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. 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 MSI-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. 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<sup>+</sup> T-cell infiltrates in common, which are responsible for a local production of interferon gamma (IFNγ). IFNγ, in turn, provokes the adaptive upregulation of the programmed death ligand 1 (PD-L1) as well on nearby tumor cells via NFκB, 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. For instance, detected strong PD-L1 expression in 36% (433/1,197) of MMR-proficient and 28% (62/223) of MMR-deficient CRC, which was associated with improved survival in MMR-proficient CRC, possibly due to concomitant increase of CD8<sup>+</sup> 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.

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. In prostate cancer and acute myeloid leukemia cohorts analyzed by The Cancer Genome Atlas (TCGA), CD274 promoter methylation (mPD-L1) correlates with gene expression and is associated with survival. 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 benefiting 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+ Illumina HiSeq), 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%202017).
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 promoter methylation (mPD-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.

| Table 1. PD-L1 promoter methylation (mPD-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. |
|---|---|---|---|---|
| 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 | 0.54* | 3.837 | <0.001* |
| 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 | 4.462 |
| 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 ≤ 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 mPD-L1 was −0.371 (range −0.455 to 0.144) among all patients enrolled in the study (Table 1). mPD-L1 levels were slightly lower in patients with nodal and distant metastases. A strong inverse correlation was observed between mPD-L1 and the patients’ age (r = −0.166; p = 0.001). In addition, mPD-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. mPD-L1, however, did not associate with MLH1 expression or MSI-status, as reported by the TCGA Research Network. Neither was mPD-L1 related to BRAF or KRAS mutational status in the limited number of specimens evaluated (Table 1).

We further investigated whether aberrant mPD-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 mPD-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, mPD-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. 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. T-cell suppression preventing excessive inflammatory response at the site of chronic inflammation depends on an intact PD-1/PD-L1 axis. 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. 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. 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.

| Variable                  | Overall survival | Recurrence-free survival |
|---------------------------|------------------|--------------------------|
|                           | Univariate       | Multivariate (n = 256)   | Univariate       | Multivariate (n = 242)   |
|                           | 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] |
| 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 [0.50–1.98] |
| 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] |
| mPD-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 ≤ 66 y vs. age > 66 y.
Overall survival: 13 events/127 censored (age ≤ 66 y), 38 events/78 censored (age > 66 y); recurrence-free survival: 20 events/113 censored (age ≤ 66 y), 21 events/88 censored (age > 66 y).
with progressive MMR-deficient metastatic CRC vs. 0% in patients with MMR-proficient CRC. Accordingly, a clinical evaluation of PD-L1/PD-1 blockade 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 mPD-L1 allows for survival prediction in CRC patients treated with PD-1/PD-L1 antagonists. The strong correlation of mPD-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 mPD-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 conflicts of interest.

Funding

This study was funded by the University Hospital Bonn.

References

1. Funkhouser WK, Jr., Lubin IM, Monzon FA, Zehnbauer BA, Evans JP, Ogino S, Nowak JA. Relevance, pathogenesis, and testing algorithm for mismatch repair-defective colorectal carcinomas: a report of the association for molecular pathology. J Mol Diagn 2012; 14:91-103; PMID:22260991; http://dx.doi.org/10.1016/j.jmoldx.2011.11.001
2. Snover DC. Update on the serrated pathway to colorectal carcinoma. Hum Pathol 2011; 42:1-10; PMID:20869746; http://dx.doi.org/10.1016/j.humpath.2010.06.002
3. Wu X, Zhang H, Xing Q, Cui J, Li J, Li Y, Tan Y, Wang S. PD-1 (+) CD8(+) T cells are exhausted in tumours and functional in draining lymph nodes of colorectal cancer patients. Br J Cancer 2014; 111:1391-9; PMID:25093496; http://dx.doi.org/10.1038/bjc.2014.416
4. Topalian SL, Taube JM, Anders RA, Pardoll DM. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nat Rev Cancer 2016; 16:275-87; PMID:27079802; http://dx.doi.org/10.1038/nrc.2016.36
5. Chen J, Jiang CC, Jin L, Zhang XD. Regulation of PD-L1: a novel role of pro-survival signalling in cancer. Ann Oncol 2016; 27:409-16; PMID:26681673; http://dx.doi.org/10.1093/annonc/mdv615
6. Zhu H, Qin H, Huang Z, Li S, Zhu X, He J, Yang J, Yu X, Yi X. Clinical significance of programmed death ligand-1 (PD-L1) in colorectal serosal adenocarcinoma. Int J Clin Exp Pathol 2015; 8:9351-9; PMID:26464688
7. Song M, Chen D, Lu B, Wang C, Zhang J, Huang L, Wang X, Timmons CL, Hu J, Liu B et al. PTEN loss increases PD-L1 protein expression and affects the correlation between PD-L1 expression and clinical parameters in colorectal cancer. PLoS One 2013; 8:e65821; PMID:23785454; http://dx.doi.org/10.1371/journal.pone.0065821
8. Dreeser RA, Hirt C, Viehl CT, Frey DM, Nebiker C, Huber X, Zlobec J, Eppenberger-Castori S, Tzankov A, Rosso R et al. Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. Eur J Cancer 2013; 49:2233-42; PMID:23478000; http://dx.doi.org/10.1016/j.ejca.2013.02.015
9. Liang M, Li J, Wang D, Li S, Sun Y, Sun T, Zhang J, Chen X, Li Q, Sun S. T-cell infiltration and expressions of T lymphocyte co-inhibitory B7-H1 and B7-H4 molecules among colorectal cancer patients in northeast China’s Heilongjiang province. Tumour Biol 2014; 35:55-60; PMID:23873101; http://dx.doi.org/10.1007/s13277-013-1006-6
10. Shi SJ, Wang LJ, Wang GD, Guo ZY, Wei M, Meng XL, Yang AG, Wen WH. B7-H1 expression is associated with poor prognosis in colorectal carcinoma and regulates the proliferation and invasion of HCT116 colorectal cancer cells. PLoS One 2013; 8:e76012; PMID:24124529; http://dx.doi.org/10.1371/journal.pone.0076012
11. Yang H, Bueso-Ramos C, DiNardo C, Estescio MR, Davanlou M, Geng QR, Fang Z, Nguyen M, Pierce S, Wei Y et al. Expression of PD-L1, PD-L2, PD-1 and CTLA4 in myelodysplastic syndromes is enhanced by treatment with hypomethylating agents. Leukemia 2014; 28:1280-8; PMID:24270737; http://dx.doi.org/10.1038/leu.2013.355
12. Gevensleben H, Holmes EE, Goltz D, Dietrich J, Salier V, Ellinger J, Dietrich D, Kristiansen G PD-L1 promoter methylation is a prognostic biomarker for biochemical recurrence-free survival in prostate cancer patients following radical prostatectomy. Oncotarget 2016; [Epub ahead of print]; PMID:27835597; http://dx.doi.org/10.18632/oncotarget.13161
13. Goltz D, Gevensleben H, Grinen S, Dietrich J, Kristiansen G, Landsberg J, Dietrich D PD-L1 (CD274) promoter methylation predicts survival in patients with acute myeloid leukemia. Leukemia 2016; [Epub ahead of print]; PMID:27840427; http://dx.doi.org/10.1038/leu.2016.328
14. Zhang B, Wang J, Wang X, Zhu J, Liu Q, Shi Z, Chambers MC, Zimmerman IJ, Shaddock XF, Kim S et al. Proteogenomic characterization of human colorectal cancer. Nature 2014; 513:82-7; PMID:25043054; http://dx.doi.org/10.1038/nature13438
15. Austin R, Smyth MJ, Lane SW. Harnessing the immune system in acute myeloid leukaemia. Crit Rev Oncol Hematol 2016; 103:67-77; PMID:27247119; http://dx.doi.org/10.1016/j.critrevonc.2016.04.020
16. Zhou Q, Munger ME, Highfill SL, Tolar J, Weigel BJ, Riddle M, Sharpe AH, Valleria DA, Azuma M, Levine BL et al. Program death-1 signaling and regulatory T cells collaborate to resist the function of adoptively transferred cytotoxic T lymphocytes in advanced acute myeloid leukemia. Blood 2010; 116:2484-93; PMID:20570856; http://dx.doi.org/10.1182/blood-2010-03-275446
17. Le DT, Yoshino T, Jäger D, André T, Bendell JC, Wang R, Kang SMP, Kossihi Minori, Diaz LA The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD et al. KEYNOTE-164: Phase II study of pembrolizumab (MK-3475) for patients with previously treated, microsatellite instability-high advanced colorectal carcinoma. J Clin Oncol 2016; 34:suppl 4S; abstr TPS787
18. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 2015; 372:1259-20; PMID:26028255; http://dx.doi.org/10.1056/NEJMoai1500596