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RHAMM in liver metastases of stage IV colorectal cancer with mismatch-repair proficient status correlates with tumor budding, cytotoxic T-cells and PD-1/PD-L1

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ABSTRACT

Background: During the last decades, the management for metastatic colorectal cancer patients has improved due to novel therapeutic approaches. A mismatch-repair deficient status seems to favour a better response to checkpoint inhibitor therapy, but the question arises whether a specific subgroup of stage IV patients with mismatch-repair (MMR) proficient status should also be considered. RHAMM (Receptor for Hyaluronic Acid Mediated Motility/HAMMR/CD168) is characterized by tumor progression and immunogenicity. Therefore, the aim of this study is to determine whether RHAMM within the CRLM of MMR-proficient patients correlate with a more immunological microenvironment, represented by cytotoxic T-cells, PD-1 and PD-1.
Methods: Two patient cohorts of liver metastases from MMR colorectal cancers were included into the study (n = 81 and 76) using ngTMA® technology and immunohistochemically analyzed for RHAMM, cytotoxic T-cells (CD8+), PD-1/PD-L1, intrametastatic budding (IMB) and perimetastatic budding (PMB).
Results: RHAMM-positive IMB was linked to a higher PD-L1 expression ($r = 0.32$; $p = 0.233$ and $r = 0.28$; $p = 0.044$) in the center and periphery of the metastasis and RHAMM-positive PMB was associated with a higher expression of PD-1 ($r = 0.33$; $p = 0.0297$), and especially PD-L1 ($r = 0.604$; $p < 0.0001$ and $r = 0.43$; $p = 0.003$) in the center and periphery of the metastasis. IMB and PMB were additionally associated with a higher count of CD8+ T-cells ($p < 0.0001$; $r = 0.58$; $p < 0.0001$; $r = 0.53$).
Conclusions: The RHAMM status can be assessed in IMB/PMB either in biopsies or in resections of colorectal cancer liver metastases. A positive RHAMM status in IMB and/or PMB may be a potential indicator for a checkpoint inhibitor therapy for stage IV colorectal cancer patients with MMR proficient status.

1. Introduction

Tumor budding is a histopathological prognostic biomarker and its standardized reporting was proposed by the International Tumor Budding Consensus Conference (ITBCC) in 2016 [1]. Tumor budding is now included in the UICC's TNM and WHO classifications [2,3] and also in important guidelines such as the European Society of Medical Oncology (ESMO) [4], National Comprehensive Cancer Network (NCCN) [5], International Collaboration on Cancer Reporting (ICCR) [6], Royal College of Pathologists (RCPath) [7] and College of American Pathologists (CAP) [8]. Tumor budding along with other clinico-pathological parameters plays a role in the management of colorectal cancer (CRC) patients especially in two clinical scenarios: first, in pT1 CRC tumor budding is associated with the presence of lymph node metastases and is therefore a potential indicator for an oncologic resection [9–11]; second, in stage II colon cancer with high grade budding (BD3), adjuvant therapy may be considered [9,12].

Based on the literature tumor budding may also be included in two additional clinical scenarios, namely the assessment of intratumoral budding (ITB) in preoperative biopsies in colon and rectal cancer [13]

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and especially in colorectal cancer liver metastases (CRLM) of stage IV CRC patients [14]. While the promising role of ITB in preoperative CRC biopsies is underlined by several studies, the assessment and function of tumor budding in CRLM requires more evidence before consideration for patient management. In a recent study, Fonseca et al. have demonstrated the prognostic impact of tumor budding in CRLM in univariate analysis [15]. Additionally, we proposed the terms intrametastatic (IMB) and perimetastatic budding (PMB) based on H&E staining for assessment of tumor budding in CRLM [14].

Over the last 20 years, the prognosis for metastatic CRC (mCRC) has improved and the median overall survival (OS) for patients with mCRC is approximately 30 months based on phase III trials and large observational series or registries [16]. Some data have shown a potential benefit of immune checkpoint blockade with pembrolizumab in patients with a deficient mismatch-repair status (dMMR) in mCRC compared to those with a proficient mismatch-repair status (pMMR) [17,18].

Nevertheless, the question arises whether mCRC patients with pMMR status may still be candidates for a checkpoint inhibitor therapy. Therefore, the objective of the present study was to evaluate biomarkers of CRLM in pMMR cases according to the following hypothesis: the Receptor for Hyaluronic Acid Mediated Motility (RHAMM/HMMR/CD168) has been shown to be a strong independent prognostic factor in CRC [19–22], its overexpression in cancer cells correlates with increased migration and invasion and its downregulation abolishes metastasis in mouse models [23]. Additionally, several studies have postulated a potential immunogenic role of RHAMM [24–26]. Taken together, the aim of this study is to determine whether RHAMM positive cells (including IMB and PMB) within the CRLM of MMR-proficient patients correlate with a more immunological microenvironment, represented by cytotoxic T-cells, PD-1 and PD-1. The specific selection of cytotoxic T-cells is based on a previous study showing the role of CD8 positive T-cells in the surroundings of tumor buds [27].

2. Material and methods

2.1. Patients

Two collectives of patients with CRLM from 2002 to 2016 were included into the study. In the first, 81 patients diagnosed at the Inselspital, University Hospital Bern, Switzerland, and for whom tissue blocks were available from metastatic lesions were entered into this study. All cases were re-reviewed for histopathology and used to construct a multi-punch tissue microarray. A second collective of 76 CRLM patients was included and used to study expression of biomarkers on whole slides. Eighteen cases were common to both cohorts. Upon re-review of the H&E slides, the tumor block with the largest metastasis was included in downstream analysis. Clinicopathological information can be found in Table 1. All the CRLM cases were mismatch-repair proficient. No information on overall or progression-free survival were included in this study. This retrospective study was approved by the local ethics committee of the Canton of Bern (#2020−00498)

2.2. Tissue microarray

For the first collective, upon re-review of tissue slides, tissue blocks corresponding to selected representative areas of the metastatic lesion were retrieved from the archives of the Institute of Pathology, University of Bern. A fresh H&E section was made and all slides were scanned (Pannoramic P250, Budapest, Hungary). Each scan was annotated in two regions of interest (ROI), namely the metastasis center and the metastasis front and both ROIs were annotated twice. In a few cases, where >1 metastasis was available, more ROIs were made. The final number of ROIs was 196. Using a tissue microarrayer, the digital ROIs from the scans were aligned with images of the tissue blocks, ROIs were cored out and transferred into two sets of next-generation Tissue Microarrays (ngTMA®). In order to account for possible tumor heterogeneity, two punches were taken from the center of the metastasis and two punches from the periphery, in each case. An H&E of each was sectioned.

Table 1
Characteristics of stage IV CRC patients in cohort 1 and 2.

		Cohort 1 (n = 81)	Cohort 2 (n = 76)
Gender	Male	55 (67.9 %)	44 (57.9 %)
	Female	26 (32.1 %)	32 (42.1 %)
Age at metastasis diagnosis	Mean, range	61.4 (26−86)	63 (40−81)
Neoadjuvant therapy	None	8 (9.9 %)	9 (11.8 %)
	Yes	21 (26.0 %)	18 (22.2 %)
	Unknown	52 (64.2 %)	49 (60.5 %)
Metastasis	Synchronous	55 (67.9 %)	40 (52.6 %)
	Metachronous	25 (30.9 %)	34 (44.7 %)
Size of largest metastasis (cm)	Mean, range	4.0 (0.5−16)	3.9 (0.2−16)
% necrosis	Mean, range	26.3 (5−60)	30.7 (0−90)
% fibrosis	Mean, range	25.5 (5−60)	30 (5−95)
Growth pattern			
% desmoplastic	Mean, range	39.9 (0−100)	55.4 (0−100)
% replacement	Mean, range	57.1 (0−100)	40.1 (0−100)
% pushing	Mean, range	3.0 (0−96)	4.5 (0−75)
% sinusoidal	Mean, range	0%	0%
% portal	Mean, range	0%	0%

2.3. Immunohistochemistry

All ngTMAs® were cut at 2.5 um. Whole slides of the CRLM in the second collective were also sectioned and stained similarly.

All immunostainings was performed by automated staining using Bond RX (Leica Biosystems) immunostainer and dewaxed in Bond dewax solution (Leica Biosystems). The antigen retrieval was performed for all sections in Tris buffer at 95° for 30 min (Leica Biosystems). For all single immunostainings, PD-1 (Cell Marque, clone NAT105, Ref 315M-95) diluted at 1:100, PD-L1 (Cell Signaling, clone E1L3N, Ref 13684) diluted at 1:400 and RHAMM (Abcam, Ref ab108339) diluted at 1:250 were in incubated at RT for 30 min. Then, all samples were incubated HRP (Horseradish Peroxidase)-polymer for 15 min and subsequent visualized using 3,3-Diaminobenzidine (DAB) as brown chromogen (Bond polymer refine detection, Leica Biosystems, Ref DS9800) for 10 min.

For double immunostaining, all primary antibodies were incubated sequentially: first step, mouse PanCK antibody (Agilent, clone AE1/AE3, Ref M351501−2) was diluted 1:400, incubated for 30 min. Then, samples were incubated in HRP and DAB, as above. On a second step, mouse CD8 antibody (Dako- Agilent, clone C8/144B, Ref M7103) was diluted 1:100, incubated for 30 min. Secondary antibody, AP (Alkaline phosphatase)-polymer for 15 min, and visualized using fast red as red chromogen (Red polymer refine Detection, Leica Biosystems, Ref DS9390).

In common for all, the samples were counterstained with Haematoxylin and mounted with Aquatex (Merck). Slides were scanned and photographed using Pannoramic 250 (3DHistech). Representative images are shown in Fig. 1.

2.4. Biomarker assessment

Tumor budding was evaluated in both the ngTMA® slides and whole slides. In the former, the number of tumor buds in each punch was recorded and then the punch with the maximum value of budding was

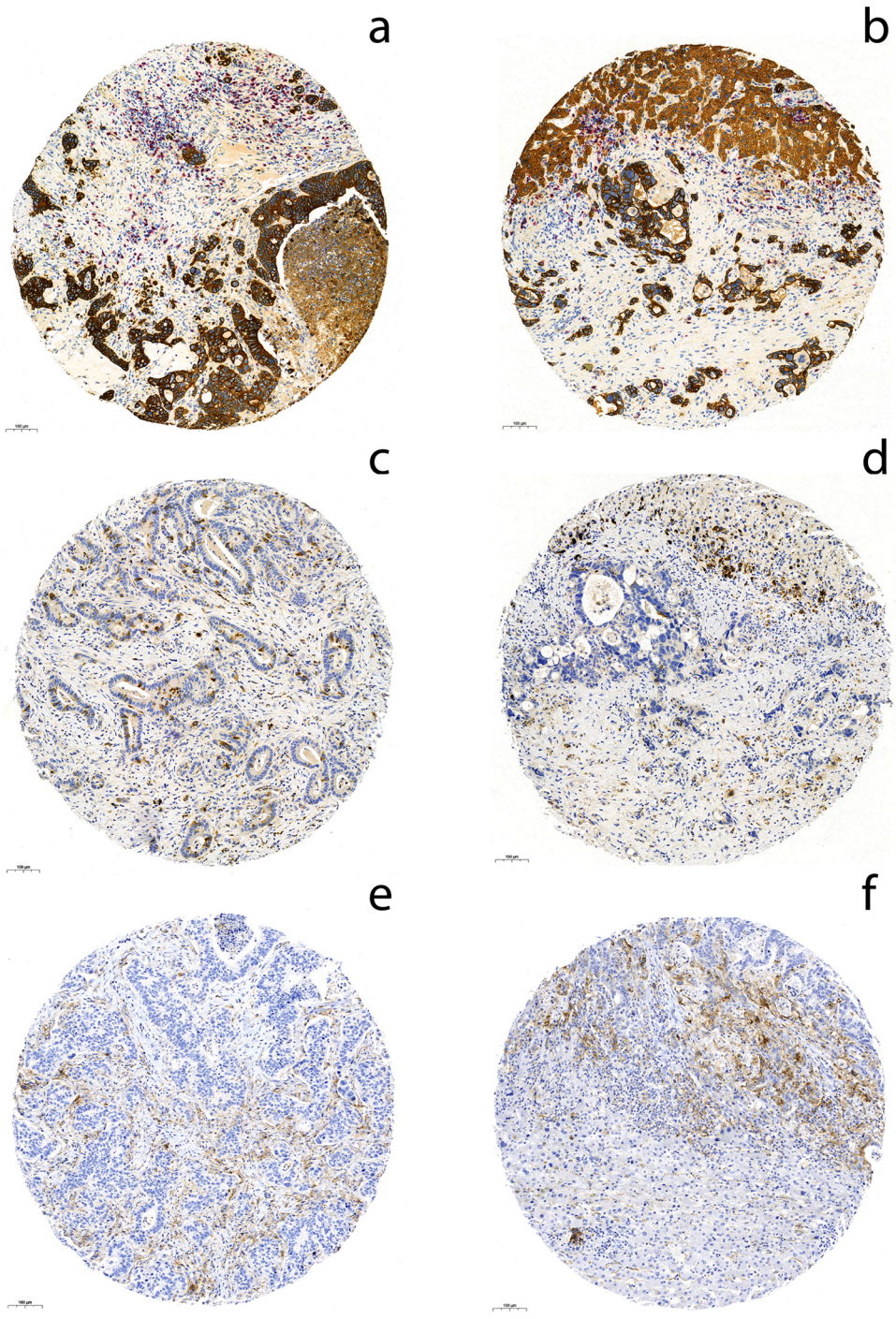

Fig. 1. Staining examples of the selected biomarkers for the present study: Presence of intrametastatic (A) and perimetastatic (B) CD8 positive T-Cells (red). Cytokeratin staining (brown) is only used for visualization, but not for assessment of tumor buds. Intrametastatic (C) and perimetastatic (D) tumor buds expressing RHAMM. PDL-1 positivity mainly in the intrametastatic (E) and perimetastatic (F) tumoral stroma in comparison to the expression of the neoplastic cell population. Scale bar 100 μm, 5x magnification.

used for analysis of both tumor budding and CD8+ T-lymphocytes. For the latter, we assessed the average number of buds in 10 high-power fields at the invasion front (perimetastatic budding, PMB) and within the main tumor body (intrametastatic budding, IMB) based on H&E staining [14]. The histopathological growth patterns (desmoplastic, pushing and replacement subtype) were scored according to the international consensus guidelines published in 2017 [28].

For RHAMM, the percentage of immunoreactive (RHAMM-positive) tumor cells over the total number of tumor cells was evaluated in both TMA punches (again, the average was taken across tumor area) and whole slides [22,29]. For PD-1 and PD-L1, a previously published scoring method based on 4 grades was used to semi-quantify the PD-L1 in the tumor stroma (from 0 to 3) and across multiple punches from the same tissue area, used the median value for analysis [30,31].

2.5. Statistics

Spearman's correlation coefficient was used to determine the strength of the relationships between all quantitative or ordinal variables. P-values were two-sided and considered statistically significant when <0.05. All analyses were carried out using SAS V9.4.

3. Results

IMB was associated with a higher count of CD8+ T-cells ($p < 0.0001$; $r = 0.58$) as well as with replacement tumor growth pattern ($p =$

0.0049). Consequently, a desmoplastic growth pattern was negatively correlated with the number of tumor buds (r=-0.38; p = 0.0004). RHAMM-positive IMB were linked to a higher PD-L1 expression (r = 0.32; p = 0.233 and r = 0.28; p = 0.044) in the center and periphery of the metastasis. PMB was also correlated with a higher CD8+ T-cell count (p < 0.0001; r = 0.53) but not with any particular growth pattern. RHAMM-positive PMB was similarly associated with a higher expression of PD-1 (r = 0.33; p = 0.0297), and especially PD-L1 (r = 0.604; p < 0.0001 and r = 0.43; p = 0.003) in the center and periphery of the metastasis and in general, the expression of RHAMM, regardless of location within the lesion was linked to a greater expression pf PD-L1 (r = 0.32; p = 0.0059 and r = 0.451; p = 0.0001; center and front of metastasis respectively). These interactions are illustrated in Fig. 2.

4. Discussion

In the era of personalized healthcare, pathology has an important role in the course of a promising biomarker to make its way from hypothesis to implementation in daily practice. In that sense, we embarked on this study using two CRLM cohorts and hypothesis-driven biomarkers to achieve a clear aim, namely outline a potential basis for multi-centric retrospective and prospective clinical trials in stage IV colorectal cancer patients with pMMR status. The obtained results are promising and worth being validated in further clinical trials. In summary, the assessment of RHAMM in IMB and/or PMB in biopsies or resections of CRLM could lead to the selection of stage IV CRC patients with pMMR status who could potentially benefit from immunotherapies. The summarized results are supported by the following clinico-pathological aspects:

First, tumor budding is an independent prognostic biomarker in all CRC stages based on robust data in the literature [32]. Nevertheless, tumor budding is included in international classifications and guidelines only for two clinical scenarios, namely as potential indicator for an oncologic resection in pT1 CRC or for a postoperative therapy in stage II CRC [9]. Recently, several studies focus on two additional clinical scenarios which need further data before their implementation in daily practice. In preoperative biopsies of rectal cancer patients, tumor budding is associated with tumor progression and decreased regression grade and is therefore along with other clinico-pathological parameters a potential indicator for neo-adjuvant therapy [33,13]. The available data on the role of tumor budding in CRLM are still in a preliminary phase and the present study would be just the third analysis on the potential role of tumor budding in CRLM. In 2018, Fonseca et al.

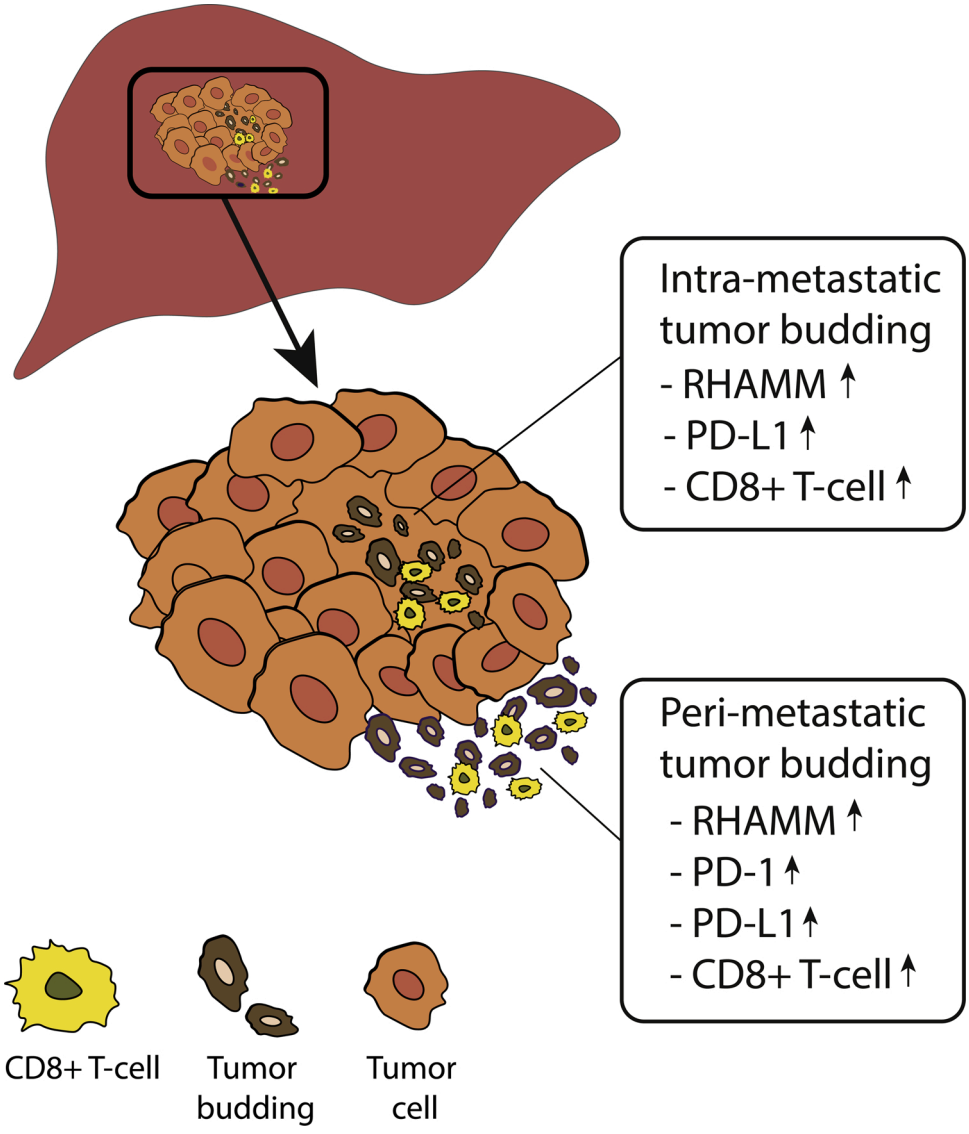

Fig. 2. Visualisation of the interaction between RHAMM, cytotoxic T-cells, PD1/PDL-1, perimetastatic and intrametastatic tumor budding in colorectal cancer liver metastases.

described for the first time the role of tumor budding in CRLM [15]. Tumor budding was associated with portal invasion, infiltrative border and survival in univariate, but not in multivariate analysis [15]. Our own research group systematically investigated on the possibilities for assessment of tumor budding in CRLM and implemented the terms IMB and PMB in 2019 [14]. We concluded that tumor budding assessment in CRLM is definitely challenging in cases without desmoplastic stroma reaction or accentuated ductular proliferation and proposed therefore to score tumor budding based on H&E staining and avoid immunohistochemistry [14]. According to the international consensus guidelines for scoring the histopathological growth patterns of liver metastasis published in 2017, the desmoplastic, pushing and replacement subtypes are the most frequently observed variants [28]. The combining of the IMB/PMB concept and the consensus patterns revealed interesting pathogenetic aspects. The growth pattern subtypes are assessed at the periphery of the metastasis; the lack of a perimetastatic desmoplastic stroma reaction does not exclude a high IMB count, which is supported by the association of IMB with replacement tumor growth pattern. A possible explanation could be a switch from PMB to IMB in case there is no desmoplastic stroma at the periphery, a mechanism that would allow to the CRLM to keep tumor progression. This hypothesis and observation should be followed and definitely needs further functional data.

Second, the biomarkers included in the study, RHAMM, cytotoxic T-cells and PD1/PD-L1, were selected according to the data in the literature. RHAMM is a multi-functional protein, typically activated in wound healing [34], and involved in different signalling pathways by binding proteins such as FAK [35], Src [36] or ERK [37,38]. Additionally, RHAMM activation leads to regulation of mitotic spindle integrity, cell cycle progression, reorganization and degradation of the extracellular matrix [23]. In CRC, RHAMM is an independent prognostic factor and its expression by tumor buds increases motility, dissemination and infiltration of lymph and blood vessels [22,29,19]. These pathogenetic features of RHAMM in CRC were supported by a functional study showing that RHAMM downregulation essentially abolishes metastases in mouse models [23]. An interesting additional aspect is the potential immunogenic aspect of RHAMM reflected by its association with an increased T-cell lymphocyte infiltration and normally detected in dMMR CRC [26, 24,25,21]. Not surprisingly, because of its link to a prominent peritumoral T-cell infiltration, dMMR status is considered a surrogate of the PD1/PD-L1 status and therefore an indicator for a checkpoint inhibitor therapy in stage IV CRC [17]. One important rationale for personalised healthcare is to stratify patients into subgroups which qualify for a specific therapeutic approach and indeed, there is enough evidence in the literature that immune infiltration and microsatellite status are independent of each other [39,40]. In that line, the obtained results in this study seem to be promising by proposing a specific subgroup of stage IV CRC patients with pMMR status and RHAMM expression in IMB/PMB which may qualify for a checkpoint inhibitor therapy.

The main goal of this manuscript is clearly not to present a prognostic or predictive analysis, but to test preliminary a potential biomarker which can be validated in well-defined stage IV CRC multicentric retrospective and prospective trials. Additionally, it has to be stated that the sample size in the present study is clearly too small and not fully characterized to draw definitive conclusions, but nevertheless the obtained results seem to be quite promising: RHAMM could be potentially assessed in IMB and PMB of CRLM biopsies or resections, respectively. The question which needs to be answered now is: do RHAMM positive CRLM cases with pMMR status benefit from immunotherapy in comparison to RHAMM negative CRLM cases?

Authors' specific contribution

Sandra Burren: Selection of cases, biomarker analysis, editing manuscript.

Katharina Reche: Selection of cases, biomarker analysis, editing manuscript.

Annika Blank: Data management, biomarker analysis, editing manuscript.

José A, Galvan: Figure management, editing manuscript.

Heather Dawson: Biomarker analysis, editing manuscript.

Martin D. Berger: Data management, editing manuscript.

Inti Zlobec: Data management, statistics, study design, editing manuscript.

Alessandro Lugli: Study design, biomarker analysis, editing manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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