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The coding microsatellite mutation profile of PMS2-deficient colorectal cancer

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2 Nielsen and Kloor contributed equally as last authors.

ABSTRACT

Lynch syndrome (LS) is caused by a pathogenic heterozygous germline variant in one of the DNA mismatch repair (MMR) genes: MLH1, MSH2, MSH6 or PMS2. LS-associated colorectal carcinomas (CRCs) are characterized by MMR deficiency and by accumulation of multiple insertions/deletions at coding microsatellites (cMS). MMR deficiency-induced variants at defined cMS loci have a driver function and promote tumorigenesis. Notably, PMS2 variant carriers face only a slightly increased risk of developing CRC. Here, we investigate whether this lower penetrance is also reflected by differences in molecular features and cMS variant patterns. Tumor DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue cores or sections (n = 90). Tumors originated from genetically proven germline PMS2 variant carriers (including 14 PMS2-deficient tumors). The mutational spectrum was analyzed using fluorescently labeled primers specific for 18 cMS previously described from genetically proven germline PMS2 variant carriers (including 14 PMS2-deficient tumors). The extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections. The cMS spectrum of PMS2-deficient CRCs did not show any significant differences from MLH1/MSH2-deficient CRCs. PMS2-deficient tumors, however, displayed lower CD3-positive T-cell infiltration compared to other MMR-deficient cancers (28.00 vs. 55.00 per 0.1 mm 2, p = 0.0025). Our study demonstrates that the spectrum of potentially immunogenic cMS variants in CRCs from PMS2 gene variant carriers is similar to that observed in CRCs from other MMR gene variant carriers. Lower immune cell infiltration observed in PMS2-deficient CRCs could be the result of alternative mechanisms of immune evasion or immune cell exclusion, similar to those seen in MMR-proficient tumors.
encoding regions can lead to shifts of the translational reading frame and thus to inactivation of the affected genes. Moreover, through shifting of the reading frame, completely new peptide sequences are synthesized that are unknown to the immune system and therefore can elicit strong immune responses of the host (Schwitalla et al., 2008; Le et al., 2017). Several coding microsatellite (cMS) variants that drive Lynch syndrome cancer progression through the inactivation of tumor suppressor genes have been previously identified (Alhopuro et al., 2012; Woerner et al., 2003). As cMS variants can contribute to cancer development, the patterns of cMS variants observed in manifest cancers reflect evolutionary selection and therefore the pathogenesis of tumor developments. This is exemplarily illustrated by marked differences in cMS variant frequency between colorectal and endometrial carcinomas (Kim et al., 2013; Hause et al., 2016).

Carriers of a pathogenic variant in the PMS2 gene have a markedly lower penetrance and later age of onset of colorectal and endometrial cancer than carriers of pathogenic MLH1 or MSH2 variants (ten Broeke et al., 2015; Senter et al., 2008; Ten Broeke et al., 2018a). The reported cumulative risk of CRC is 11–20% for PMS2 carriers, which is in sharp contrast to a cumulative risk of 35–55% up to age 70 for MLH1/MSH2 carriers (Barrow et al., 2017). Notably, prospective studies have now reported that the cumulative risk of colorectal cancer for PMS2 variant carriers undergoing colonoscopic surveillance and polypectomies is 0% (Ten Broeke et al., 2018a). This is in contrast to MLH1/MSH2 carriers with risks of CRCs arising between follow-up colonoscopies to be up to 46% and 43% respectively. Even MSH6 carriers who also have a milder phenotype are at risk (15%) of such incident cancers (Moller et al., 2017). Consequently, the functional significance of pathogenic PMS2 variants during the pathogenesis of cancers has been questioned and it is perceivable that PMS2 deficiency may play a different and potentially less prominent role in tumorigenesis.

A previous study by Alpert and colleagues reported that CRCs with isolated PMS2 loss (i.e. without concomitant MLH1 loss) displayed less histological features associated with MSI (i.e. tumor-infiltrating lymphocytes (TILs), Crohn’s-like lymphocytic reaction, mucinous or signet ring cell component and medullary growth pattern) (Alpert et al., 2018). Their data also suggested that these tumors might display a less aggressive behavior, with an odds ratio of 0.87 (95% CI: 0.89–27.04) for disease-specific death when the analyses were restricted to germline variant or probable Lynch syndrome cases only. They hypothesized that these observations might be related to a lower degree of immune activation potentially resulting from a smaller number of immunogenic frameshift peptide neoantigens produced in PMS2-deficient tumors.

We have recently developed a novel tool to quantify immunogenic CMS mutations in MSI cancer (Balhausen et al., 2020). In the present study, we used this approach to compare CMS variant patterns in PMS2-deficient CRCs from confirmed carriers to those observed in CRCs from MLH1 and MSH2 variant carriers.

2. Material and methods

2.1. Tumor specimens

Tumor material from 10 confirmed pathogenic germline PMS2 variant carriers was collected at Leiden University Medical Centre. Tumor material from 41 MLH1, 21 MSH2, and 4 PMS2 carriers was collected at the Department of Applied Tumor Biology, Institute of Pathology, University Hospital Heidelberg, as a center of the German Consortium for Familial Intestinal Cancer. Informed consent was obtained from all participating patients. The study was approved by the Institutional Ethics Committee (Protocol number: S-583/2016). Additional data on age at diagnosis, tumor histology and tumor stage was extracted from pathology reports when available.

2.2. Tumor workup and DNA isolation

Tissue blocks were collected, and DNA was isolated from three tissue cores of variable length (0.3 mm diameter, or 0.7 mm in case of tissue with a low cell count) or from whole tissue sections after manual microdissection using the DNasey FFPE Kit (Qiagen, Germany).

2.3. Microsatellite analysis

For the characterization of cMS patterns, we performed fragment length analysis using fluorescently labeled primers specific for a selected series of 18 coding microsatellites previously described as mutational targets in MSI cancer development. (Woerner et al., 2010) Selection criteria were (Schwitalla et al., 2008) frequency of variant in MMR-deficient cancers, (Le et al., 2017) evidence of a functional driver role of variants suggested by a pathogenic variant frequency higher than expected from microsatellite length, and (Alhopuro et al., 2012) potential significance as source of immunogenic frameshift peptide neoantigens supported by MHC binding prediction algorithms. PCR products were visualized on an ABI3130xl sequencer, and the obtained results were processed using a newly developed algorithm to obtain quantitative estimation of the frequency of the mutant alleles in tumor specimens (ReFrame) (Balhausen et al., 2020). CMS analyses demonstrated in this study are partially based on our previous report with a re-analysis of mutation data with regard to differences between MMR variant carriers.

2.4. Immunohistochemical analysis of CD3

From 11 PMS2-, 13 MLH1- and 14 MSH2-deficient tumors formalin fixed paraffin embedded (FFPE) tumor blocks were available for further analysis of immune cell infiltration. For immunohistochemical detection of CD3-positive cells, 4 μm thick sections of the tumors were stained with an antibody specific for CD3 (1:60 dilution, Acris, Cat. No.: DM112, clone PS1). In short, sections were deparaffinized and endogenous peroxidase was inactivated with H2O2 in methanol solution (0.3%). After this, heat-induced antigen retrieval was performed in a 10 mM citrate buffer (pH 6). Sections were incubated with the anti-CD3 antibody overnight at 4 °C followed by incubation with poly-HRP and staining with 3,3’-Diaminobenzidine. Slides were counterstained with hematoxylin. For immune cell scoring 4 areas of interest (0.1 mm² each) were randomly placed in the tumor center, CD3-positive immune cell infiltration was scored as the mean number of CD3-positive immune cells of the 4 areas (Fig. 1).

2.5. Statistical analysis

Gene-wise variant rates were compared in three steps: First, all cMS showing a prevalence of at least 15% non-wild type alleles in a certain tumor were classified as mutant. The pathogenic variant status was compared between tumors from PMS2 carriers and those from MLH1 and MSH2 carriers grouped together. For this, Wilcoxon-Mann-Whitney test was used and raw p-values were adjusted over all genes using Holm method. Finally, quantitatively analyzed cMS patterns of PMS2-deficient CRCs were separately tested against those with MLH1- or MSH2-deficiency. The global p-value states if there is a significant difference for at least one of the two pairwise comparisons (PMS2 vs. MLH1 or PMS2 vs. MSH2) in the relative effects. The pairwise p-values give the results for local pairwise comparison. Then, a two-sample Kolmogorov-Smirnov test was applied for the pairwise comparisons in order to test for equal distribution.
Pairwise p-values for both approaches were adjusted over all genes using Holm method.

Additionally, T-cell infiltration measured by mean number of CD3-positive T cells was compared between PMS2 and MLH1/MSH2 groups. Wilcoxon-Mann-Whitney test was applied in order to test the difference in median CD3-positive T-cell infiltration between two groups. Results were also compared with a sporadic MSI CRC cohort (n = 47).

3. Results

3.1. Clinical characteristics

A description of clinical characteristics of the analyzed cohorts is available in Table 1. Of note, PMS2-deficient CRCs displayed a higher tumor stage compared to the MLH1/MSH2 cohorts (p = 0.0351).

3.2. Comparison of cMS variant frequency between PMS2- and non-PMS2-deficient CRCs

Tumors from 14 PMS2, 35 MLH1 and 20 MSH2 carriers were analyzed for cMS variant patterns (Table 2). Interpretable results were obtained for a median number of 58 tumors per cMS gene and ranged from 51 to 65 tumors per gene. Five out of 18 analyzed cMS showed variants in all analyzable PMS2-deficient CRCs: ACVR2 (n = 12), AIM2 (n = 12), BANP (n = 12), C4orf6 (n = 10), NFE2L2 (n = 12). Common functionally relevant target cMS presented with similar variant frequencies in PMS2 vs. non-PMS2-deficient CRCs, including ACVR2 (PMS2: 12/12, 100% vs non-PMS2: 46/51, 90.2%, p = 1.00), AIM2 (PMS2: 12/12, 100% vs. non-PMS2: 42/48, 87.5%, p = 0.69), and TGFBR2, the most commonly analyzed cMS target in MMR-deficient colorectal cancer (PMS2: 9/11, 81.8% vs. non-PMS2: 41/44, 93.2%, p = 1.00).

No significant differences in cMS variant frequencies were observed between CRCs from PMS2 carriers and CRCs from MLH1 and MSH2 carriers after adjusting for multiple testing (Table 2).

In order to evaluate whether PMS2-deficient tumors may show a quantitative difference in cMS variants compared to MLH1- and MSH2-deficient CRCs, we quantitatively analyzed cMS variant profiles (Table 3). After adjusting for multiple testing, there were no significant differences between cMS mutated allele ratios of PMS2- and MSH2-deficient CRCs. Of borderline significance, 10 PMS2-deficient CRCs had a higher cMS mutant allele ratio in C4orf6 than 47 MLH1- and MSH2-deficient CRCs (0.434 vs. 0.253, p = 0.07).

3.3. Quantitative analysis of intratumoral CD3-positive lymphocyte infiltration

As a next step, we analyzed the density of tumor-infiltrating lymphocytes in tumors from PMS2 (n = 11), MLH1 (n = 13), and MSH2 (n = 14) carriers. The analysis of CD3-positive lymphocyte infiltration revealed significantly lower median CD3-positive T-cell counts per 0.1 mm² in PMS2-deficient CRCs when compared to MLH1- and MSH2-deficient CRCs (28.00 vs. 55.00, p = 0.0025, Fig. 2).

In order to account for a potential overrepresentation of high stage tumors in the PMS2 cohort, we performed a stratified analysis on UICC tumor stage. The difference in CD3-positive T-cell count remained statistically significant for the comparison of UICC III + IV for the PMS2 vs. the non-PMS2 cohort (19.50 vs. 82.37, p = 0.0286, Fig. 3).

3.4. Analysis of B2M mutation status

To assess, whether the observed difference of lower CD3-positive T-cell counts in PMS2 associated cancers correlated with a difference in mutation frequency of B2M, the mutation frequency of B2M was analyzed by sanger sequencing. Of interest, the B2M mutation frequency of 10 PMS2 associated cancers (10%, 1/10 B2M mutation frequency) and 32 MLH1- or MSH2 associated cancers (43.8%, 14/32 B2M mutation frequency) did not differ significantly (p = 0.2556, Table 1), although a trend towards fewer mutations in B2M was observed in the PMS2 associated cohort. As a next step, the influence of tumor stage on B2M mutation frequency was analyzed. Interestingly, the B2M mutation frequency of 4 PMS2 associated high stage (UICC III and IV) cancers

---

**Table 1**

<table>
<thead>
<tr>
<th>Description of the cohort.</th>
<th>PMS2 (n = 14)</th>
<th>MLH1 (n = 47)</th>
<th>MSH2 (n = 21)</th>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>7 (50%)</td>
<td>19 (46%)</td>
<td>11 (52%)</td>
</tr>
<tr>
<td>Female</td>
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<td>19 (46%)</td>
<td>9 (43%)</td>
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<td>Age at CRC diagnosis</td>
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<tr>
<td>Median (IQR)</td>
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<td>48.0 (41.2–62.3)</td>
<td>47.0 (42.5–53.9)</td>
</tr>
<tr>
<td>Mean (SD)</td>
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<td>51.3 (13.4)</td>
<td>49.2 (9.5)</td>
</tr>
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<td>8</td>
<td>5</td>
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<tr>
<td>Location</td>
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<td>21 (51%)</td>
<td>7 (33%)</td>
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<tr>
<td>Distal</td>
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<td>11 (27%)</td>
<td>6 (29%)</td>
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<tr>
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<td>9 (22%)</td>
<td>8 (38%)</td>
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<td>MSI</td>
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<tr>
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<tr>
<td>Stable</td>
<td>12 (85%)</td>
<td>41 (100%)</td>
<td>21 (100%)</td>
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<td>0</td>
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<tr>
<td>B2M status</td>
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<tr>
<td>Wildtype</td>
<td>11 (79%)</td>
<td>23 (56%)</td>
<td>11 (52%)</td>
</tr>
<tr>
<td>Mutated</td>
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<td>11 (26.8%)</td>
<td>4 (19%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (14%)</td>
<td>7 (17%)</td>
<td>6 (28%)</td>
</tr>
</tbody>
</table>
deficient CRCs. In contrary to what we expected, we observed very similar somatic mutation patterns in PMS2-deficient CRCs versus other MMR-genes such as MLH1 from Lynch syndrome CRCs caused by germline variants affecting other MMR-genes such as MSH2 versus MLH1. This difference remained significant even after adjusting for the UICC III/IV subgroup, which was more common in the PMS2 group.

Alpert et al. proposed an interesting hypothesis that a lower amount of mutational neoantigens may underlie the limited T-cell infiltration in PMS2-deficient compared to other MMR-deficient CRCs. This finding may also be causally related to the observation that CRCs with isolated PMS2 loss showed trends towards presenting with more distant metastasis and higher disease-specific death rates when compared to tumors due to pathogenic variants in other MMR genes. Similarly to Alpert et al., we also observed significantly lower CD3-positive T-cell infiltration in PMS2-deficient compared to other MMR-deficient CRCs. This difference remained significant even after adjusting for the UICC III/IV subgroup, which was more common in the PMS2 group.

4. Discussion

Carriers of a pathogenic PMS2 variant represent a distinct group among Lynch syndrome patients, denoted mainly by a low penetrance, making PMS2 a moderately penetrant gene at most (ten Broeke et al., 2015; Senter et al., 2008; Ten Broeke et al., 2018a; Goodenberger et al., 2016). However, recent work has suggested that tumors with PMS2 deficiency may demonstrate a more aggressive phenotype (Alpert et al., 2018). This finding may also be causally related to the observation that CRCs with isolated PMS2 loss showed trends towards presenting with more distant metastasis and higher disease-specific death rates when compared to tumors due to pathogenic variants in other MMR genes. Similarly to Alpert et al., we also observed significantly lower CD3-positive T-cell infiltration in PMS2-deficient compared to other MMR-deficient CRCs. This difference remained significant even after adjusting for the UICC III/IV subgroup, which was more common in the PMS2 group.

Alpert et al. proposed an interesting hypothesis that a lower amount of mutational neoantigens may underlie the limited T-cell infiltration in PMS2-deficient CRCs. This implies that PMS2-deficient CRCs may develop through a pathogenetic mechanism with an impact of MMR-deficiency similar to other Lynch syndrome-associated cancers. Our results therefore support the hypothesis that MMR deficiency is a significant driving force of tumor development in the PMS2-deficient tumors analyzed, rather than representing merely an epiphenomenon.

In general, Lynch-associated CRCs appear to have better prognosis, which could be a consequence of increased immune activation due to CMS variant-induced frameshift neoantigens (Schwitalle et al., 2008; Kloor and von Knebel Doeberitz, 2016). However, a lower frequency of MSI-related features such as increased tumor-infiltrating lymphocytes (TILs) and Crohn-like infiltrate has previously been reported in PMS2-deficient tumors, suggesting a lower degree of immune activation (Alpert et al., 2018). This finding may also be causally related to the observation that CRCs with isolated PMS2 loss showed trends towards presenting with more distant metastasis and higher disease-specific death rates when compared to tumors due to pathogenic variants in other MMR genes. Similarly to Alpert et al., we also observed significantly lower CD3-positive T-cell infiltration in PMS2-deficient compared to other MMR-deficient CRCs. This difference remained significant even after adjusting for the UICC III/IV subgroup, which was more common in the PMS2 group.
these tumors. However, we could not confirm this hypothesis, as no significant differences between PMS2-deficient CRCs and MLH1- or MSH2-deficient CRCs were detected. As our data did not provide any evidence for a decreased amount of cMS mutation-induced frameshift peptides in PMS2-deficient CRCs on the DNA level, alternative explanations should be considered. It has recently been shown that loss of PMS2 appears to occur rather as a secondary event following neoplasia-inducing variant such as pathogenic APC variants. First, KRAS variants in PMS2-deficient CRCs, in contrast to those (G12D, G13D) typically observed in other Lynch syndrome-associated CRCs, rarely fit to the inducing variant such as pathogenic APC variants. According to our finding of only few B2M mutations among PMS2-deficient CRCs (Ahadova et al., 2016; Ahadova et al., 2018b). Such tumors may develop more rapidly and potentially without an adenoma as precursor lesion that can be prevented by a polypectomy (Ahadova et al., 2016; Ahadova et al., 2018b). Indeed, prospective cohorts report a low or even absent CRC risk for PMS2 carriers undergoing regular surveillance and polypectomies if needed (Moller et al., 2017; Moller et al., 2015). Third, normal PMS2 expression was detected in 16 adenomas from PMS2 carriers, again underlining that PMS2 deficiency rather occurs as a secondary event (ten Broeke et al., 2019).

All these findings suggest that PMS2-deficient CRCs may resemble MMR-proficient CRCs in regards to tumor initiation and early evolution. Accordingly, immune evasion phenomena of PMS2-deficient CRCs may follow mechanisms recently described for MMR-proficient CRCs, for example local immune suppression related to Wnt signaling activation (Grasso and Giannakis, 2018). This hypothesis is further supported by our finding of only few B2M mutations among PMS2-deficient CRCs compared to MLH1- and MSH2-deficient CRCs. Immune evasion by inactivation of B2M is a well described event in MMR-deficient tumors (Kloor et al., 2010), which, however, occurs preferentially in an activated local immune environment, typically characterized by a high density of tumor-infiltrating lymphocytes (Echterdiek et al., 2016).

![CD3+ cell counts in MSI CRCs](image1)

**Fig. 2.** Mean CD3-positive T-cell counts per 0.1 mm² are depicted as box plots for CRCs of MLH1, MSH2 and PMS2 variant carriers as well as a control group of sporadic MSI CRCs ("spor. MLH1"). Asterisks mark significantly lower median CD3-positive T-cell counts per 0.1 mm² in PMS2-deficient CRCs compared to those of MLH1 and MSH2 variant carriers.

CD3 infiltration by UICC stage

![CD3 infiltration by UICC stage](image2)

**Fig. 3.** CD3-positive T-cell counts per 0.1 mm² are depicted as box plots for MSI CRCs of MLH1 and MSH2 variant carriers ("nonPMS2") and PMS2 variant carriers, each divided into low stage (UICC I+II) and high stage (UICC III+IV) groups. CD3-positive T-cell count per 0.1 mm² after correction for tumor stage (UICC III+IV PMS2 vs. UICC III+IV MLH1/MSH2: 19.50 vs. 82.37, p = 0.0286).

Janikovits et al., 2018). Immunohistochemical characterization of immune cell infiltration suggest that this strong immunoselective pressure is lacking in PMS2-deficient CRCs.

Our results also imply that differences in cMS mutational spectrum do not directly explain the lower penetrance of pathogenic PMS2 variants. One theory about the lower penetrance of PMS2 variants is that the MLH3 or PMS1 proteins may take over part of the function of the PMS2 protein by forming a heterodimer with MLH1 in the absence of PMS2 (Peltomaki, 2003). Interestingly, the high prevalence of cMS variants in PMS2-deficient CRCs do not provide any evidence for a limited degree of MMR deficiency in these tumors after clinical manifestation. However, our study was limited by the number of analyzed tumor specimens and by focusing predominantly on CMS variants with putative driver function. For this reason, subtle or genome-wide differences may have been missed, as our panel was not designed to assess a general estimation of the quantitative level of MSI in PMS2-deficient tumors. Therefore, further studies on larger sample sets are required to validate our observation of a similar CMS spectrum for all MMR-gene variant carriers. Future studies will also be required to evaluate whether PMS2-deficient CRC patients with metastasized disease may benefit from immune checkpoint blockade using anti-PD-1 or anti-PD-L1 antibodies which
have shown very promising results in MMR-deficient cancer patients. In conclusion, we observed surprisingly high amounts of somatic cMS variants in PMS2-deficient CRCs comparable to those observed in CRCs from MLH1 and MSH2 carriers. At the same time, we confirmed previous findings of limited T-cell infiltration in these tumors. Our data suggest that a low abundance of MMR-deficiency-induced mutational neoantigens does not seem to be an explanation for the limited local immune infiltration. Instead, we suggest that PMS2-deficient cancers, prior to inactivation of PMS2, may have acquired alternative mechanisms of immune cell exclusion typical of MMR-proficient CRCs. Larger studies including clinical follow-up data should further explore the influence of the immune system on PMS2-deficient CRCs. If confirmed, a lower immune response in PMS2 carriers that develop CRC may have consequences for metastatic potential and overall prognosis.

Conflict of interest statement

The authors declare no potential conflicts of interest.

Ethics approval and consent to participate

The Institutional Ethics Committee, University Hospital Heidelberg (Protocol number: S-583/2016) approved the study. Informed consent was retrieved from all patients.

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Author contributions

Providing tissue specimens: M.v.K.D., M.N., M.K.
Revision and final approval of the manuscript: all authors.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2021.104668.

References


