

The molecular basis of EPCAM expression loss in Lynch syndrome-associated tumors

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Germline deletions affecting the Epithelial cell adhesion molecule (EPCAM) gene lead to silencing of MSH2 and cause Lynch syndrome. We have recently reported that lack of EPCAM expression occurs in many, but not all tumors from Lynch syndrome patients with EPCAM germline deletions. The differences in EPCAM expression were not related to the localization of EPCAM germline deletions. We therefore hypothesized that the type of the second somatic hit, which leads to MSH2 inactivation during tumor development, determines EPCAM expression in the tumor cells. To test this hypothesis and to evaluate whether lack of EPCAM expression can already be detected in Lynch syndrome-associated adenomas, we analyzed four carcinomas and two adenomas from EPCAM germline deletion carriers for EPCAM protein expression and allelic deletion status of the EPCAM gene region by multiplex ligation-dependent probe amplification. In four out of six tumors we observed lack of EPCAM expression accompanied by biallelic deletions affecting the EPCAM gene. In contrast, monoallelic retention of the EPCAM gene was observed in the remaining two tumors with retained EPCAM protein expression. These results demonstrate that EPCAM expression in tumors from EPCAM deletion carriers depends on the localization of the second somatic hit that inactivates MSH2. Moreover, we report lack of EPCAM protein expression in a colorectal adenoma, suggesting that EPCAM immunohistochemistry may detect EPCAM germline deletions already at a precancerous stage.

Modern Pathology (2012) 25, 911–916; doi:10.1038/modpathol.2012.30; published online 2 March 2012

Keywords: colorectal cancer; EPCAM/TACSTD1; immunohistochemistry; Lynch syndrome; multiplex ligation-dependent probe amplification (MLPA)

Lynch syndrome, clinically referred to as hereditary non-polyposis colorectal cancer (HNPCC), is one of the most common hereditary cancer predisposition syndromes that is associated with a high-lifetime risk of colorectal carcinomas and extracolonic tumors including endometrial cancers. Lynch syndrome is responsible for about 3% of colorectal

cancers.¹ Germline mutations underlying Lynch syndrome affect the DNA mismatch repair genes² such as *MLH1*³ or *MSH2*,⁴ and less frequently *MSH6*⁵ or *PMS2*.⁶ Lynch syndrome-associated tumors are characterized by DNA mismatch repair deficiency, which results from a second somatic event inactivating the respective remaining functional mismatch repair gene allele.⁷ As a consequence, tumorigenesis is promoted by secondary mutations that accumulate at short repetitive sequences, a phenotype termed high-level microsatellite instability.

In Lynch syndrome diagnostics, the detection of high-level microsatellite instability in tumor tissue is supplemented by mismatch repair protein immunohistochemistry, which allows predicting the mismatch repair gene affected by a germline mutation.⁸

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Received 6 September 2011; revised 16 November 2011; accepted 16 November 2011; published online 2 March 2012

Deletions affecting the polyadenylation site located in exon 9 of the Epithelial cell adhesion molecule (*EPCAM*) gene located upstream of *MSH2* have recently been identified as a novel cause of Lynch syndrome.⁹ *EPCAM* deletions lead to a transcriptional read-through, silencing *MSH2*⁹ and are estimated to cause Lynch syndrome in ~20–25% of patients with *MSH2*-negative cancers, but no detectable *MSH2* germline mutation.¹⁰ This corresponds to ~2–3% of Lynch syndrome patients.¹⁰

A recent study demonstrated that concomitant lack of *EPCAM* and *MSH2* protein expression is a feature highly specific for cancers from *EPCAM* deletion carriers, suggesting *EPCAM* immunohistochemistry as a potential tool for the identification of Lynch syndrome patients with *EPCAM* germline deletions.¹¹ However, *EPCAM* protein expression was retained in some cancers from *EPCAM* deletion carriers. There was no relation between *EPCAM* protein expression status in cancers and the localization of the *EPCAM* germline deletion.¹¹

We here hypothesized that the localization of the second somatic hit leading to *MSH2* inactivation determined *EPCAM* expression in the tumor cells. To that end, we analyzed *EPCAM* protein expression and allelic deletion status in the *EPCAM* gene by

multiplex ligation-dependent probe amplification (MLPA) using DNA isolated from tumor tissue and corresponding blood samples in *EPCAM* germline deletion carriers.

Materials and methods

Patients' Characteristics and Tumors

Clinical specimens used in this study were collected at the Department of Applied Tumor Biology, Institute of Pathology, University Hospital Heidelberg in the context of the German HNPCC Consortium funded by the Deutsche Krebshilfe (German Cancer Aid). Tumors and corresponding blood samples were obtained from five Lynch syndrome patients with known germline deletions affecting a genetic region encompassing exon 9 of the *EPCAM* gene. Family history and clinicopathological data are provided in Figure 1. Patients 1 and 2 were related, sharing the same germline deletion, and patients 3, 4, and 5 were from independent families. Exon-wise sequencing of the *MLH1* and *MSH2* genes had been performed in all index patients (patients 1, 3, 4, and 5), and no pathogenic germ line mutations were detected in these genes. Results of *EPCAM*

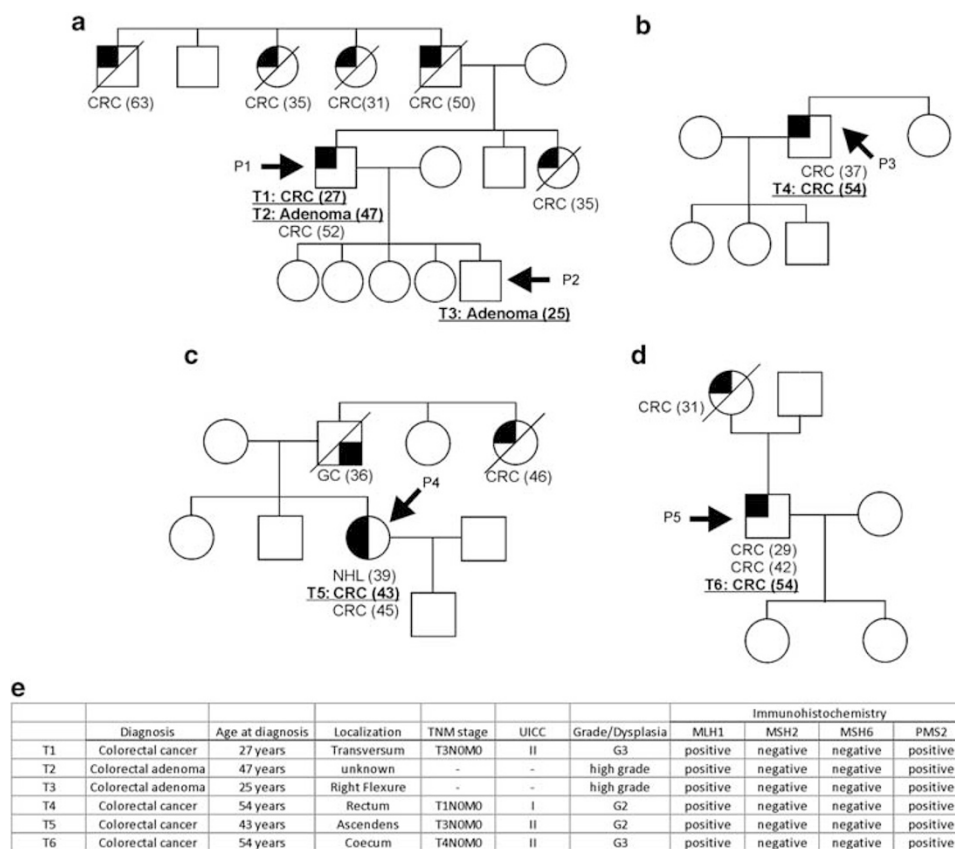


Figure 1 Family history of included patients (a–d). Index patients are marked by arrows (P1–P5), analyzed tumors are underlined and bold (T1–T6). Clinical and histopathology information is provided in (e). Tumors are abbreviated as follows: Adenoma, colorectal adenoma; CRC, colorectal cancer; GC, gastric cancer; NHL, non-Hodgkin lymphoma.

immunohistochemistry of tumors from patients 3, 4, and 5 (colorectal carcinomas) and patient 1 (small bowel carcinoma) have been presented previously.¹¹ In addition, two colorectal adenomas (patient 1 and 2) were included in the present study.

The study was approved by the Institutional Ethics Committee, and written informed consent was obtained from all patients included in this study.

DNA Isolation

DNA was extracted from whole blood and tumor specimens using the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry of MSH2 and EPCAM was performed using mouse monoclonal antibody clones FE11 (dilution 1:200, Calbiochem, Gibbstown, NJ, USA) and BER-Ep4 (1:100, Dako, Glostrup, Denmark), respectively, following standard procedures. Briefly, tissue sections (2 μ m) from formalin-fixed paraffin embedded tissue were mounted on aminopropylsilane-coated slides (SuperFrost, Menzel, Braunschweig, Germany). Following deparaffinization and rehydration, slides were boiled in 10 mM citrate buffer (pH 6) for 15 min for antigen retrieval. After cooling for 20 min, endogenous peroxidase activity was blocked by incubation with 0.6% H₂O₂ in methanol during 20 min. Sections were blocked with 10% normal horse serum (Vectastain Elite ABC kit, Vector, Burlingame, CA, USA), before the application of the primary antibodies, which was followed by incubation over night at 4°C. After washing, biotinylated secondary antibody (1:50 dilution, horse anti-mouse IgG, Vectastain Elite ABC kit, Vector) was applied, followed by incubation at room temperature for 30 min, and the application of the AB reagent according to the manufacturer's instructions (Vectastain Elite ABC kit). Visualization was performed using AEC chromogen (Dako), and sections were counterstained with hematoxylin (AppliChem, Darmstadt, Germany) and mounted with Aquatex (Merck, Darmstadt, Germany).

Multiplex Ligation-Dependent Probe Amplification

MLPA is a method for the detection of genomic deletions by quantitative hybridization and subsequent multiplex amplification of probes specific for different regions of a gene or multiple genes of interest.¹² Here, MLPA was used to detect deletions affecting the *EPCAM* and/or *MSH2* genes.

We performed MLPA by using the SALSA MLPA kits P072-B1 and P003-B1 (MRC Holland,

Amsterdam, The Netherlands), following the manufacturer's instructions. The kits contain probes for sequences within exons 3, 8, and 9 of *EPCAM*, exons 1–16 of *MSH2* and three probes for sequences in the intergenic region between *EPCAM* and *MSH2*. MLPA was performed using DNA isolated from formalin-fixed tumor tissues to evaluate potential somatic deletions. DNA isolated from corresponding blood samples was used to quantify the germline allelic status. DNA of a healthy donor who did not harbor any deletions affecting the genomic region encompassing *EPCAM* and *MSH2* was used as reference.

MLPA analysis was performed twice for each sample. Amplified products were visualized on an ABI3100 sequencer (Applied Biosystems, Darmstadt, Germany). Peak areas were quantified according to the manufacturer's suggestions for intra-sample and inter-sample normalization. Briefly, peak areas of each probe were divided by the sum of the reference probe peak areas for each of the samples separately to ensure intra-sample normalization. Intra-normalized probe ratios were then divided by intra-normalized probe ratios of the reference sample. Ratios lower than 0.8 were regarded as heterozygous deletions, lower than 0.3 as biallelic deletions.

Exon-wise sequencing of *MSH2* was performed for the colorectal adenoma from patient 1 as described previously.¹³

Results

EPCAM Protein Expression

EPCAM protein expression was absent in four out of six tumors (two out of three CRCs, one small bowel cancer, one out of two colorectal adenomas) from heterozygous *EPCAM* germline deletion carriers and retained in the remaining two tumors. EPCAM loss was spatially congruent with MSH2 loss in all specimens. Representative staining results of the colorectal adenoma from patient 1 are shown in Figure 2.

Deletions of *EPCAM* and EPCAM Protein Expression

MLPA analysis from tumor tissue revealed biallelic deletions affecting the *EPCAM* gene in four out of six analyzed tumors. In the remaining two tumors, no biallelic *EPCAM* deletions were detected, and the allelic profile obtained for the *EPCAM* gene region was identical in DNA isolated from tumor tissue and matched blood samples.

All tumors showing biallelic deletions in the *EPCAM* gene region were negative for EPCAM protein expression, whereas EPCAM protein expression was retained in the tumors with a retained second *EPCAM* allele (CRC from patient 5 and colorectal adenoma from patient 1, Figure 3). In one

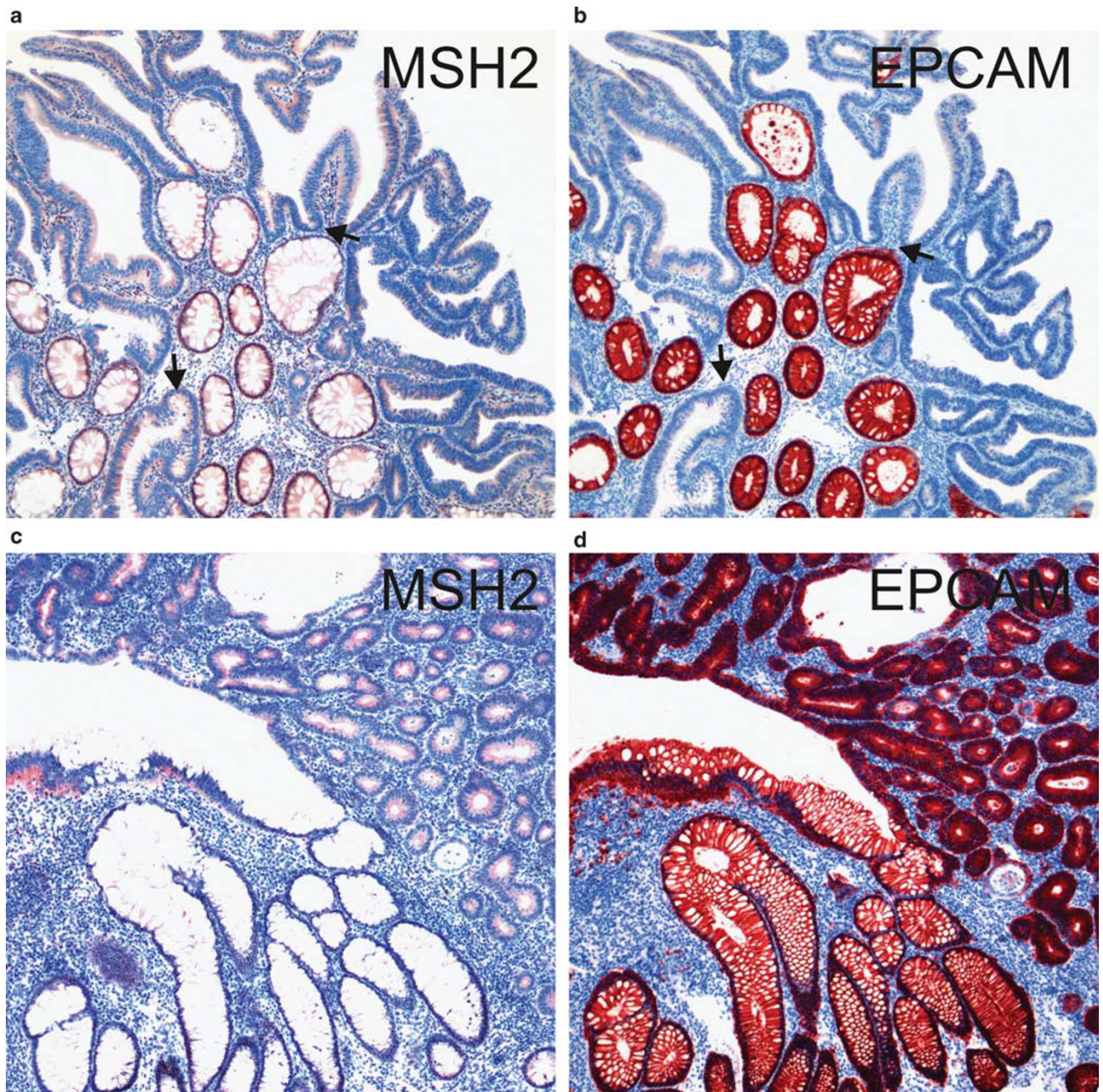


Figure 2 Concomitant loss of EPCAM (a) and MSH2 (b) protein expression is observed in a colorectal adenoma from an EPCAM germline deletion carrier with Lynch syndrome (patient 2, $\times 100$ magnification). EPCAM loss colocalized with MSH2 loss (arrows), suggesting synchronous inactivation of EPCAM and MSH2 through the same somatic event. The adenoma from patient 1 ($\times 100$ magnification, lower panel) shows lack of MSH2 (c), but retained EPCAM expression (d).

of these tumors (colorectal adenoma from patient 1), the heterozygous mutation c.942 + 3A > T, which is known to inactivate *MSH2*,¹⁴ was identified as the second somatic hit causing loss of MSH2.

In three out of four tumors, germline deletions were exactly mirrored by the corresponding somatic deletions (Figure 3, tumors from patient 1, 2, and 3). In one tumor (tumor from patient 4) the somatic deletion affecting *EPCAM* spanned a smaller area than the germline deletion (Figure 3).

Discussion

Concomitant loss of EPCAM and MSH2 protein expression is a typical feature of tumors from *EPCAM* germline deletion carriers with Lynch syndrome.¹¹ In this study, we correlated EPCAM protein expression with the second somatic hit that leads to the inactivation of *MSH2* in Lynch syndrome patients. Only if the second hit affected the *EPCAM* gene resulting in a biallelic *EPCAM*

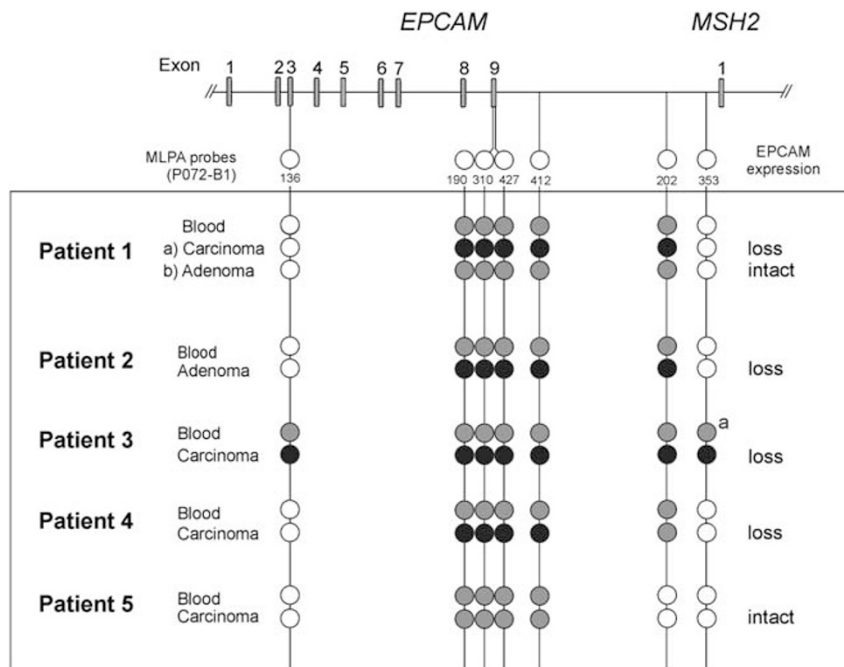


Figure 3 Results of *EPCAM* deletion analysis. MLPA probes are shown in relation to the genomic region encompassing *EPCAM* and the 5' region of *MSH2*. MLPA probes are denoted by fragment lengths (SALSA MLPA kit P072-B1, MRC Holland). White circles: biallelic presence, gray circles: heterozygous deletion, black circles: biallelic deletion. a—Exons 1–15 of the *MSH2* gene showed heterozygous deletion in blood and biallelic deletion in the tumor, exon 16 was biallelically retained in blood and tumor.

deletion, we observed a loss of EPCAM protein expression. EPCAM loss was spatially congruent with the loss of MSH2 protein expression in all analyzed lesions (Figure 2), suggesting that one somatic event led to the simultaneous inactivation of both EPCAM and MSH2.

In tumors with retained EPCAM expression, the MLPA profile obtained for the *EPCAM* gene region was identical to the profile of corresponding normal blood cells. These data demonstrate that the lack of EPCAM protein expression observed in tumors from *EPCAM* deletion carriers requires biallelic *EPCAM* deletion, which results from a combination of germline alteration and second somatic hit. This, *vice versa*, implies that heterozygous *EPCAM* germline deletions are not necessarily associated with loss of EPCAM expression in tumor tissue. The detection of a somatic mutational event causing MSH2 inactivation in one of the EPCAM-positive tumors (adenoma from patient 1) explains why some tumors from *EPCAM* germline deletion carriers show loss of MSH2, but retained EPCAM expression.¹¹

The crucial role of the second hit in determining EPCAM protein expression is further illustrated by the comparative analysis of three tumors from two related individuals who shared the same EPCAM deletion (patients 1 and 2, Figure 3). Consistent with EPCAM protein expression status, *EPCAM* MLPA revealed biallelic deletions affecting the *EPCAM* gene in the EPCAM-negative tumors, but monoallelic retention of *EPCAM* in the EPCAM-positive adenoma.

EPCAM immunohistochemistry has been suggested as a potential diagnostic tool in Lynch syndrome.¹¹ However, the dependence of EPCAM expression on both, germline and somatic alterations explains why EPCAM immunohistochemistry can yield inconspicuous results in a subset of tumors from *EPCAM* deletion carriers, namely if the second somatic *MSH2*-inactivating event does not affect the *EPCAM* gene.

The frequent occurrence of somatic deletions affecting the *EPCAM* gene as a second hit in tumors from *EPCAM* deletion carriers suggests that the localization of somatic events inactivating mismatch repair genes in Lynch syndrome is not random, but related to the underlying germline mutation. This is in line with the previous observation that large deletions affecting the *MLH1* or *MSH2* gene are particularly frequent in tumors from germline deletion carriers.¹⁵ Moreover, in three out of four tumors showing biallelic deletions affecting *EPCAM* the germline and somatic alteration was identical. This strongly supports the hypothesis that gene conversion is a common mechanism of mismatch repair gene inactivation in Lynch syndrome-associated tumors,¹⁵ demonstrating that this mechanism of locus-restricted recombination is also responsible for *MSH2* inactivation in tumors from *EPCAM* deletion carriers.

EPCAM MLPA proved applicable for the detection of heterozygous and homozygous deletions in DNA isolated from paraffin-embedded tumor tissue. Our data confirm that MLPA is suitable for germline deletion analysis in DNA isolated from

paraffin-embedded tissue,^{15,16} thus enabling molecular diagnostics in families affected by Lynch syndrome or other inherited disease conditions, if no blood is available for analysis.

In summary, we demonstrate that lack of EPCAM protein expression in tumors from *EPCAM* deletion carriers requires biallelic *EPCAM* deletion, resulting from the combination of a germline and a second somatic deletion, both affecting the *EPCAM* gene. Lack of EPCAM expression may already occur in precancerous lesions from *EPCAM* deletion carriers. Our study provides novel information about EPCAM immunohistochemistry in diagnostics of Lynch syndrome and elucidates the mechanism underlying alterations of EPCAM protein expression in *EPCAM* deletion carriers.

Acknowledgements

The excellent technical assistance of Beate Kuchenbuch, Stefanie Kellner, Petra Höfler, and Daniel Baumgärtner is gratefully acknowledged. This work was funded in part by Grants of the Deutsche Krebshilfe and the Deutsche Forschungsgemeinschaft (DFG, Grant number BL-554/3).

Author contributions

Conception and design: CH, MK, HB; financial support: PS, MvKD, HB; administrative support: PS, MvKD; provision of study materials or patients: MK, CE, HG, MT, PS, MvKD, HB; collection and assembly of data: CH, MK, AV, CE, HG, MT, GB, HB; data analysis and interpretation: CH, MK, AV, HB; manuscript writing: CH, MK, AV, GB, PS, MvKD, HB; final approval of the manuscript: CH, MK, AV, CE, HG, MT, GB, PS, MvKD, HB.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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