High numbers of PDCD1 (PD-1)-positive T cells and B2M mutations in microsatellite-unstable colorectal cancer

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ABSTRACT
DNA mismatch repair (MMR)-deficient cancers accumulate high numbers of coding microsatellite mutations, which lead to the generation of highly immunogenic frameshift peptide (FSP) neoantigens. MMR-deficient cells can grow out to clinically manifest cancers either if they evade immune cell attack or if local T-cells get exhausted. Therefore, a subset of MSI cancer patients responds particularly well to treatment with immune checkpoint inhibitors. We analyzed whether immune evasion in MMR-deficient cancer mediated by loss of HLA class I or II antigens is related to local immune cell activation status. Microsatellites located in Beta2-microglobulin (B2M) and the HLA class II-regulatory genes RFX5 and CIITA were analyzed for mutations in MMR-deficient colorectal cancers (n = 53). The results were related to CD3-positive and PDCD1 (PD-1)-positive T-cell infiltration. PDCD1 (PD-1)-positive T-cell counts were significantly higher in B2M-mutant compared to B2M-wild type tumors (median: 22.2 cells per 0.25 mm² vs. 2.0 cells per 0.25 mm², Wilcoxon test p = 0.002). Increasing PDCD1 (PD-1)-positive T-cell infiltration was significantly related to an increased likelihood of B2M mutations (OR = 1.81). HLA class II antigen expression status was significantly associated with enhanced overall T-cell infiltration, but not related to PDCD1 (PD-1)-positive T-cells. These results suggest that immune evasion mediated by B2M mutation-induced loss of HLA class I antigen expression predominantly occurs in an environment of activated PDCD1 (PD-1)-positive T cell infiltration. If B2M mutations interfere with anti-PDCD1 (PD-1)/CD274 (PD-L1) therapy success, we predict that resistance towards anti-PDCD1 (PD-1) therapy may counterintuitively be particularly common in patients with MMR-deficient cancers that show high PDCD1 (PD-1)-positive T cell infiltration.

Introduction
Cancers of the microsatellite instability (MSI) phenotype are caused by deficiency of the DNA mismatch repair (MMR) system. MSI cancers are distinguished among other features by their high immunogenicity. This likely results from an exceptionally high mutational load: MMR deficiency leads to the generation of multiple insertion/deletion mutations in coding microsatellites and consequently to mutation-induced frameshift peptide (FSP) neoantigens.1 MSI is observed in 15% of all colorectal cancers and in a variety of extracolonic malignancies such as endometrial cancer, of which up to 30% are MSI.2-4 MSI cancers can develop sporadically, most frequently through somatic epigenetic inactivation of the MMR gene MLH1.5 Alternatively, they occur in the context of the hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome that is caused by germline mutations of MMR genes, most frequently MLH1 or MSH2.6

MSI colorectal cancers commonly present with a dense lymphocyte infiltration, typically of an activated and cytotoxic phenotype.7-10 FSP-specific tumor-infiltrating lymphocytes from MSI cancers in fact retain the potential of lysing MSI tumor cells.11 Despite the evidence of local anti-tumoral immune responses, MSI tumors often grow out to large and clinically relevant size, which suggests the existence of immuno-suppressive and/or immuno-evasive mechanisms. In fact up to 30% of all MSI colorectal cancers display mutations in the B2M gene with a consecutive loss of HLA class I antigens that prevents recognition as well as killing of B2M-mutant cells by cytotoxic T cells.12,13 B2M mutations, as a likely mechanism of immune evasion, are thought to provide affected MSI tumor cells with a major selection advantage as it has been reported for other tumor types such as malignant melanoma.14,15 More recently, an additional mechanism has been proposed to contribute to the immune evasion of MSI tumor cells: loss of functional HLA class II antigen presentation machinery occurs in approximately one third of all MSI CRCs as a consequence of mutations inactivating the HLA class II-regulatory genes RFX5 and CIITA.16 an alteration that is also associated with increased local T cell infiltration of the tumor.17

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Immune checkpoint molecules such as PDCD1 (PD-1) have gained attention as targets for novel immune therapy approaches. The physiological role of PDCD1 (PD-1) is to preserve the equilibrium between co-stimulatory and co-inhibitory signals that regulate T cell activity and maintain self-tolerance. In tumors, PDCD1 (PD-1) can limit the potency of anti-tumor immune responses (T cell exhaustion) upon activation by one of its ligands (CD274 [PD-L1], PDCD1LG2 [PD-L2]). This may facilitate the outgrowth of even highly antigenic tumors in the presence of tumor-antigen specific immune cells. Antibody-mediated blockade of PDCD1 (PD-1) pathway blockade. We therefore analyzed the local infiltration by one of its ligands (CD274 [PD-L1], PDCD1LG2 [PD-L2]). This may facilitate the outgrowth of even highly antigenic tumors in the presence of tumor-antigen specific immune cells. Antibody-mediated blockade of PDCD1 (PD-1) or CD274 (PD-L1) has recently emerged as a promising novel immune therapy approach. In tumors, PDCD1 (PD-1) can limit the potency of anti-tumor immune responses (T cell exhaustion) upon activation by one of its ligands (CD274 [PD-L1], PDCD1LG2 [PD-L2]). This may facilitate the outgrowth of even highly antigenic tumors in the presence of tumor-antigen specific immune cells. Antibody-mediated blockade of PDCD1 (PD-1) or CD274 (PD-L1) has recently emerged as a promising novel method of cancer immunotherapy, particularly in MSI cancer patients. Although a substantial proportion of approximately half of advanced MSI cancer patients showed objective responses to anti-PDCD1 (PD-1) antibody therapy, even including radiographic complete responses in almost 20%, about one fourth of patients had progressive disease under therapy. So far, markers predicting the success of anti-PDCD1 (PD-1)/CD274 (PD-L1) therapy among MSI cancer patients are not yet established. Similarly, studies are lacking that investigate the relationship of PDCD1 (PD-1)-positive T cell infiltration in MSI cancers with immune evasion phenomena such as alterations of HLA class I or II-mediated antigen presentation. Such more comprehensive information about the tumor immune status could potentially be highly relevant in the identification of patients expected to benefit from immune therapy by PDCD1 (PD-1)/CD274 (PD-L1) pathway blockade. We therefore analyzed the local infiltration of MSI colorectal cancers by PDCD1 (PD-1) positive T cells and investigated the effect of local PDCD1 (PD-1) positive T cell infiltration on B2M mutation status and HLA class II antigen expression pattern of the respective tumors.

### Results

**B2M mutation status and HLA class II expression status of MSI colorectal cancers**

In order to examine a potential influence of the infiltration of MSI colorectal cancer lesions with immune cells on B2M mutation status and/or HLA class II antigen expression status of the tumors, immunohistochemical staining was performed. Representative staining results are shown in Fig. 1. In total, we analyzed a series of 56 MSI colorectal cancers (sporadic MSI cancer, n = 38, Lynch syndrome-associated cancer, n = 18). Patients’ characteristics are summarized in Table 1. Of the analyzed tumors, 19 (33.9%) displayed a mutation of the B2M gene. B2M mutations tended to be more frequent in Lynch syndrome-associated cancers compared to sporadic MSI cancers (9/18 vs. 10/38), but statistical significance was not achieved (p = 0.13, Fisher’s exact test).

All tumors classified as B2M-mutant by Sanger sequencing showed negative staining, and all tumors classified as B2M wild type showed homogenous positive staining. HLA class II antigen expression was ‘0’ in 19 (33.9%), ‘1’ in 10 (17.9%) and ‘2’ in 27 (48.2%). 4 of the 19 (21.1%) tumors classified as lack of or barely detectable expression of HLA class II antigen displayed a mutation in the RFX5 gene.

| Number of patients | Total CRCs | Sporadic CRCs | Hereditary CRCs | B2M-mt | B2M-wt |
|--------------------|------------|---------------|----------------|--------|--------|
| Number of patients | 56         | 38 (67.9%)    | 18 (32.1%)     | 19 (33.9%) | 37 (66.1%) |

Table 1. Characteristics of MSI colorectal cancer patients.

Percentages are given in brackets.
Tumor infiltration with CD3-positive T cells and PDCD1 (PD-1)-positive T cells

Microsatellite-unstable colorectal cancer lesions were first analyzed for general lymphocyte infiltration by staining with the pan T cell marker CD3. Overall, the tumors showed CD3-positive T cell infiltration at a median number of 118.9 cells per 0.25 mm². A significantly higher density of CD3-positive T cells was observed in hereditary compared to sporadic MSI colorectal cancers (median: 143.1 cells per 0.25 mm² vs. 92.5 cells per 0.25 mm², p = 0.009). Analyzing the total infiltration of PDCD1 (PD-1)-positive T cells revealed a median number of 5.2 cells per 0.25 mm². Comparison of PDCD1 (PD-1)-positive T cell infiltration between hereditary and sporadic MSI CRCs also showed a significantly elevated number of PDCD1 (PD-1)-positive cells in hereditary tumors (median: 31.0 cells per 0.25 mm², her., vs. 2.7 cells per 0.25 mm², spor., p = 0.006).

Relation between immune cell infiltration and B2M mutation status

We investigated the association of general tumor lymphocyte infiltration with tumor B2M mutation status and did not observe a statistically significant change in distribution of the intratumoral CD3-positive T cell infiltration with respect to B2M mutation status (median: 101.2 B2M-wild type vs. 139.0 B2M-mutant, p = 0.23, Fig. 2a). As a next step, we examined a potential relation of the infiltration of the tumors with PDCD1 (PD-1)-positive T cells and B2M mutation status of the tumor cells. A significantly higher number of PDCD1 (PD-1)-positive T cells was observed in B2M-mutant compared to B2M-wild type MSI colorectal cancers (median: 22.2 cells per 0.25 mm² B2M-wild type vs. 2.0 cells per 0.25 mm² B2M-mutant, p = 0.002, Wilcoxon test, Fig. 2b).

In a multivariate model, PDCD1 (PD-1)-positive T cell infiltration was identified as the only statistically significant predictor being positively associated with the presence of B2M mutation (odds ratio for doubling of PDCD1 [PD-1]-positive T cell counts, OR = 1.69, Table 2). Also after variable selection, PD-1-positive T cell infiltration remained a significant predictor in the model and showed a similar effect (OR = 1.48).

Relation between immune cell infiltration and HLA class II antigen expression

We observed a significantly higher overall T cell infiltration in tumors with higher HLA class II antigen expression (median 150.0 cells per 0.25 mm² HLA class II antigen 2 vs. 133.9 cells per 0.25 mm² HLA class II antigen 1 vs. 50.8 cells per 0.25 mm² HLA class II antigen 0, p < 0.001, Fig. 3a). PDCD1 (PD-1)-positive T cell infiltration also tended to be higher in tumors with higher HLA class II antigen expression; however, the proportion of PDCD1 (PD-1)-positive T cells among all T cells was not significantly related to HLA class II antigen expression (Fig. 3b, p = 0.0802, Jonckheere-Terpstra test).

In a multivariate model, CD3-positive T cell infiltration was identified as the only statistically significant predictor being positively associated with higher HLA class II antigen expressions (odds ratio for doubling of CD3-positive T cell counts, OR = 2.48, for ‘lack of or barely detectable’ vs. ‘intermediate’ as well as for ‘intermediate’ vs. ‘high’, Table 3). Also after variable selection, CD3-positive T cell infiltration remained a significant predictor.
Table 2. Multivariate logistic regression model for B2M mutation status.

|                          | OR (95%-CI) | P-value |
|--------------------------|-------------|---------|
| PD-1-positive T cell infiltration (2-fold change) | 1.69 (1.03-2.78) | 0.04   |
| CD3-positive T cell infiltration (2-fold change) | 1.04 (0.53-2.03) | 0.92   |
| Age (diff. of 10 years) | 0.52 (0.26-1.07) | 0.08   |
| Gender (female vs. male) | 0.20 (0.02-1.67) | 0.14   |
| CRC type (sporadic vs. hereditary) | 0.28 (0.02-4.35) | 0.36   |
| HLA II antigen expression (increase by one level) | 0.91 (0.12-6.76) | 0.92   |
| BRAF mutation status (wildtype vs. mutated) | 4.74 (0.72-30.97) | 0.10   |

Abbreviations: OR, Odds ratio; CI, confidence interval

Table 3. Multivariate ordered logistic regression model for HLA class II antigen expression.

|                          | OR (95%-CI) | P-value |
|--------------------------|-------------|---------|
| PD-1-positive T cell infiltration (2-fold change) | 0.92 (0.64-1.31) | 0.63   |
| CD3-positive T cell infiltration (2-fold change) | 2.48 (1.31-4.70) | 0.005  |
| Age (diff. of 10 years) | 0.62 (0.24-1.51) | 0.13   |
| Gender (female vs. male) | 0.61 (0.12-3.13) | 0.56   |
| CRC type (sporadic vs. hereditary) | 0.23 (0.02-2.22) | 0.20   |
| B2M mutation status (wildtype vs. mutated) | 1.08 (0.24-4.77) | 0.92   |
| BRAF mutation status (wildtype vs. mutated) | 1.33 (0.27-6.43) | 0.73   |

Abbreviations: OR, Odds ratio; CI, confidence interval

Discussion

A central observation of our study is that alterations to tumor antigen presentation molecules are related to the local lymphocyte infiltration patterns. Our results suggest that mutations in the B2M gene, which are the most frequent cause of complete breakdown of HLA class I-mediated antigen presentation in MSI colorectal cancer, are specifically related to a high number of tumor-infiltrating PDCD1 (PD-1)-positive T cells.

In contrast, we did not observe any significant difference of overall T cell counts between B2M-wild type and B2M-mutant tumors. This is in line with several previous studies that did not observe any correlation between immune cell infiltration and B2M mutation status, except the study by de Miranda et al. who found evidence of higher counts of certain T cell subsets infiltrating B2M-mutant compared to wild type tumors. However, increasing PDCD1 (PD-1)-positive T cell infiltration was significantly related to an increased likelihood of B2M mutation.

Our results for the first time establish B2M mutation as a consequence of high PDCD1 (PD-1)-positive T cell counts in MSI cancers. Potentially underlying this is the influence of the local immune cell milieu on the phenotype of the emerging tumors, as postulated by the immunoediting model. In an environment of dense infiltration with activated PDCD1 (PD-1)-positive T cells, reflective of a more active local immune milieu and thus a stronger immunoselective pressure, emerging HLA class I-positive tumor cell clones may be eliminated more effectively. This probably favors the outgrowth of poorly immunogenic, B2M-mutant MSI colorectal cancer cells. These no longer present FSP-derived neoantigens to the immune system via HLA class I antigens. This model is in line with the previous observation by Giannakis et al. who reported an increased likelihood of mutations affecting B2M or other genes involved in HLA class I-mediated antigen presentation in colorectal cancers densely infiltrated by lymphocytes.

In fact, PDCD1 (PD-1)-positive T cell infiltration remained the only significant predictor of B2M mutation status in a multivariate model. No significant relationship between the proportion of PDCD1 (PD-1)-positive T cells and HLA class II antigen expression on tumor cells was observed. However, in concordance with the previously reported association of HLA class II antigen expression with CD4-positive T cell infiltration, overall T cell density was significantly increased in tumors with higher HLA class II antigen expression.

Lynch syndrome-associated cancers showed significantly elevated immune cell infiltration compared to sporadic MSI cancers, which is in line with previous findings. In addition, we here for the first time report a significantly higher absolute

Figure 3. Distribution of specific T cell infiltration data in tumors with HLA class II antigen expression status high, intermediate or lack of or barely detectable A We observed a significant increase in overall T cell infiltration in tumors with higher HLA class II antigen expression B PDCD1 (PD-1)-positive T cell infiltration tended to increase with higher HLA class II antigen expression, but the difference did not reach statistical significance.
and relative amount of PDCD1 (PD-1)-positive T cells in Lynch syndrome vs. sporadic MSI cancer specimens. This suggests that the recurrent exposure of immune cells towards MSI-related antigens in Lynch syndrome, e.g. through mismatch repair-deficient crypts\textsuperscript{1,40,41} may lead to T cell activation and consequently an enhanced number of activated T cells in clinically manifest tumors.

The recurrent stimulation of the immune system with MSI-induced FSP neoantigens in Lynch syndrome may thus be the reason for the higher frequency of B2M mutations in Lynch syndrome-associated compared to sporadic MSI colorectal cancers\textsuperscript{42} This supports the concept that stimulating the immune system against MSI-related FSP antigens, e.g. by a vaccine\textsuperscript{1} may not only delay tumor formation in Lynch syndrome, but also lead to a better outcome, because B2M-mutant cancers have an excellent prognosis after surgical removal.\textsuperscript{43,44}

The presence of functional HLA class I antigens and therefore the presence of functional B2M is a prerequisite for recognition and killing of tumor cells by cytotoxic CD8-positive T cells.\textsuperscript{45} Therefore, B2M mutations are expected to interfere with the success of immune checkpoint modulator therapy, e.g. using pembrolizumab\textsuperscript{14,46-48} as has recently been observed as a mechanism of secondary resistance in a melanoma patient and in MSI cancers.\textsuperscript{27,49} Accordingly, our results predict that upfront resistance towards immune checkpoint blockade may be particularly common among MSI cancers with high PDCD1 (PD-1)-positive T cell infiltration, because they commonly show B2M mutation-induced loss of HLA class I antigen presentation. This, somewhat counterintuitively, suggests that MSI cancers with low PDCD1 (PD-1)-positive T cell counts will likely respond better to anti-PDCD1 (PD-1) therapy than tumors with high PDCD1 (PD-1)-positive T cell counts.

B2M mutations have only rarely been observed in MSI colorectal cancers presenting with hematogenous metastasis formation, for example to the liver.\textsuperscript{13,43} Possible mechanisms underlying this observation are enhanced NK cell killing of B2M-mutant, HLA class I antigen-negative MSI cancer cells\textsuperscript{50} or, alternatively, impaired tumor cell/platelet interaction after loss of HLA class I antigen expression.\textsuperscript{51} However, B2M mutations still allow metastasis formation through local or lymphatic tumor cell spread. In fact, peritoneal metastasis through non-hematogenous tumor cell spread is a common manifestation in MSI colorectal cancer of stage M1.\textsuperscript{52,53} B2M mutation status and infiltration with activated PDCD1 (PD-1)-positive T cells may be of particular predictive significance for checkpoint blockade in this clinical constellation.

In summary, our results suggest that high amounts of PDCD1 (PD-1)-positive tumor-infiltrating T cells, representing a highly active local immune milieu, may favor the outgrowth of emerging B2M-mutant, HLA class I-negative tumor cell clones. This is in line with the hypothesis that B2M mutations in MSI cancer occur as a direct result of DNA mismatch repair deficiency-induced mutations and subsequent immunoselective pressure. It is strongly suggested that all future clinical trials using PDCD1 (PD-1)/CD274 (PD-L1) checkpoint modulators should account for B2M mutation status and immune cell infiltration of the primary tumor and all available metastatic lesions as potential predictors of therapy efficacy.
Patients and methods

Patients and tumor specimens

Tumor samples represent a consecutive series of MSI colorectal cancers recorded in the Department of Applied Tumor Biology, Institute of Pathology, University Hospital Heidelberg as part of the German HNPCC Consortium. Informed consent was obtained from all patients included in this study. The Ethics Committee of the University of Heidelberg approved the study. None of these patients underwent neoadjuvant radio- and/or chemotherapy prior to surgery.

Microsatellite instability status was determined using the Bethesda standard marker panel (BAT25, BAT26, D2S123, D5S346 and D17S250/Mfd15) and CAT25 as described previously. Tumors from patients with a germline mutation of the DNA mismatch repair genes were classified as ‘hereditary’. Tumors exhibiting MLH1 promoter methylation and/or the BRAF V600E mutation were classified as ‘sporadic’.

Microdissection and DNA isolation

Three to six tissue sections (2 μm, formalin-fixed and paraffin-embedded) were deparaffinized and stained with hematoxylin and eosin according to standard protocols. Tumor areas were manually microdissected from HE-stained sections, and genomic tumor DNA was isolated using the Qiagen DNeasy Tissue Kit (Qiagen, Cat. No.: 69506) according to the manufacturer’s instructions.

Immunohistochemical staining

Immunohistochemical staining was performed on 2 μm paraffin sections. After deparaffinization in xylol and rehydration in a decreasing series of ethanol baths, the slides were boiled 3 × 5 minutes in 10 mM citrate buffer (pH 6.0) for antigen retrieval. After boiling, the slides cooled down for 20 minutes, followed by blocking the endogenous peroxidase activity with 0.06% H2O2 (v/v in methanol) for 20 minutes. Subsequently the slides were incubated with 10% horse serum (Vector Laboratories, Cat. No.: S-2000) v/v in phosphate-buffered saline for 30 min at room temperature to prevent nonspecific antibody binding.

Mouse monoclonal antibodies specific for CD3 (1:60 dilution, Acris, Cat. No.: DM112, clone PS1), PDCD1 (PD-1) (1:50 dilution, Abcam, Cat. No.: ab52587, clone NAT105), HLA-DR, HLA-DQ, HLA-DP (1:200 dilution, clone LGII-612.14) and Beta-2-microglobulin (B2M) (1:50 dilution, clone L368) were used as primary antibodies at 4 °C overnight. Monoclonal antibodies LGII-612.14 and L368 were kind gifts from Prof. Saldano Ferrone and developed and characterized as described previously. After washing with PBS and incubation with biotinylated anti-mouse IgG antibodies for 30 min at room temperature, AB reagent was, following another washing step, applied for 30 minutes at room temperature (Vectastain Elite ABC HRP kit; Vector Laboratories, Cat. No.: PK-6102). Finally, antigen detection was performed by a color reaction with 3,3-di-amo-benzidine (DAB+ chromogen; Dako North America, Cat. No.: K3468) and counterstaining with Mayer’s hematoxylin (AppliChem, Cat. No.: A4840).

Determination of B2M and RFX5/CIITA mutation status

Mutation analysis of the B2M gene was performed for all tumor specimens by using Sanger sequencing as described previously. For all samples categorized as ‘lack of or barely detectable’ HLA class II antigen expression, Sanger sequencing of the RFX5 and CIITA genes was performed as previously described. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Cat. No.: 28106). For the sequencing reaction the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Cat. No.: 4337450) was used. The products for sequencing were precipitated, dissolved in 12 μl Hi-Di Formamide (Applied Biosystems, Cat. No.: 4311320) and analyzed on an ABI3130xl genetic analyzer (Applied Biosystems, Darmstadt, Germany).

Microscopic evaluation and quantification of immune cell infiltration

HLA class II antigen expression was categorized in three levels. 0 = ‘lack of or barely detectable’, less than 25% of the tumor tissue stained positive, 1 = ‘intermediate’, 25–75% positive tumor cells, 2 = ‘high’ more than 75% positive tumor cells. Immune cell infiltration of CD3-positive T cells and PDCD1 (PD-1)-positive T cells was evaluated (by J.J.) in three to five representative tumor regions (t = number of evaluated tumor regions), utilizing a 10 × 10 ocular grid (0.25 mm²) at a magnification of × 200 and a Leica DMRBE microscope (Leica Camera AG, Wetzlar, Germany). Numbers of positive cells per 0.25 mm² (n) were calculated as the mean value of the positive cells in the individual regions (n_i) using the following formula:

\[
n = \frac{1}{t} \times \sum_{i=1}^{t} \frac{n_i}{0.25 \text{mm}^2}
\]

Pictures were scanned using a Hamamatsu NanoZoomer Digital Pathology system (Hamamatsu, Hamamatsu City, Japan). A subset of immunohistochemically stained tissue sections (20 tumor regions) was examined by a second observer (M.K.) with respect to CD3-positive T cells. With a Spearman’s rank correlation coefficient of 0.96 (one-sided 95%-CI: 0.87-1), the results of the manual counting can be considered reliable.

Statistical analysis

Wilcoxon’s rank sum test was applied to compare the distribution of immune cell infiltration depending on B2M mutation status and type of MSI (hereditary or sporadic). With respect to HLA class II antigen status the Jonckheere-Terpstra test was used. Fisher’s exact test was applied to compare distributions of categorical data.

For the multivariate analysis with respect to the B2M mutation status, a logistic regression model was set up including age (continuous), gender, MSI type (sporadic/hereditary), CD3-positive T cells per 0.25 mm², PDCD1 (PD-1)-positive T cells per 0.25 mm², HLA class II antigen expression and BRAF mutation status as covariates. For the HLA class II antigen expression, an ordered logistic model was applied to the categorized version of the HLA class II antigen expression. Other than
replacing the HLA class II antigen expression by the B2M mutation status, the same covariates were considered. Due to a low number of cases (53 cases for B2M mutation status; 37 wild type vs. 15 mutant; 49 cases for HLA class II antigen; 14 level 0 vs. 10 level 1 vs. 25 level 2), a backward selection was added to the multivariate analysis. The threshold for the selection was chosen at $p = 0.5$ according to Steyerberg et al. as a tradeoff between selection bias and overestimation bias.

All analyses were performed on the basis of the mean of the immune cell counts over all tumor regions where measurements have been taken. $P$ values smaller than 0.05 were considered statistically significant. The statistical analysis was performed within the statistical software environment R.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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