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CTLA4 methylation predicts response to anti–PD-1 and anti–CTLA-4 immunotherapy in melanoma patients

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Recent years have witnessed the groundbreaking success of immune checkpoint blockage (ICB) in metastasized malignant melanoma. However, biomarkers predicting the response to ICB are still urgently needed. In the present study, we investigated CTLA4 promoter methylation (mCTLA4) in 470 malignant melanoma patients from The Cancer Genome Atlas (non-ICB cohort) and in 50 individuals with metastasized malignant melanomas under PD-1/CTLA-4–targeted immunotherapy (ICB cohort). mCTLA4 levels were quantified using the Infinium HumanMethylation450 BeadChip (non-ICB cohort) and methylation-specific quantitative real-time PCR in DNA formalin-fixed and paraffin-embedded tissues (ICB cohort). Methylation levels were associated with molecular and clinicopathological variables and analyzed with respect to response (irRECIST) and overall survival. CTLA-4 mRNA and mCTLA4 showed a significant inverse correlation (non-ICB cohort: Spearman’s $\rho = -0.416$, $P < 0.001$). In ICB-treated melanoma patients, low mCTLA4 was further strongly correlated with response to therapy ($P = 0.009$, ANOVA) and overall survival (hazard ratio = 2.06 [95% CI: 1.29–3.29], $P = 0.003$). Our data strongly support the assumption that mCTLA4 predicts response to both anti–PD-1 and anti–CTLA-4 targeted ICB in melanoma and provides paramount information for the selection of patients likely to respond to ICB.

Introduction

Immune checkpoint blockage (ICB) targeting the cytotoxic T lymphocyte–associated protein 4 (CTLA-4) or the programmed cell death 1 (PD-1) pathway, either alone or in combination, has proven efficacy in the treatment of various malignancies. Patients with metastatic malignant melanoma show high response rates with long-lasting tumor control in a subgroup of patients (1–4). ICB as adjuvant treatment in high-risk melanoma has further led to prolonged survival (5–7). Despite its remarkable success, resistance to primary therapy limits the therapeutic efficacy of ICB treatment. Moreover, ICB causes immune-related side effects requiring long-term medical management. Robust predictive biomarkers predicting responsiveness to ICB are therefore of paramount importance.

Research on predictive biomarkers for response to immunotherapies currently focuses on the expression of immune checkpoints, particularly PD-L1, mutational load and genomic stability, and immune cell infiltrates. Microsatellite instability and mismatch repair deficiency, for instance, have received FDA approval as predictive biomarkers for anti–PD-1 immunotherapy with pembrolizumab in microsatellite instability–high (MSI-H) or mismatch repair–deficient (dMMR) pediatric and adult solid tumors. The current understanding of response to ICB suggests that patients with a preexisting antitumor T cell response benefit from ICB. PD-L1 expression on tumor cells is associated with tumor-infiltrating T cells and can be induced by interferon (IFN) response signatures (8, 9). Therefore, numerous studies have evaluated PD-L1 expression on melanoma cells by immunohistochemistry, supporting a role as predictive biomarker. However, PD-L1–negative melanomas have also been shown to achieve durable clinical benefit from ICB, indicating the limited usefulness of PD-L1 expression as a robust biomarker (reviewed in ref. 10). A meta-analysis by Khunger et al. (11) of 6,664 patients with various malignancies including melanomas revealed an overall poor clinical performance of PD-L1 expression as a predictive biomarker for PD-1–targeted immunotherapies.
In contrast to PD-1/PD-L1 expression, CTLA-4 expression on tumor cells has barely been investigated, and its role in melanomas remains unknown. In vitro, human melanoma cell lines show a high CTLA-4 expression regulated by the IFN-γ/JAK/STAT1–mediated signaling pathway (12). RNA sequencing (RNA-Seq) transcriptome data from 20 melanoma patients after anti–CTLA-4 immunotherapy revealed a correlation between an IFN-γ response signature (including CTLA-4) and a long-term benefit from anti–CTLA-4 treatment. The authors consequently supported the notion that CTLA-4 might serve as a potential biomarker for the response to anti–CTLA-4 immunotherapy (12). In melanoma cells, CTLA-4 has also been identified as a direct transcriptional target of the Wnt/β-catenin signaling pathway, and the activation of this pathway has been described as a tumor cell–intrinsic factor contributing to ICB resistance. Interestingly, β-catenin induces an increase in CTLA-4 mRNA and total CTLA-4 protein levels without concomitant changes in cell surface expression (13, 14). It has therefore been suggested that intracellular CTLA-4 might have a so far unknown function in melanoma cells. These data highlight the potential of CTLA-4 as a therapeutic target beyond its function in T cell biology. The fact that CTLA-4 expression in melanoma and immune cells is dynamically regulated in the tumor microenvironment hampers an accurate assessment. Additionally, tumor heterogeneity, sampling variability, and different assays and antibodies affect the accuracy of immune checkpoint expression analysis as potential biomarkers. In contrast to gene and protein expression, DNA methylation is a biologically and chemically more stable marker that can be accurately quantified, e.g., in formalin-fixed and paraffin-embedded (FFPE) tissues. Consequently, methylation serves as a biomarker for screening, diagnosis, prognosis, and prediction in various clinical settings (15–19). To better understand the regulation of CTLA-4 expression via promoter methylation in melanomas, we investigated mCTLA4 and CTLA-4 expression in a large cohort of 470 melanoma patients with detailed clinicopathological follow-up and molecular characterization provided by The Cancer Genome Atlas (TCGA) (20). The utility of mCTLA4 as predictive biomarker for response to ICB was furthermore investigated retrospectively in a cohort of 50 metastatic melanoma patients treated with anti–PD-1 or/and anti–CTLA-4 antibodies.

**Results**

**Association of CTLA4 methylation and mRNA expression (non-ICB cohort).** Since aberrant promoter methylation is frequently observed in cancer leading to the activation or repression of genes, we analyzed the correlation between CTLA-4 mRNA expression and mCTLA4 in 468 malignant melanoma samples from the TCGA Research Network. Methylation assessed by 2 beads (cg08460026 and cg05074138) from the Infinium HumanMethylation450 BeadChip located within the transcriptional start site of CTLA4 (Figure 1) was included in the analysis. Mean methylation of the analyzed loci showed a significant inverse correlation with CTLA-4 mRNA expression (Spearman’s $\rho = -0.416$, $P < 0.001$). Tumor content (percentage nuclei that are tumor cells) was 84.8% [95% CI: 83.2–86.3]. Since mean methylation in the cohort was 43.1% [95% CI: 41.5–44.7] (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.96793DS1), a subgroup of tumor cells are inevitably unmethylated. Since immune checkpoint genes are frequently coexpressed, we analyzed the correlation of CTLA-4 mRNA with PD-1 mRNA expression levels. Here, PD-1 and CTLA-4 mRNA were shown to be significantly coexpressed ($\rho = 0.579$, $P < 0.001$, $n = 468$).

**Association of CTLA4 methylation and mRNA expression with immune cell infiltrates and IFN-γ signature (non-ICB cohort).** Clinicopathological parameters (e.g., tumor thickness, ulceration, absence of tumor infiltrating lymphocytes) and molecular features like BRAF mutations are known prognostic factors in melanoma
patients. To identify established prognostic markers and additional parameters that correlate with CTLA-4 mRNA and mCTLA4, we performed a detailed analysis of clinicopathological parameters and their association with CTLA-4 mRNA expression and mCTLA4 (Supplemental Table 1). Of note, our analyses revealed significantly lower mCTLA4 levels in tumors with an activating BRAF mutation. As expected, increased CTLA-4 mRNA expression significantly correlated with lymphocyte scores (ρ = 0.328, P < 0.001), and CTLA-4 mRNA expression was higher in samples with low tumor cell purity (correlation with sample purity score: ρ = –0.479, P < 0.001). In concordance, CTLA-4 mRNA expression significantly correlated with RNA signatures of immune cells (B cells, CD4+ and CD8+ T cells, neutrophils, macrophages, and dendritic cells) as provided by Li et al. (21) (Supplemental Table 1). Accordingly, a significant inverse correlation was found between mCTLA4 and CD8+ T cell and dendritic cell RNA signatures.

Since the infiltration of CD8+ T cells is associated with the activation of the IFN pathways and IFN-γ/JAK/STAT1-mediated signaling pathways have been shown to induce CTLA-4 expression, we analyzed IFN-γ/JAK/STAT1 pathway–associated genes with respect to CTLA-4 mRNA expression. CTLA-4 mRNA showed a significant positive correlation with the mRNA of IFN-γ and the IFN-γ-regulated genes STAT1, STAT2, JAK2, and IRF9 (IFN-γ: Spearman’s ρ = 0.566, STAT1: ρ = 0.462, STAT2: ρ = 0.226, JAK2: ρ = 0.336, and IRF9: ρ = 0.352; all P < 0.001, n = 468). mCTLA4 correlated inversely with IFN-γ mRNA expression (ρ = –0.133, P = 0.004, n = 468).

**Figure 2. Kaplan-Meier analysis of overall survival in melanoma patients (non-ICB cohort) stratified according to CTLA4 methylation and mRNA expression.** Patient samples were dichotomized based on optimized cutoffs (43.27% methylation and 13.12 normalized mRNA counts [n.c.]). P values refer to log-rank test.

Prognostic value of CTLA4 methylation and mRNA expression (non-ICB cohort). As CTLA-4 expression so far has not been tested as a prognostic factor, we investigated the prognostic impact of mCTLA4 and CTLA-4 mRNA expression in the non-ICB melanoma cohort. For survival analyses, death was considered the endpoint, and overall survival was defined as the interval from primary diagnosis to death. In Cox proportional hazards analysis, log2-transformed CTLA-4 mRNA showed significant association with overall survival (hazard ratio [HR] = 0.92 [95% CI: 0.86–0.98], P = 0.009). For log2-transformed mCTLA4, no prognostic impact was shown (HR = 1.11 [95% CI: 0.86–1.44], P = 0.43). For Kaplan-Meier (KM) analysis, we further approximated optimized cutoffs for the categorization of patient samples as hyper- and hypomethylated. An optimized mCTLA4 cutoff (43.27%) allowed for the stratification of patients into long-term and short-term overall survivors (likelihood ratio [LH] = 7.76, P = 0.005 in KM [Figure 2A]; HR = 1.57 [95% CI: 1.14–2.16], P = 0.006 in Cox proportional hazards analysis). Analogously, optimizing the CTLA-4 mRNA cutoff (13.12 normalized counts) confirmed the prognostic value of CTLA-4 mRNA expression (LH = 14.58, P < 0.001 in KM [Figure 2B]; HR = 0.50 [95% CI: 0.34–0.72], P < 0.001). In concordance with the correlation of CTLA-4 and IFN-γ-associated gene expression and CD8+ T cell infiltrates, CTLA-4 mRNA expression was associated with a more favorable prognosis. However, the overall performance of mCTLA4 and CTLA-4 expression as a prognostic biomarker was rather weak, as indicated by relatively low HRs.
Association of CTLA4 methylation with response and survival in patients treated with anti–PD-1 and anti–CTLA-4 immunotherapy (ICB cohort). The predictive value of mCTLA4 for ICB was analyzed retrospectively in a cohort of 50 pretreatment melanoma samples. Mean mCTLA4 levels were slightly lower compared with the non-ICB cohort; however, variances may have resulted from different analytic techniques. CTLA-4 mRNA was omitted from further analysis since the FFPE tissue quality did not allow for quantitative mRNA analysis. For detailed clinicopathological parameters and mCTLA4 levels in the treatment cohort see Supplemental Table 2. We found significant differences in mean mCTLA4 levels in tumors from patients with progressive disease (PD), stable disease (SD), and response to therapy (partial response [PR]/complete response [CR]; \( P = 0.009 \), 1-way ANOVA; Figures 3 and 4A). One-way ANOVA with Bonferroni’s post hoc pairwise comparisons revealed a trend towards higher mCTLA4 in tumors from patients with PD compared with SD (mean difference \( \Delta \) = 21.2% ± 8.5%, \( P = 0.050 \)) and significant differences between PD and PR/CR (\( \Delta = 33.3\% ± 12.4\% \), \( P = 0.031 \)).

For survival analyses, death was considered the clinical endpoint, and overall survival was defined as the interval from primary dose of immune checkpoint inhibition to death. HR was first calculated using univariate Cox proportional hazards models based on log2-transformed mCTLA4 levels without the introduction of a cutoff in order to avoid an overfitted model. Elevated mCTLA4 levels were significantly associated with a higher risk of death (HR = 2.06 [95% CI: 1.29–3.29], \( P = 0.003 \)). We further performed KM with the log-rank test (LH). Patient samples were classified into mCTLA4_high and mCTLA4_low samples.
respectively, based on an optimized cutoff (13.98%). The prognostic value of categorized mCTLA4 showed significantly longer overall survival in patients with low promoter methylation (LH = 11.23, \( P = 0.001 \) in KM; Figure 4B). The Cox proportional hazards analysis revealed an HR = 6.16 [95% CI: 1.84–20.7] (\( P = 0.003 \)) for patients with high mCTLA4 levels.

Significantly lower mCTLA4 levels were found in lymph node metastases compared with cutaneous or subcutaneous metastases (\( \Delta = 35.7% \pm 9.8\% \), \( P = 0.004 \) by 1-way ANOVA and Bonferroni’s post hoc test; Supplemental Table 2). We performed survival subanalyses in these 2 sample groups in order to ensure that survival differences were associated with response to treatment and not type of metastasis. Analyzing mCTLA4 as continuous log2-transformed variable without dichotomization revealed a significantly worse survival in patients with higher methylation in both subcohorts with comparable HRs (lymph node metastases: \( n = 14 \), HR = 4.40 [95% CI: 1.06–18.33], \( P = 0.042 \); cutaneous or subcutaneous metastases: \( n = 17 \), HR = 4.37 [95% CI: 1.52–12.56], \( P = 0.006 \)).

**Discussion**

Biomarkers that predict response to ICB are urgently needed. In the present study, mCTLA4 was shown to be inversely correlated with CTLA-4 mRNA expression, suggesting an epigenetic regulation mechanism via...
DNA methylation in melanomas. Furthermore, increased m\textit{CTLA4} was a weak prognostic biomarker for poor overall survival in melanoma patients not treated with ICB, potentially indicating a higher ability of hypermethylated tumors for immune evasion. In patients under anti–PD-1 or combined anti–PD-1 and anti–CTLA-4 ICB, however, m\textit{CTLA4} strongly predicted patients’ survival and response to treatment. In accordance with our data, RNA-Seq transcriptome data of tumor tissue from 20 melanomas revealed that patients with a long-term survival benefit from anti–CTLA-4 treatment had significantly higher CTLA-4 expression levels compared with nonresponders (12). In this cohort, CTLA-4 expression was associated with the expression of IFN-γ target genes (e.g., \textit{STAT1} and \textit{IRF1}) and other immune checkpoints (e.g., PD-L1, TIM-3, and LAG-3). In the present study, we also identified an IFN-γ response gene signature associated with high levels of CD8+ T cell RNA signature and low m\textit{CTLA4} levels in the analyzed large non-ICB cohort. In both our study and the investigation conducted by Mo et al. (12), however, whole tumor tissue was used for analyses. It was therefore impossible to identify either melanoma cells or tumor-associated immune cells as the source of CTLA-4.

In the non-ICB cohort, on average 84.75% of nuclei were tumor nuclei while mean methylation was only 43.1%. Accordingly, one or both \textit{CTLA4} alleles are unmethylated in a significant number of melanoma cells. Several studies have reported the expression of CTLA-4 in melanoma cell lines and melanoma tissue of patients (12, 13, 22). Still, the effect of CTLA-4 expression in tumor cells remains barely understood. A potential functional role of CTLA-4 in melanoma cells might lie in the induction of caspase-dependent apoptosis, which is induced following the interaction with CD80 or CD86 recombinant ligands in vitro (22). CTLA-4 overexpression in melanocytes was discovered in the context of erythematous inflammatory response in UVB-irradiated mouse skin, suggesting a survival mechanism upon DNA damage (23). CTLA-4 expression by tumor cells may also represent a mechanism by which tumors evolve to an immune evasion phenotype and inhibit adaptive and innate immunity. Tumor cell–intrinsic activation of β-catenin signaling can induce CTLA-4 expression and has been associated with a non–T cell–inflamed microenvironment and resistance to ICB (24). A direct biological link between m\textit{CTLA4} and immune evasion would support the assumption that m\textit{CTLA4} represents a truly predictive biomarker instead of a treatment-unrelated prognostic biomarker.

Apart from CTLA-4 expression in melanoma cells, CTLA-4 expression on immune cells appears to be functionally relevant for the response to ICB. Daud and colleagues recently used a novel multiparameter flow cytometry assay for the characterization of tumor-infiltrating immune cells in freshly isolated melanomas (25). The authors identified a unique immune cell population of partially exhausted cytotoxic CD8+ T cells. CTLA-4 expression by tumor cells would support the assumption that m\textit{CTLA4} represents a truly predictive biomarker instead of a treatment-unrelated prognostic biomarker.

T cell exhaustion is accompanied by stable epigenetic changes that limit the capability for reinvigoration through ICB (27, 28). Epigenetic alterations, including DNA methylation changes, are a hallmark of T cell differentiation (29–31). De novo DNA methylation is involved in T cell exhaustion and limits ICB-mediated T cell rejuvenation (32). Consequently, DNA methylation might also be suitable as a quantitative surrogate biomarker for T cell exhaustion. Since CTLA-4 expression has been shown to correlate inversely with promoter methylation in various malignancies (33–35), CTLA4 hypomethylation in tumors might be a surrogate biomarker for the state of T cell exhaustion. Such a second mechanism might add to the ability of m\textit{CTLA4} to assess the amount of CTLA-4–expressing and ICB-responding tumor cells, thereby explaining its high performance as a predictive biomarker.

Of interest, our data revealed a significant association of m\textit{CTLA4} with \textit{BRAF} mutational status. This is consistent with the recently published observation that \textit{BRAFV600E} or \textit{NRAS}-mutant cell lines expressed CTLA-4 at much greater levels than wild-type cell lines.

Combination therapies of ICB and BRAF inhibition have already proven efficacy in murine models (36) and anti–PD-1 or anti–CTLA-4 ICB is currently being tested together with BRAF inhibitors in several clinical trials. A potential crosstalk between CTLA-4 and MAPK signaling advocates testing m\textit{CTLA4} as a predictive biomarker for BRAF/MEK inhibitors and ICB combination therapies in melanomas with \textit{BRAF} mutation.

DNA methylation can be robustly and accurately determined even in limited amounts of FFPE tissues (37–39) and therefore constitutes a prime method for the assessment of biomarkers in human cancers. Furthermore, this methodology opens the possibility to assess methylation and mutation biomarkers at the
same time. Most technologies applied to assess DNA methylation biomarkers depend on a previous deamination of DNA. Despite the reduced complexity of the DNA due to the conversion, DNA mutations can be detected. Even C-to-T transitions are preserved in either of the 2 DNA strands. Next-generation sequencing of bisulfite-converted DNA therefore might allow for the simultaneous testing of methylation and mutation biomarkers in the future. Additional genetic predictive biomarkers, i.e., tumor mutational burden, microsatellite instability (MSI), and mismatch repair (MMR) deficiency, could be multiplexed with methylation testing and tested in combination in various tumor entities. ICB is currently tested as combination therapy with PARP- and BRAF-/MEK-targeted therapies. Predictive biomarker tests for such combinatory therapies might include MAPK-pathway (RAS/RAF/MEK/ERK) and DNA repair enzyme (BRCA) mutation analyses together with CTLA4 methylation testing.

The present study is the first to our knowledge to demonstrate the predictive value of CTLA4 methylation in a treatment cohort of melanoma patients with long-term follow-up in response to immune checkpoint inhibition. Our study was conceptualized using a cohort of ICB-treated patients comprising anti–PD-1 monotherapy and sequential as well as combined anti–PD-1/anti–CTLA-4 ICB. Since the latter therapeutic regimes hold major effects on treatment responses, this inhomogeneity adds to the major weaknesses of this study. Methylation data, however, were obtained from pretreatment samples only, ensuring that treatment itself had no impact on mCTLA4 value. Further studies analyzing matched pretherapeutic samples and samples obtained from tumors progressing under immunotherapy from the same patients are required to show that increasing mCTLA4 associates with adaptive therapy resistance.

Coexpression of immune checkpoint molecules frequently occurs on cancer-specific T cells (reviewed in ref. 40). In the present study, coexpression of PD-1 and CTLA-4 was shown in the non-ICB cohort and might provide a rationale for the predictive value of mCTLA4 as a biomarker for single and combination therapies targeting PD-1 as well as CTLA-4. Consequently, we strongly recommend the prospective validation of mCTLA4 as a companion predictive biomarker for ICB in running clinical trials.

Methods
Patients (non-ICB cohort). A total of n = 470 primary solid and metastatic malignant melanoma tissue samples provided by the TCGA (TCGA SKCM cohort) were included. Solid normal tissues and additional metastatic tumor tissues were excluded. Only 1 sample per patient was analyzed, and metastatic tumor tissue samples were favored in cases with more than 1 sample. Clinicopathological data were obtained from the TCGA Research Network. Molecular data were adopted from a previously published study (20). Clinicopathological data and molecular data are summarized in Supplemental Table 1. Sample purity and ploidy estimates provided by the TCGA Research Network were calculated using the ABSOLUTE algorithm (41). Quantitative data on infiltrating lymphocytes (B cells, dendritic cells, neutrophils, macrophages, CD4+ and CD8+ T cells) were adopted from Li et al. (21) who used an RNA-Seq signature as surrogate for immune cell infiltrates.

Data on lymphocyte distribution (0–3; 0 = no lymphocytes within the tissue, 1 = lymphocytes present involving < 25% of the tissue cross-sectional area, 2 = lymphocytes present in 25% to 50% of the tissue, 3 = lymphocytes present in > 50% of tissue), lymphocyte density (0–3; 0 = absent, 1 = mild, 2 = moderate, 3 = severe), and lymphocyte (0–6, score defined as the sum of the lymphocyte distribution and density scores) were adopted from the TCGA Research Network (20).

Patients (ICB cohort). A total of 50 patients diagnosed with metastasized melanomas and treated with checkpoint inhibition at the University Hospital Bonn between October 2014 and April 2017 were included in the cohort. Pretreatment samples of 10 primary tumors, 17 cutaneous or subcutaneous metastases, 14 lymph node metastases, and 8 distant metastases were analyzed. Of the 50 melanoma patients included in the study, 17 patients harbored BRAF mutations, 32 individuals were BRAF wild type, and the mutational status of 1 patient was unknown. The majority of patients received therapeutic regimes with anti–PD-1 inhibition (Supplemental Table 2). In case of adverse effects, the regimen was switched to anti–CTLA-4 inhibition. One patient received ipilimumab as monotherapy. Response patterns were reported based on immune-related response criteria (irRECIST). Altogether, 27 patients (54%) suffered from PD, 17 patients presented with SD (34%), 5 patients had PR (10%), and 1 patient achieved CR under therapy (2%).

mRNA expression analysis (non-ICB cohort). mRNA data provided by the TCGA Research Network (http://cancergenome.nih.gov/) were generated by means of the Illumina HiSeq 2000 RNA Sequencing.
Version 2 analysis (Illumina, Inc.). Level 3 expression data were downloaded from the TCGA web page. Normalized counts per genes were calculated using the SeqWare framework via the RSEM algorithm (42). Data on mRNA expression were available from \( n = 468 \) samples.

**Methylation analysis (non-ICB cohort).** Gene methylation data generated by the TCGA Research Network were downloaded from the UCSC Xena browser (www.xena.ucsc.edu). TCGA methylation analysis was performed using the Infinium HumanMethylation450 BeadChip (Illumina, Inc.). Methylation levels (\( \beta \) values) were calculated as \( \beta = (\text{Intensity}_\text{Methylated})/(\text{Intensity}_\text{Methylated} + \text{Intensity}_\text{Unmethylated} + \alpha) \) (43). The constant offset \( \alpha \) was set to 0. The \( \beta \) values (values between 0 and 1) were multiplied with the factor 100\% in order to show percentage methylation (0 to 100\%). Two beads (cg08460026 and cg05074138) targeting loci within the transcriptional start site of \( CTLA4 \) (Figure 1) were investigated. Mean \( \beta \) values from both beads were computed for each patient sample. Methylation data were procurable from all \( n = 470 \) included patient samples.

**Methylation analysis (ICB cohort).** The InnuCONVERT Bisulfite All-In-One Kit (Analytik Jena) was used to prepare bisulfite DNA from FFPE tissues. \( m_{CTLA4} \) was quantified by means of a methylation-specific real-time \( m_{CTLA4}/ACTB \) duplex PCR (qMSP) assay (\( m_{CTLA4} \) oligonucleotides: 6-FAM-AAGTCGTTGGTATTGTTAC-BHQ-1, probe; GTTTTTTTTGTTTTGGTTTTACGA, reverse primer; TACTTTAATTACCTTTTCGACG, forward primer) under PCR buffer and cycling conditions as described previously (44).

**Statistics.** Statistical analyses were conducted using SPSS, version 23.0 (SPSS Inc.). Correlations were calculated using Spearman’s rank correlation (Spearman’s \( \rho \)). Mean value comparisons were performed with Wilcoxon-Mann-Whitney \( U \) (2 groups) or Kruskal-Wallis ( \( >2 \) groups) test. Multiple comparisons between groups were further tested with 1-way ANOVA and Bonferroni’s post hoc test. Survival analyses were performed using the KM method, likelihood ratios, and Cox proportional hazards regression. \( P \) values refer to log-rank and Wald tests. For KM analysis, methylation levels and mRNA expression levels were dichotomized based on an optimized cutoff. Cox proportional hazards analyses were performed with dichotomized (based on optimized cutoffs) and continuous methylation and mRNA expression data (log2-transformed). Two-sided \( P \) values < 0.05 were considered statistically significant.

**Study approval.** The study was approved by the Institutional Review Board at the University Hospital of Bonn (vote no. 187/16).

**Author contributions**
DG, HG, JL, and DD designed the study, analyzed data, and wrote the manuscript. TJV and JD analyzed and acquired data. CG conducted the experiments. FB, GK, and JL provided patient samples.

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