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ORIGINAL ARTICLE – CANCER RESEARCH

Predictive value of PD-L1 based on mRNA level in the treatment of stage IV melanoma with ipilimumab

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Abstract

Introduction PD-L1 is established as a predictive marker for therapy of non-small cell lung cancer with pembrolizumab. Furthermore, PD-L1 positive melanoma has shown more favorable outcomes when treated with anti-PD1 antibodies and dacarbazine compared to PD-L1 negative melanoma. However, the role of PD-L1 expression with regard to response to checkpoint inhibition with anti-CTLA-4 is not clear, yet. In addition, the lack of standardization in the immunohistochemical assessment of PD-L1 makes the comparison of results difficult. In this study, we investigated the PD-L1 gene expression with a new fully automated technique via RT-PCR and correlated the findings with the response to the anti-CTLA-4 antibody ipilimumab.

Materials and methods Within a retrospective multi-center trial, PD-L1 gene expression was evaluated in 78 melanoma patients in a total of 111 pre-treatment tumor samples from 6 skin cancer centers and analyzed with regard to response to ipilimumab. For meaningful statistical analysis, the cohort was enriched for responders with 30 responders and 48 non-responders. Gene expression was assessed by quantitative RT-PCR after extracting mRNA from formalin-fixed paraffin embedded tumor tissue and correlated with results from immunohistochemical (IHC) stainings.

Results and discussion The evaluation of PD-L1 expression based on mRNA level is feasible. Correlation between PD-L1 expression as assessed by IHC and RT-PCR showed varying levels of concordance depending on the antibody employed. RT-PCR should be further investigated to measure PD-L1 expression, since it is a semi-quantitative method with observer-independent evaluation. With this approach, there was no statistical significant difference in the PD-L1 expression between responders and non-responders to the therapy with ipilimumab. The evaluation of PD-L1 expression based on mRNA level is feasible. Correlation between PD-L1 expression as assessed by IHC and RT-PCR showed varying levels of concordance depending on the antibody employed. RT-PCR should be further investigated to measure PD-L1 expression, since it is a semi-quantitative method with observer-independent evaluation. With this approach, there was no statistical significant difference in the PD-L1 expression between responders and non-responders to the therapy with ipilimumab.

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Keywords PD-L1 · Ipilimumab · Predictive marker · Prognostic marker · Checkpoint inhibitors · Melanoma · Biomarker

Introduction

Checkpoint inhibiting antibodies targeting cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell-death protein 1 (PD-1) are a major breakthrough in cancer treatment (Ugurel et al. 2016). In advanced melanoma response rates for ipilimumab, an anti-CTLA-4 antibody, anti-PD-1 antibodies (e.g., pembrolizumab and nivolumab), and the combination of ipilimumab and nivolumab are 12–19, 33–44, and 55%, respectively (Robert et al. 2015; Larkin et al. 2015). In addition, efficacy for anti-PD1 antibodies has also been demonstrated in several other cancer entities such as non-small cell lung carcinoma (NSCLC) or renal cell carcinoma (Brahmer et al. 2012, 2015; Atkins et al. 2015; Garon et al. 2015; Winkler et al. 2016; Nghiem et al. 2016). However, only a subset of patients benefit from checkpoint inhibitor therapy and treatment induces considerable toxicity (Heinzerling and Goldinger 2017). To date, for melanoma patients, there are no known parameters that would allow the identification of potential responders or non-responders prior to therapy, and it is not possible to predict which patients will suffer from (severe) adverse events. To identify potential responders and avoid unnecessary morbidity due to side effects induced by the therapy, investigations for predictive biomarkers are ongoing (Rizvi et al. 2015; Patel and Kurzrock 2015; Spencer et al. 2016; Böger et al. 2016; Loo and Daud 2016). Mechanisms of resistance remain largely elusive, but several factors have been suggested to predict tumor remission induced by checkpoint inhibitors including presence of tumor-infiltrating lymphocytes, T cell receptor clonality, and mutational load leading to the formation of so-called neoantigens (Galon et al. 2012; Brahmer et al. 2012; Ji et al. 2012; Taube et al. 2014, 2015; Spranger et al. 2016). Lately, immune gene signatures have been characterized, which are associated with resistance (Zaretsky et al. 2016).

In patients with advanced non-small cell lung cancer (NSCLC) PD-L1 expression is a predictive marker for response to anti-PD-1 with an ORR to pembrolizumab of 45.5% in patients with high PD-L1 expression and 8.1% in patients with low expression that even lead to a restricted approval in PD-L1 positive NSCLC patients (Garon et al. 2015). In melanoma, patients with tumors expressing PD-L1 were more likely to respond to anti-PD-1 antibodies than patients with PD-L1 negative tumors (Brahmer et al. 2012; Taube et al. 2014). However, in melanoma, it is rather prognostic than predictive, as PD-L1 positive patients also show a better survival when treated with dacarbazine (Robert et al. 2014; Garon et al. 2015; Larkin et al. 2015). Furthermore, in melanoma, PD-L1 expression is low, difficult to evaluate, and heterogeneous within the tumor (Taube et al. 2012) across tumor sites and over time (Madore et al. 2015). Immunohistochemical evaluation of PD-L1 expression in tumor and tumor margin as a potential biomarker to predict the outcome of checkpoint inhibition with anti-PD-1 antibodies has been included in various studies (Gandini et al. 2016). However, lack of standardization with different antibodies (22C3 for pembrolizumab studies and 28-8 for nivolumab studies), different thresholds [1 and 50% positive tumor cells (Gadiot et al. 2011; Taube et al. 2012; Patel and Kurzrock 2015)], and suboptimal negative predictive value results in poor reliability of PD-L1 assessment by immunohistochemistry for anti-PD1 treatment in melanoma (Gibney et al. 2016). For ipilimumab, it is unclear whether PD-L1 positivity is predictive for response. Similar to PD-1, CTLA-4 is an inhibitory receptor expressed on T lymphocytes that upon binding of PD-L1 down-regulates pathways of T cell activation (Curran et al. 2010; Bour-Jordan et al. 2011; Flies et al. 2011). In the Checkmate-067 study, response rates and progression free survival in patients expressing PD-L1 $\geq$5 or <5% are not conclusive for the prediction of benefit from ipilimumab (Larkin et al. 2015). While the subgroup of patients with PD-L1-negative tumors (<5% IHC positive cells) showed a greater survival benefit when receiving the combination of nivolumab and ipilimumab compared to nivolumab, only the subgroup of patients with PD-L1-positive tumors demonstrated similar prolongation of PFS for combined therapy and monotherapy with nivolumab (Larkin et al. 2015).

To date, immunohistochemistry is used to detect PD-L1 protein expression. However, results of immunohistochemistry are contradicting with different antibodies reporting PD-L1 expression as an independent poor prognostic factor in one study (Hino et al. 2010) and associated with a better survival in another (Gadiot et al. 2011). Harmonization approaches are under way. To overcome these difficulties, we investigated the assessment of PD-L1 expression at the mRNA level using a fully automated technique for RNA extraction from formalin-fixed paraffin embedded (FFPE) tissue and subsequent quantitative real-time PCR (qRT-PCR). Our study investigated whether PD-L1 gene expression in pre-treatment tumor tissue was correlated with the response to ipilimumab in patients with metastatic melanoma.

Materials and methods

Tumor specimens

Archives in the University Hospitals Erlangen, Tübingen, Zurich, Kiel, Essen, and Regensburg were analyzed for

pre-treatment tumor samples from patients with metastatic stage IV melanoma who had received ipilimumab according to study protocol or to the prescription guidelines with predominantly four doses of 3 mg/kg IV every 3 weeks (Table S1). For the investigation of PD-L1 as a predictive biomarker for response to ipilimumab, exclusively biopsies performed before the first administration of ipilimumab were analyzed. All samples were resected metastases (including cutaneous, subcutaneous, lymph node, visceral, and brain metastases) or primary tumors. No cytology specimens or core biopsies were included. All specimens were reviewed histologically for tumor content and immunohistochemical stained for different melanoma markers including HMB45, S100, SOX10, Melan A, and Mage A3. In addition, tumor content was quantitatively assessed. For analyses, only slides with at least 5% tumor content were included. FFPE tissue blocks were cut according to a standard operating procedure to obtain RNAse free tissue sections. As controls, also 19 samples from healthy skin were investigated for PD-L1 gene expression.

mRNA extraction and gene expression analysis

mRNA was extracted from 2 to 3 5 µm sections of FFPE tissue blocks. The extraction was performed by a fully automated method using the VERSANT® Tissue Preparation Reagents Kit on Tissue Preparation System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) using silica-coated iron oxide beads, as previously described (Ostalecki et al. 2013; Naschberger et al. 2016). Subsequent qRT-PCR was performed on an ABI PRISM 7900HT (Applied Biosystems/Life Technologies, Carlsbad, California USA) as previously described (Bohmann et al. 2009). All PCR assays were performed in triplicates and their mean was used for evaluation. In case of deviation of more than two CT (Cycle Threshold) values in-between triplicates, analyses were repeated.

The absence of residual DNA amounts in the undiluted RNA extractions was controlled by a progestagen-associated endometrial protein (PAEP) gene-specific quantitative PCR without the preceding reverse transcription step using the reagents from the SuperScript® III Platinum® One-Step qRT-PCR kit with ROX and Platinum Taq DNA polymerase (Life Technologies).

For RNA normalization, different housekeeping genes were compared. Since RPL37A is best established with the VERSANT Tissue Preparation System and performed very similar to GAPDH and RN18S1, normalization was performed with RPL37A (Tramm et al. 2013). For the analyses, the RNAs were diluted to an RPL37A-CT value of 24. Relative expression of PD-L1 was determined as the change in the quantification cycle, $\Delta Cq = 40 - (CqPD\text{-}L1 - CqRPL37A)$ as performed by Bohmann et al. (2009).

Gene expression was assessed for each tumor sample using a commercially available specific primer set for PD-L1 (Context Sequence: CAAAGAAGCAAAGTGATACACATTT Applied Biosystems/Life Technologies). Specimens, with non-detectable PD-L1 or undetermined values, are represented by a CT value of 39.99. Positive and negative controls were used in each PCR plate (384-well plates, Applied Biosystems/Life Technologies). Nuclease-free water was used as no template control. Non-stimulated human umbilical vein endothelial cells, which do not express PD-L1, served as additional negative control (Mazanet and Hughes 2002; Rodig et al. 2003; Pittet et al. 2011). mRNA isolated from peripheral blood mononuclear cells (PBMC) and dendritic cells (DC) was used as positive controls. The control PCR was performed with the same master mix, as applied for the tumor measurement. To evaluate the primer performance, efficacy was controlled with a dilution series of mRNA isolated from PBMCs. The initial concentration of 5 ng per well was diluted in six steps to a ratio of 1:1024 (final concentration of 0.0005 ng per well). Efficacy of the qRT-PCR was calculated with the slope of the standard curve (Bustin et al. 2009). The slope of the standard curve was −3.43; therefore, the efficacy of the qRT-PCR was 97.9%.

The measured data were analyzed by the ABI Prism SDS 2.1 Software (Applied Biosystems/Life Technologies) according to the manufacturer's instructions.

Immunohistochemical staining

Immunohistochemical analysis of 38 cases was performed assessing PD-L1 expression. For 13 slides, IHC was performed in Princeton, NJ, US, with a commercially non-available rabbit antibody from BMS (clone 28-8). For 25 slides, IHC was performed in Erlangen with the commercially available CD274 antibody GTX117446 (GeneTex Inc., Irvine, CA, USA). IHC was performed according to the standard protocols. Specimen with >5% membranous staining was categorized PD-L1-"positive".

Data analyses

The mean normalized CT value of PD-L1 expression in all tumor specimens was applied as threshold for classification between PD-L1 high and PD-L1 low. In addition, patients were classified into responders and non-responders according to their clinical response to ipilimumab. Patients with complete response (CR), partial response (PR), mixed response (MR), and stable disease (SD) were considered responders, while patients with progressive disease (PD) were included in the non-responder group. To qualify as responder, patients had to demonstrate stable disease (SD)

for at least 3 months. All patients were staged radiologically via RECIST/immune-related (IR) response criteria.

The preparation of tissue sections, extraction of mRNA, performance of qRT-PCR, and the analyses of the acquired data were done in Erlangen. The study was approved by the local ethics committee of the University Erlangen.

The difference of PD-L1 gene expression was assessed between responders and non-responders and also between tumor specimens and healthy skin. To analyze the statistical significance the two-sample *t* test was applied and carried out with IBM SPSS® (Version 20).

Results

In this multi-center retrospective case–control study, 111 pre-treatment melanoma tumor specimens from 78 patients who were subsequently treated with ipilimumab were included in the study. Patient characteristics are shown in Table 1. Patients had been heavily pretreated before undergoing therapy with ipilimumab with more than 48% receiving at least three prior treatment regimens (Figure S1). More than 61% of the patients received further treatment after therapy with ipilimumab. This percentage did not differ between responders and non-responders.

In total, 47 samples of 30 responders and 64 samples of non-responders were analyzed for PD-L1 expression. Site of tumor probes is given in Table 1. A methodology using formalin-fixed paraffin embedded tissue sections was established (see "Materials and methods").

To analyze the role of PD-L1 with regard to response to ipilimumab, all 111 tumor samples were analyzed for PD-L1 gene expression and showed a mean normalized CT value of 29.21 (95% CI 28.89–29.53). As a next step, this mean was applied as threshold for the classification of tumor specimen as PD-L1 high or PD-L1 low with 53 ($n = 59$) and 47% of the tumor specimens ($n = 52$) in each group, respectively. The mean normalized CT value of PD-L1 gene expression in the 19 control tissue samples from healthy patients was 28.34 (95% CI 27.70–28.98). Thus, PD-L1 expression of regular skin was significantly lower than PD-L1 expression in melanoma tissue ($p = 0.016$; Fig. 1).

Findings from immunohistochemistry were correlated with RT-PCR expression data measuring PD-L1 expression in a random subset of 38 cases. Slides were stained with two different anti-PD-L1 antibodies, the BMS/DAKO antibody clone 28-8 in Princeton, and the commercially available CD274 antibody (GeneTex Inc. GTX117446) in Erlangen (Figure S2). Immunohistochemical findings were consistent with RT-PCR in 36% (4/11 cases) with two specimen which were not evaluable due to high melanin content for the BMS/DAKO staining. For the commercially available CD274 antibody (GeneTex Inc. GTX117446), 80% of cases (20/25) showed consistent results (Figure S3).

Table 1 Clinical characteristics of ipilimumab treated patients

	Responder	Non-responder
Number of patients	30 (39%)	48 (61%)
Number of samples	47 (42%)	64 (58%)
Clinical response to ipilimumab		
CR	2 (3%)	0
PR	13 (17%)	0
SD	10 (13%)	0
MR	5 (6%)	0
PD		48 (61%)
Sex		
Male	17 (22%)	32 (41%)
Female	13 (17%)	16 (20%)
Age: Mean (years)	61 (42–82)	62 (30–86)
Origin of tissue sample		
Primary tumor	2	1
Cutaneous metastasis	17	18
Subcutaneous metastasis	3	8
Lymph node metastasis	7	20
Visceral metastasis	7	13
Brain metastasis	2	1
Other origin	9	3

Subsequently, differential PD-L1 gene expression of tumor samples of responders and non-responders to ipilimumab was assessed to investigate its role as predictive marker. There was no statistically significant difference between the two cohorts ($p = 0.688$; Fig. 2). Since lymph node metastases could potentially bias results due to inflammatory cells expressing PD-L1, a subset analysis was performed excluding all lymph node metastases. With this aim, 84 tumor probes of 66 patients were re-assessed to omit potential bias. Again, no significant difference in gene expression of PD-L1 between ipilimumab responders and non-responders could be seen ($p = 0.199$).

Discussion

This study established an alternative, quantitative method to determine PD-L1 using automated extraction of mRNA from FFPE tissue and subsequent RT-PCR. Since FFPE material is available for most melanoma patients, this technique allows standardized, reproducible testing in research and clinical routine (Bohmann et al. 2009; Müller et al. 2011; Ostalecki et al. 2013). RPL37A is described as a reliable reference gene for melanoma samples using FFPE material in the literature and served as a stable housekeeping gene (Pfister et al. 2011; Tramm et al. 2013).

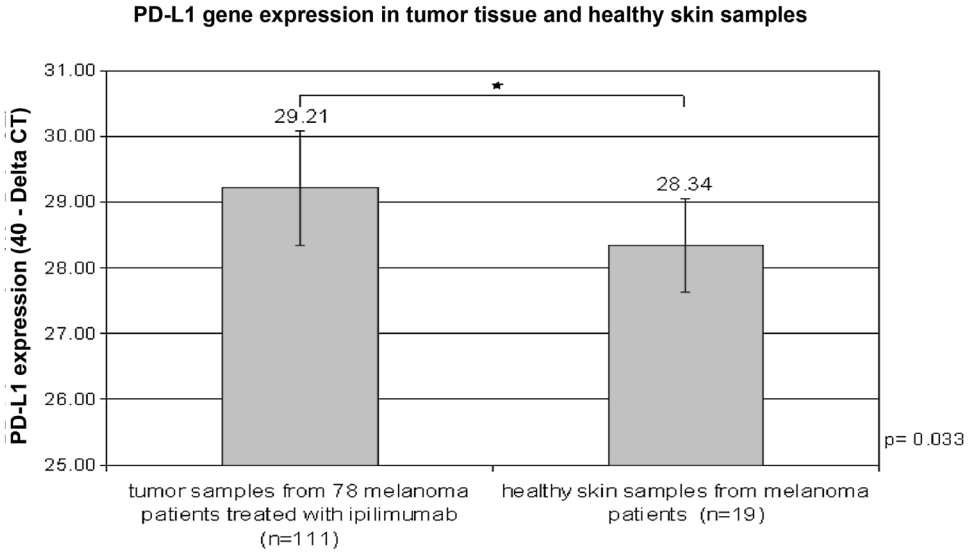

Fig. 1 Expression of PD-L1 in tumor samples from melanoma patients and healthy skin samples is shown. PD-L1 expression is significantly lower in healthy skin samples than in tumor samples. *Y error bars* indicate standard deviation; *indicates statistical difference at $p < 0.05$

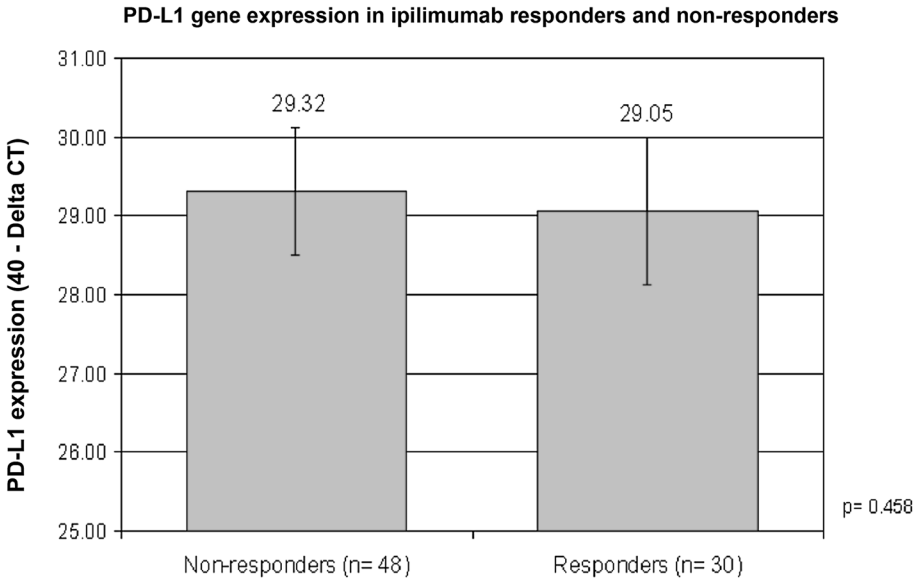

Fig. 2 Expression of PD-L1 in tumor samples from ipilimumab responders and non-responders. Patients were classified according to their clinical response to ipilimumab. No statistically significant difference between the two cohorts was observed. *Y error bars* indicate standard deviation

The importance of establishing new methods to evaluate the PD-L1 status arises from the lack of standardization in the immunohistochemical staining, which may account for the discordant results. Previous studies showed that valid and reproducible measurement of PD-L1 protein expression by immunohistochemistry in melanoma can be difficult, since positive staining is scarce, there are no clearly defined thresholds for positivity, and several commercial antibodies show lack of specificity (Patel and Woodman 2011; Gadiot et al. 2011; Taube et al. 2012; Chen et al. 2013). This has recently led to the blueprint initiative an attempt to find a common approach for the assessment of PD-L1 expression. In the nivolumab/ipilimumab combination trial (NCT01024231) with a threshold of 5% positive tumor cells, around 25% of patients were classified as PD-L1 positive (Larkin et al. 2015), whereas in the study comparing pembrolizumab with ipilimumab (NCT01866319), 80% of patients were PD-L1 positive at a threshold of 1% tumor cells (Robert et al. 2014). Dealing with a similar patient population, this illustrates the need for standardization. In addition, in melanoma, high melanin content makes the assessment of to assess slides difficult. Another potential issue with immunohistochemistry could be protein degradation which could lead to false-negative results. Even though Taube et al. could not show a significant relationship between specimen age and tumor PD-L1 expression in their study using IHC technique (Taube et al. 2014) in our hands, older samples very rarely are positive for PD-L1 (data not shown). However, PD-L1 expression is regulated by various factors including interferon-gamma (Spranger et al. 2013; Gowrishankar et al. 2015; Taube et al. 2015; Chen et al. 2016) and can thus show variation

over time with, e.g., discordant results in about half of longitudinally taken tumor samples of patients (Madore et al. 2015). For this reason, also new methods in the assessment of PD-L1 status may lead to discordant results.

In addition, we could not prove a predictive value of PD-L1 in stage IV melanoma in regard of the response to ipilimumab. With response rates of 10–15% (Wolchok et al. 2010; Hodi et al. 2010; Hamid et al. 2011; Robert et al. 2011) and long-term responses (Korn et al. 2008; Prieto et al. 2012; Wolchok et al. 2013), ipilimumab was the first drug to increase overall survival of metastatic melanoma. However, even though only a subgroup benefits, the majority of patients suffers from drug-related adverse effects and the treatment imposes significant costs (Voskens et al. 2012, 2013). Therefore, the identification of biomarkers could prevent unneeded exposure to a potentially harmful agent, select patients who will more likely respond to the treatment and reduce costs. Single reports exist on predictive markers such as forkhead box P3 and indolamin-2,3-dioxygenase (IDO), a post-treatment increase in tumor-infiltrating lymphocytes (Hamid et al. 2011) and expression of immune-related genes such as Th1 cytokines and chemokines (CCL4, CCL5, CXCL9, CXCL10, and CXCL11), marker for CD8+ cytotoxic T cells (CD8A and cytolytic molecules: Granzyme B and Perforin1), and other immune-related genes such as IDO1 (the gene encoding for IDO; [3]). Recent research showed the association of clinical outcome following ipilimumab treatment in patients with metastatic melanoma and peripheral blood biomarkers. Keldermann et al. demonstrated that low LDH correlated with favorable outcome following ipilimumab (Kelderman et al. 2014). Patients with a neutrophil-to-lymphocyte ratio <5 showed improved overall survival (Kelderman et al. 2014; Ferrucci et al. 2016) as well as patients with an elevated absolute eosinophil count (Delyon et al. 2013). Nevertheless, so far, no standard for pre-therapeutic testing could be established (Singh and Salama 2016; Zhu et al. 2016). Studies on the tumor microenvironment in melanoma show that a subset of patients shows CD8+ T cells in melanoma tumors without them being immunologically rejected, since they also show high expression of PD-L1, indoleamine-2,3-dioxygenase (IDO) and regulatory T cells (Treg). Mice experiments suggest that PD-L1 (similar to IDO and Treg) might follow CD8+ T cell infiltration as an intrinsic negative feedback loop mediated by interferon-γ (Spranger et al. 2013). Thus, cancer therapy targeting negative regulatory immune checkpoints might be preferentially beneficial in patients with a T-cell rich tumor microenvironment (Spranger et al. 2013).

Our data show that pre-treatment PD-L1 gene expression does not represent a biomarker for response to ipilimumab, since no significant difference in PD-L1 expression between responders and non-responders to ipilimumab was detected. Studies to fully comprehend the role of PD-L1 as predictive for anti-PD1/PD-L1 therapy and/or prognostic marker are ongoing. For example, various levels of expression can be found in different melanoma metastases originating from the same patient (Madore et al. 2015). Furthermore, additional variables, such as the presence of pre-existing intratumoral CD8+ T cells and tumor mutational load, may be important components to assess the potential for anti-PD-1 therapies (Tumeh et al. 2014; Rizvi et al. 2015).

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Compliance with ethical standards

Ethical approval The study was approved by the local ethics committee of the University Erlangen.

Conflict of interest BW received research founding and honoraria from Bristol-Myers Squibb (BMS). SG is a member of a BMS advisory board and received honoraria and a travel grant from BMS and Merck Sharp and Dohme (MSD). DS receives research funding from Bristol-Myers Squibb and has a consultant or advisory board relationship with BMS. RC is a member of a Sysmex Corporation advisory board. CH received honoraria, and travel grants from BMS. He was also a member of a BMS advisory board. RD receives research funding from BMS and has a consultant or advisory board relationship with BMS. MS obtained funding from Siemens Healthcare Diagnostics GmbH and a research Grant for colorectal carcinoma from Siemens Healthcare Diagnostics. This is not in conflict with the present study and did not affect the interpretation of the results of the present studies. LH is a member of a BMS advisory board and of the former ipilimumab reference center. She has also received travel grants from BMS. KCK is a member of a BMS advisory board and of the former ipilimumab reference center. All the remaining authors have declared no conflicts of interest.

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