann–Whitney *U* test.
**Kruskal–Wallis test.
IHC: immunohistochemistry; OS: overall survival; RFS: recurrence free survival.

group and thereby removed these issues. Age strata were introduced using median age (66 y). Follow-up regarding OS was available for 364 patients, 34 cases (8.9%) were censored before the earliest event in a stratum and were therefore omitted from Cox proportional hazard analysis. Strata showed 22 deaths (age $\leq$ 66 y) and 48 deaths (age > 66 y). Follow-up regarding RFS was accessible for 313 patients, thereof only 1 was censored before the earliest event in a stratum and the case was excluded from Cox proportional hazard analysis. Strata provided 30 recurrences for each age group. *p*-values less than 0.05 were considered statistically significant.

First, we investigated whether PD-L1 upregulation in CRC is regulated by promoter methylation. Gene expression data from 383 tumor samples were analyzed with respect to PD-L1 expression and mPD-L1. Median m*PD-L1* was −0.371 (range −0.455 to 0.144) among all patients enrolled in the study (Table 1). m*PD-L1* levels were slightly lower in patients with nodal and distant metastases. A strong inverse correlation was observed between m*PD-L1* and the patients' age ($r = -0.166$; $p = 0.001$). In addition, m*PD-L1* inversely correlated with PD-L1 mRNA expression ($r = -0.112$, $p = 0.031$), indicating that PD-L1 expression may be regulated by promoter methylation on a cellular level in CRC.

A strong association was observed between PD-L1 mRNA expression and MLH1 expression detected by immunohistochemistry, MSI status, and *BRAF*-mutation. m*PD-L1*, however, did not associate with MLH1 expression or MSI-status, as reported by the TCGA Research Network. Neither was m*PD-L1* related to *BRAF* or *KRAS* mutational status in the limited number of specimens evaluated (Table 1).

We further investigated whether aberrant m*PD-L1* is associated with adverse outcome in CRC patients. In addition, we tested whether OS and RFS were associated with differential PD-L1 expression. In univariate Cox proportional hazard analysis, increased m*PD-L1* was significantly associated with reduced OS and RFS (HR = 21.1 [95% CI: 2.92–152], $p = 0.003$ for OS; HR = 35.3 [95% CI: 5.05–247], $p < 0.001$ for RFS; Table 2). PD-L1 mRNA expression, however, was unrelated to patients' outcome. In age-stratified multivariate Cox proportional hazard analyses including tumor (pT), nodal (pN), distant metastasis (pM) categories as well as MSI-status, and PD-L1 mRNA, m*PD-L1* added significant prognostic information with regard to OS and RFS (HR = 17.7 [95% CI: 1.33–225], $p = 0.030$ for OS; HR = 84.7 [95% CI: 7.85–915], $p < 0.001$ for RFS; Table 2).

Of the 383 patients under investigation, 32 patients (8%) were treated with an additional pharmaceutical therapy, whereas 23 patients (6%) did not receive any further treatment. For the majority of patients (328/383, 86%), however, data on additional treatment were not available, and no details were obtainable for the few patients having undergone further therapy. Therefore, the influence of adjuvant therapies, in particular epigenetic drugs like 5-azacytidine or histone deacetylase (HDAC) inhibitors, which might potentially influence PD-L1 expression and/or methylation, could not be evaluated in the present study.

As another limitation of our study, data on PD-L1 protein expression were not available to us. Zhang *et al.*, however, have recently published a proteomic characterization of the cohort under investigation.[14] They demonstrated that protein abundance could not be reliably predicted from DNA- or RNA-level measurements. Although mRNA and protein levels were modestly correlated, over two-thirds of these correlations were not statistically significant. Thus, further studies are required to explore the correlation of PD-L1 protein expression, mRNA expression, and promoter methylation in CRC.

Immunotherapies targeting immune checkpoint molecules, especially PD-1/PD-L1, may foster innate immune responses antagonizing tumor growth.[15] T-cell suppression preventing excessive inflammatory response at the site of chronic inflammation depends on an intact PD-1/PD-L1 axis.[16] In the presence of activated T cells, in return, tumor cells upregulate PD-L1, the major mediator of immunosuppression, resulting in inhibition of T_{helper} cell response and "T-cell exhaustion" via the PD-1 pathway.[4] Targeting the immune system as novel therapeutic modality has proven efficacy in CRC. It has previously been shown that PD-1/PD-L1 immune checkpoint inhibition can be a promising therapeutic option for CRC patients.[17] Results from the phase II KEYNOTE-016 study showed that the monoclonal anti-PD-1 antibody pembrolizumab provided an objective response rate of 40% in patients

Table 2. Univariate and multivariate Cox proportional hazard analyses of overall survival and recurrence-free survival in patients with CRC. Bold numbers indicate statistically significant correlations with p-values less than 0.05.

	Overall survival					Recurrence-free survival				
	Univariate			Multivariate ($n = 256$)		Univariate			Multivariate ($n = 242$)	
Variable	*n*	*p*-value	Hazard Ratio [95% CI]	*p*-value	Hazard ratio [95% CI]	*n*	*p*-value	Hazard ratio [95% CI]	*p*-value	Hazard ratio [95% CI]
Stratifying by age $\leq$ 66 y vs. age > 66 y*										
Sex (women vs. men)	330	0.23	0.74 [0.45–1.21]	0.76	1.09 [0.62–1.93]	313	0.14	0.67 [0.39–1.14]	0.93	0.97 [0.50–1.89]
pT category	318	<**0.001**	3.96 [2.18–7.18]	**0.005**	3.07 [1.40–6.72]	301	<**0.001**	3.70 [2.01–6.82]	0.14	1.89 [0.81–4.40]
pN category	316	<**0.001**	1.79 [1.36–2.38]	0.34	1.23 [0.81–1.86]	300	<**0.001**	1.86 [1.34–2.57]	0.98	1.01 [0.61–1.65]
pM category	258	<**0.001**	5.40 [2.87–10.2]	**0.003**	2.96 [1.45–6.05]	243	**0.001**	3.76 [1.79–7.91]	**0.006**	3.35 [1.41–7.94]
MSI-H vs. non-MSI-H	329	0.37	0.85 [0.60–1.21]	0.76	0.93 [0.57–1.51]	312	0.36	0.83 [0.56–1.23]	0.69	0.90 [0.53–1.52]
m*PD-L1*	319	**0.003**	21.1 [2.92–152]	**0.030**	17.7 [1.33–225]	302	<**0.001**	35.3 [5.05–247]	<**0.001**	84.7 [7.85–915]
PD-L1 mRNA	330	0.44	0.94 [0.79–1.11]	0.89	0.98 [0.78–1.24]	313	0.35	0.91 [0.76–1.11]	0.66	1.06 [0.82–1.37]

*Stratifying by age $\leq$ 66 y vs. age > 66 y.

Overall survival: 13 events/127 censored (age $\leq$ 66 y), 38 events/78 censored (age > 66 y:); recurrence-free survival: 20 events/113 censored (age $\leq$ 66 y), 21 events/88 censored (age > 66 y).

with progressive MMR-deficient metastatic CRC vs. 0% in patients with MMR-proficient CRC.[18] Accordingly, a clinical evaluation of PD-L1/PD-1 blockage in CRC patients in an ongoing phase III clinical trial is currently evaluating the efficacy of pembrolizumab in MMR-deficient CRC patients (ClinicalTrials.gov Identifier: NCT02563002).

Further studies are needed to determine whether m*PD-L1* allows for survival prediction in CRC patients treated with PD-1/PD-L1 antagonists. The strong correlation of m*PD-L1* with PD-L1 mRNA expression and outcome in addition to the relevance of PD-L1/PD-1 axis as an immunotherapeutic target suggests that DNA methylation might be a predictive biomarker for respective immunotherapies. The epigenetic regulation of PD-L1 demonstrated in the present study further suggests that m*PD-L1* as prognostic and potential predictive biomarker needs to be evaluated in the context of epigenetic drugs, which could be suited to modulate the PD-L1 expression and thereby sensitize tumors to immunotherapy. As DNA methylation can be measured accurately and robustly in various sample types, including minute amounts of formalin-fixed and paraffin-embedded tissues, it is well suited for clinical routine diagnostics. From our point of view, *PD-L1* gene methylation needs to be considered as a companion biomarker for immunotherapies, and we would strongly recommend the integration of its analysis in ongoing clinical trials.

Disclosure of potential conflicts of interest

A patent on *CD274* methylation as a predictive and prognostic biomarker is pending (inventor: Dimo Dietrich). All other authors state no conflict of interest.

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