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Loss of mismatch repair protein immunostaining in colorectal adenomas from patients with hereditary nonpolyposis colorectal cancer

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Colorectal adenomas occur at younger age, at increased frequency and have a greater tendency for malignant transformation in patients with hereditary nonpolyposis colorectal cancer (HNPCC). We performed immunostaining for the mismatch repair proteins MLH1, PMS2, MSH2 and MSH6 in 35 colorectal adenomas from 26 patients with HNPCC and identified loss of immunostaining in 23/35 (0.66) adenomas. Loss of mismatch repair protein immunostaining was particularly frequent in large (>5 mm) (14/16) and proximally located (13/15) adenomas, whereas the gene mutated—MLH1 or MSH2—and the type of mutation did not seem to affect the results. We conclude that loss of mismatch repair protein immunostaining is detected at a lower rate in adenomas than in carcinomas associated with HNPCC. Adenomatous tissue can thus be used for immunostaining of mismatch repair proteins in clinical investigations of HNPCC, but whereas loss of immunostaining may pinpoint the gene affected and thereby guide mutation analysis, retained staining cannot exclude that the adenoma developed as part of the syndrome due to reduced sensitivity. However, the analysis has a greater chance of being informative if large and proximally located adenomas are selected.
Modern Pathology (2005) 18, 1095–1101. doi:10.1038/modpathol.3800392; published online 25 February 2005

Patients affected by hereditary nonpolyposis colorectal cancer (HNPCC) are at increased risk for several tumor types with the greatest risks for colorectal cancer (80% life-time risk) and endometrial cancer (40–60% risk for females). Control programmes for HNPCC-patients include colonoscopies, which aim at early identification and removal of precursor lesions. Adenomas are not numerous in HNPCC-individuals but occur at a higher frequency than in the general population. The adenomas also occur at younger age, have a predilection for the proximal colon, are larger and do more often display high-grade dysplasia and villous components.[1–3] The hallmark of HNPCC-associated tumors—defective mismatch repair—is in the tumor tissue reflected as microsatellite instability and loss of immunostaining for the mismatch repair protein affected.[4,5] Mismatch repair defects are found in about 15% of sporadic colorectal cancer and in >90% of HNPCC-associated cancers.[6,7] Whereas the mismatch repair defect is generally acquired at later stages in sporadic colorectal tumorigenesis, it has been described to occur at an early stage in HNPCC tumorigenesis. Consequently, a microsatellite instability-high phenotype has been reported in 0–4% of sporadic adenomas compared to 15–20% of sporadic carcinomas.[8–11] Whereas the mismatch repair defects in sporadic colorectal tumors are mainly associated with somatic hypermethylation of the MLH1 promoter, HNPCC tumors develop as a result of germline mutations in these genes.[7,12]

Data on the frequency of microsatellite instability in HNPCC-associated adenomas are still quite scarce, but indicate that defective mismatch repair can be found in 24–93% of the adenomas.[3,13–19] Detection of defective mismatch repair through analysis of microsatellite instability and/or loss of

mismatch repair protein immunostaining in color-ectal carcinomas predicts HNPCC with high sensitivity and specificity.[4,5,20] Clinically, analysis of defective mismatch repair in an HNPCC-associated adenoma is sometimes requested since this may be the only tumor tissue available and to pinpoint the gene that mutation analysis should focus on. We therefore assessed immunohistochemical expression of the mismatch repair proteins MLH1, PMS2, MSH2, and MSH6 in 35 adenomas from 26 HNPCC patients.

Materials and methods

Patients

Among the 26 patients, 23 carried disease-causing mismatch repair gene mutations; eight different mutations in *MLH1* (five of which were missense mutations) and two mutations in *MSH2* (both of which were truncating). The remaining three patients (adenomas SA30, S1E and SA6) had family histories suggestive of HNPCC and loss of immuno-staining for MSH2 in the tumor tissue, but no mutation had yet been identified (Table 1). Regarding type of germline mutation, 20 adenomas were derived from patients with truncating mutations or intragenic deletions and 12 from patients carrying missense mutations. Of the 35 adenomas, 30 were detected at biannual colonoscopies (although some of the larger tumors were detected at the first control) and five adenomas were found in surgical resections performed because of synchronous colo-rectal cancer. Adenoma size was 2–25 mm, and for five of the adenomas size was not available since they consisted of only a few adenomatous gland formations. Of the 35 adenomas, 15 were proximal,

Table 1 Clinicopathological data and findings of defective mismatch-repair

Adenoma number	Sex/age (years)	Size (mm)	Histology	Dysplasia grade	Adenoma location	Immunohistochemical staining				MSI status	Germline mutation	
						MLH1	PMS2	MSH2	MSH6		Gene	Codon
SA1	F/36	—	TA	LG	P	−	−	+	+	ND	*MLH1*	S44F
SA4	F/66	3	TA	LG	P	−	−	+	+	ND	*MLH1*	S44F
SA16	F/35	10	TA	LG	P	−	−	+	+	MSI	*MLH1*	G67R
SA18	F/40	10	TA	LG	P	−	−	+	+	MSI	*MLH1*	del exon 16
SA21	F/60	3	TA	LG	P	−	−	+	+	ND	*MLH1*	S44F
SA27A	F/56	—	TA	LG	P	−	−	+	+	ND	*MLH1*	M35R
SA27B	F/58	—	TVA	LG	P	−	−	+	+	ND	*MLH1*	M35R
SA37	M/48	7	TA	LG	P	−	−	+	+	MSI	*MLH1*	del exon 16
SA38	F/64	3	TA	LG	P	−	−	+	+	ND	*MLH1*	S44F
SA48	M/38	3	TA	LG	P	−	−	+	+	ND	*MLH1*	del exon 16
SA15	F/45	7	TA	LG	D	−	−	ND	+	MSI	*MLH1*	S44F
SA24A	F/46	2	TA	LG	D	+	+	+	+	ND	*MLH1*	del exon 16
SA24B	F/50	2	TA	LG	D	−	−	+	+	ND	*MLH1*	del exon 16
SA38	F/64	—	TA	LG	D	−	−	+	+	ND	*MLH1*	S44F
L2B	F/47	3	TA	LG	D	+	+	+	+	ND	*MLH1*	W714X
L2C	F/48	12	TVA	LG	D	−	−	+	+	MSI	*MLH1*	W714X
SA47	M/44	3	TA	LG	P	+	+	+	+	ND	*MLH1*	Splice exon 3
SA3	F/37	3	TA	LG	D	+	+	+	+	MSS	*MLH1*	del exon 16
SA7	M/48	5	TVA	LG	D	+	+	+	+	MSS	*MLH1*	del exon 16
SA11	M/35	3	TA	LG	D	+	+	+	+	ND	*MLH1*	I68N
SA31	M/64	5	TA	LG	D	+	+	+	+	MSS	*MLH1*	S44F
SA34	M/45	5	TA	LG	D	+	ND	+	+	MSS	*MLH1*	del exon 16
SA40	M/64	6	TA	LG	D	+	+	+	+	MSS	*MLH1*	R265C
C1A	M/78	12	TA	LG	P	+	+	−	−	MSI	*MSH2*	del A, 529
C1B	M/78	8	TA	HG	P	+	+	−	−	MSI	*MSH2*	del A, 529
C1C	M/78	10	TA	LG	P	+	+	+	+	MSS	*MSH2*	del A, 529
C2A	M/67	10	TA	LG	P	+	+	−	−	MSI	*MSH2*	del A, 529
C2B	M/67	13	SA	HG	D	+	+	−	−	MSI	*MSH2*	del A, 529
C3A	F/44	20	TVA	LG	D	+	+	−	ND	MSI	*MSH2*	del A, 529
C3B	F/72	25	TA	HG	D	+	+	−	−	MSI	*MSH2*	del A, 529
SA30	M/45	8	TA	HG	D	+	+	−	−	MSI	*MSH2*	Loss of MSH2 IH
S1E	F/38	6	TA	LG	D	+	+	−	−	MSI	*MSH2*	Loss of MSH2 IH
SA32A	M/40	10	TVA	LG	D	+	+	−	−	MSI	*MSH2*	R406X
SA32B	M/50	—	TA	LG	D	+	+	+	+	ND	*MSH2*	R406X
SA6	M/27	3	TA	LG	D	+	+	+	+	ND	*MSH2*	Loss of MSH2 IH

TA, tubular adenoma; TVA, tubulo-villous adenoma; SA, serrated adenoma; LG, low-grade; HG, high-grade; D, distal; P, proximal; ND, no data available; MSI, microsattelite instable; MSS, microsatellite stable; IH, immunohistochemistry.
The shaded areas indicate different cases, some of which contain multiple tumors.

that is, located proximal to the splenic colon flexure, and 20 were distal. Multiple (2–3) adenomas from the same patients were analyzed in seven cases and these adenomas are labelled A–C (Table 1).

Immunohistochemistry

Immunostaining was performed on fresh 4-μm sections of formalin-fixed, paraffin-embedded tissue blocks and mounted on DakoCytomation ChemMate Capillary Gap Microscope slides (DakoCytomation A/S BioTek Solutions, Mt Laurel, NJ, USA) and dried at room temperature overnight followed by incubation at 60°C for 1–2 h. The tissue sections were deparaffinized in xylol and rehydrated through descending concentrations of ethanol. Antigen retrieval was achieved by microwave treatment in 1 mM EDTA, pH 9.0, at 800 W for 8 min followed by 15 min at 300 W. The slides were then allowed to cool for at least 20 min in the EDTA solution. IHC staining was performed in an automated immuno-stainer (TechMate 500 Plus, DakoCytomation), according to the manufacturer's instructions. The antibodies and dilutions were as follows: mouse monoclonal IgG antibodies to MLH1 (clone G168-15, dilution 1:100; PharMingen, San Diego, CA, USA), MSH2 (clone FE-11, dilution 1:100; Oncogene research products, Boston, MA, USA), MSH6 (clone 44, dilution 1:1000; BD Transduction Laboratories, Lexington, KY, USA), and PMS2 (clone A16-4, dilution 1:500, BD PharMingen). For MLH1 and MSH2 we used the detection kit LSAB™ (labelled streptavidin biotin) (DakoCytomation). MSH6 and PMS2 were stained with the EnVision™ Detection kit (DakoCytomation) with an extra enhancing step. After incubation with the primary antibody Rabbit anti-mouse immunoglobulins (DakoCytomation, dilution 1:400) were applied to the sections and they were incubated for 20 min. All tissue sections were counterstained with hematoxylin for 1 min, rinsed in running tap water for 10 min, dehydrated in ascending concentrations of alcohol and mounted. Evaluation was performed independently by two of the authors (MN and BH). A positive staining was defined as an unequivocal nuclear staining in the neoplastic cells, and nuclear staining was required in the stromal components of the tumor. A tumor cell population (which in all cases included all cells in the adenomatous components of the specimens) without nuclear staining in the presence of staining in normal epithelial, stromal, or inflammatory cells or within infiltrating lymphoid cells were classified as having lost the expression (Figures 1 and 2).

Results

Loss of mismatch repair protein immunostaining was detected in 23/35 (0.66) adenomas and affected MLH1 in 14 tumors, PMS2 in 14, MSH2 in nine, and MSH6 in nine tumors (Figure 1, Table 1). Different results were found in the different subgroups. Among adenomas larger than 5 mm, 14/16 (0.88) showed loss of expression compared to 9/19 (0.47) of the adenomas 5 mm or less. Also tumor location correlated with loss of immunostaining; 13/15 (0.87) proximal adenomas showed loss compared to 10/20 (0.5) distal adenomas. In the biannual nonbaseline colonoscopy surveillance group 15 of 25 (0.6) adenomas showed loss of mismatch repair protein immunostaining, compared to 8/10 (0.8) of the remaining adenomas, but the mean adenoma size in the former group was 5 mm compared to 12 mm in the latter group. Considering the types of mutations, there was no major difference between mutations in *MLH1* (loss in 14/23, 0.61) and *MSH2* (loss in 9/12, 0.75) or according to type of mutation with loss in 7/12 (0.58) adenomas with missense mutations compared 12/20 (0.6) truncating mutations or intra-genic deletions. We assessed microsatellite instability status (using the markers BAT25, BAT26, BAT40, BAT34, D5S346, and D2S123) in 16 of the 17 adenomas larger than 5 mm, and found MSI in 14/16 (0.88) of these adenomas. The findings of microsatellite instability did in all tumors correspond to the immunohistochemical expression pattern; that is, all 14 adenomas with loss of staining showed microsatellite instability and the two adenomas with retained mismatch repair protein immunostaining showed a microsatellite stable phenotype (Table 1). Discordant immunostaining patterns in colorectal adenomas from the same patient (Figure 2) were found in four patients (cases SA24, SA32, C1, and L2) and affected MLH1 and MSH2 in two cases each (Table 1).

Discussion

Defective mismatch repair has been described in a wide range (24–93%) of HNPCC-associated adenomas, but because of a very low (0–4%) frequency in sporadic adenomas and high frequencies of germline mismatch repair gene mutations among patients with microsatellite instable adenomas, such defects in adenomas have been suggested to predict HNPCC with high accuracy.[10] Since adenomatous tissue must sometimes be used for assessment of mismatch repair defects in HNPCC investigations, we aimed at determining the frequency of loss of immunostaining for the mismatch repair proteins in adenomas from HNPCC-patients. Overall, loss of immunostaining was detected in 23/35 adenomas from 26 HNPCC-patients. All adenomas showing loss of mismatch repair protein immunostaining showed a concomitant loss of either MLH1/PMS2 or of MSH2/MSH6 (Table 1) and the analysis of microsatellite instability performed did in all cases correlate with the mismatch repair protein staining (Table 1). The overall rate of loss of immunostaining in our study, 0.66, is similar to that found by Rijcken *et al*,[3] who demonstrated loss of immunostaining for mismatch
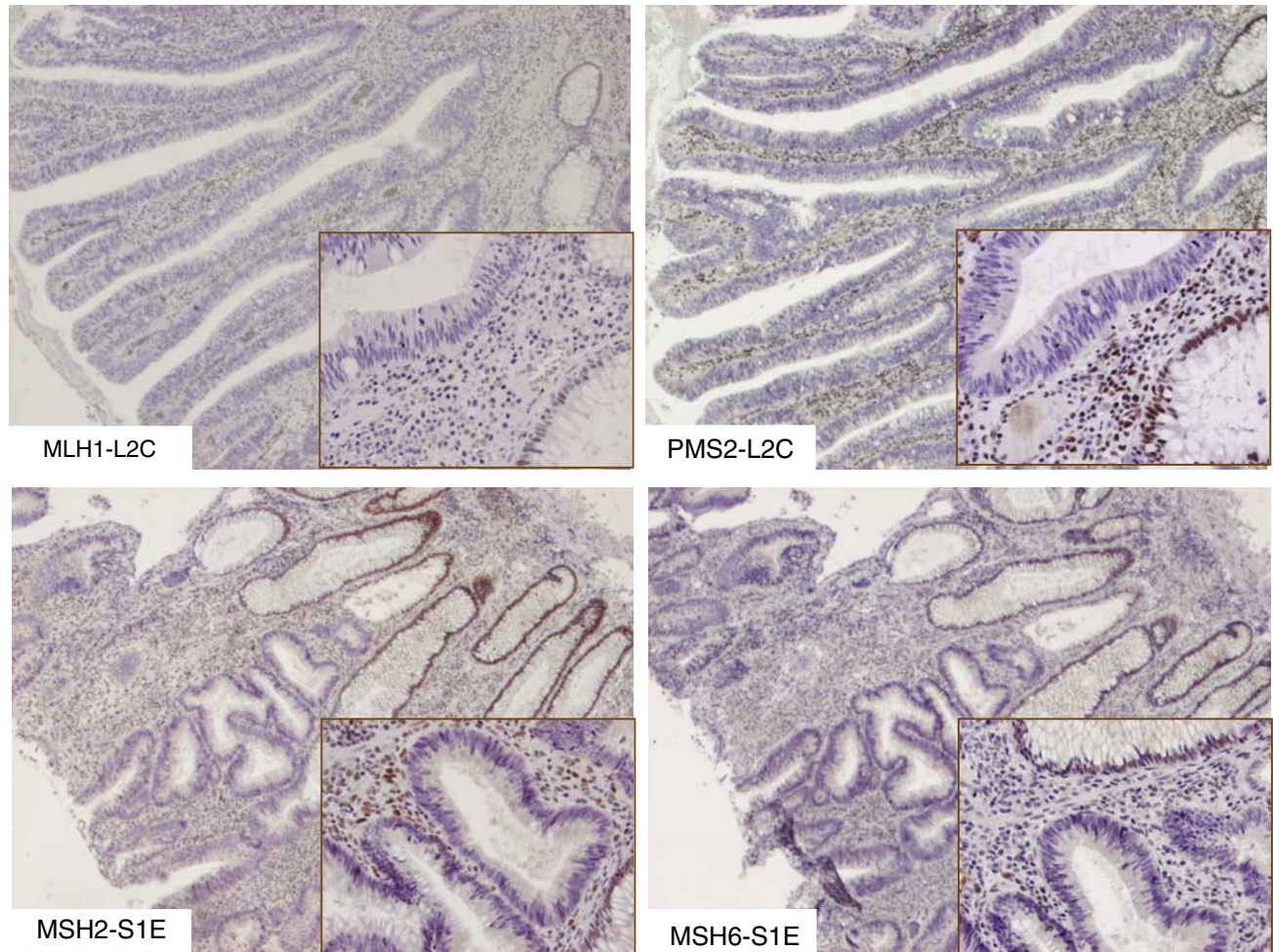
Figure 1 Loss of immunostaining in adenomas from HNPCC-patients with disease-causing mutations. *Top*: Loss of MLH1 and PMS2 staining in adenoma L2C from a patient with a nonsense mutation in *MLH1*. *Bottom*: Loss of MSH2 and MSH6 immunostaining in adenoma S1E from a patient with a frameshift mutation in *MSH2*.

repair proteins in 15/25 (0.6) HNPCC-associated microsatellite instability-high adenomas. We found the highest rates of loss of immunostaining in large (>5 mm) and proximal adenomas, in which the fractions of immunohistochemical loss of staining for the mismatch repair proteins were 0.88 and 0.87. Indeed, among the adenomas which did not reveal loss of immunostaining, 10/12 were located in the recto-sigmoid part of the colon and the only proximal adenoma with retained expression developed at high age and may therefore represent a sporadic lesion (Table 1).

A correlation between high-grade dysplasia and mismatch repair defects have been described.[3,18] In our series, all 13 adenomas with retained expression in our study showed low-grade dysplasia, and the four adenomas with high-grade dysplasia showed loss of staining (Table 1). In the series by Rijcken et al,[3] the 10 HNPCC-adenomas with retained expression were small and had low-grade dysplasia. The observation that loss of immunostaining for the mismatch repair proteins occurs at higher rates

in larger and highly dysplastic adenomas may either reflect a growth advantage inferred by the defective mismatch repair or may suggest that inactivation of the mismatch repair system occurs during tumor progression rather initiation. If the adenomas defective in mismatch repair have a growth advantage, this may explain why these tumors are larger and show frequent high-grade dysplasia, and we did indeed identify loss of immunohistochemical mismatch repair protein staining in several adenomas that were only a few millimeters in size. Alternatively, tumor initiation could depend on, for example, a mutation in the *APC* gene, whereafter the defective mismatch repair accelerates tumor progression, presumably through an accumulation of somatic frameshift alterations in various cancer-associated genes.[1] Although studies of molecular alterations in adenomas have not identified any major differences between sporadic and HNPCC-associated adenomas, and if also the HNPCC-associated adenomas achieve the mismatch repair-defective phenotype through somatic
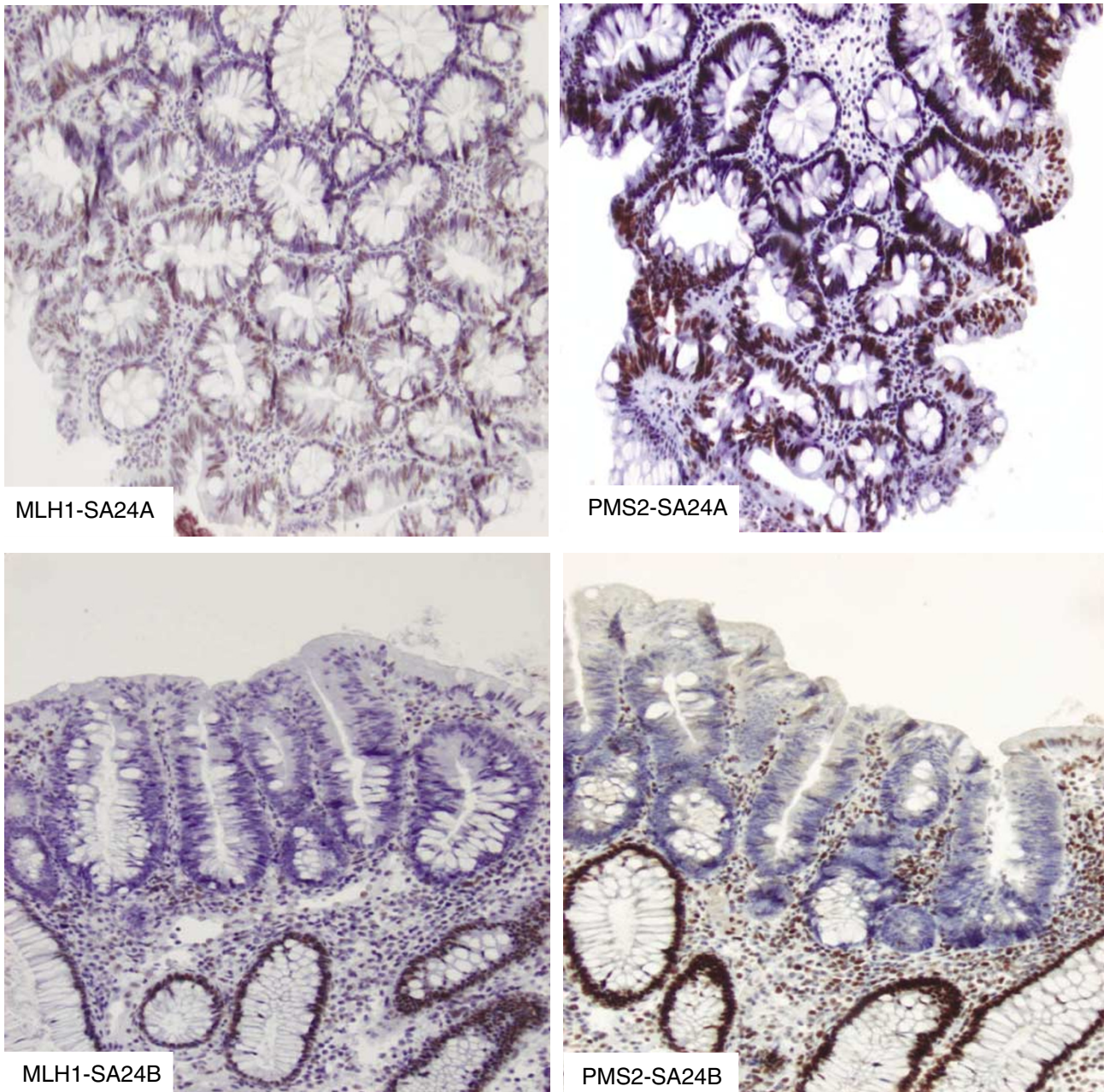
Figure 2 Discordant staining patterns in two adenomas from a patient with del of exon 16 in *MLH1*. Adenoma SA24A shows retained staining for MLH1 and PMS2, whereas adenoma SA24B shows loss of immunostaining for MLH1 and PMS2.

hypermethylation of the *MLH1* promoter, this could contribute to the relative predominance of proximal carcinomas in HNPCC.[21]

Hyperplastic polyps are generally small (they rarely exceed 1 cm in size), commonly occur within the large bowel, and have traditionally been viewed as non-neoplastic. However, clonal genetic changes—including mutations of *KRAS* and *BRAF* and microsatellite instability—have been demonstrated in hyperplastic polyps.[11,15,18,19,22–24] These lesions have been associated with an increased risk of malignancy in the hyperplastic polyposis syndrome, but may signify an increased risk of colo-

rectal cancer also in other individuals.[18,22] In HNPCC as well as in familial colorectal cancer, a strong correlation has been found between adenomas and hyperplastic polyps, suggesting similar mechanisms for their initiation.[25,26] We investigated 12 hyperplastic polyps that had occurred in the 26 patients in the study and found retained immunostaining for all four mismatch repair proteins in all polyps (data not shown), which is in accordance with previous studies and suggests that the value of immunostaining in these lesions is of limited clinical value.[27] Serrated adenomas have been suggested to represent more specific precursors

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of microsatellite instable tumor development and show distinct molecular features, such as microsatellite instability and a low frequency of *KRAS* mutations, but the serrated pathway is also heterogenous.[28] Only one serrated adenoma was included in our series and this tumor occurred in conjunction with an *MSH2* mutation and showed loss of MSH2/MSH6 protein expression.

In conclusion, immunostaining for defective mismatch repair proteins in HNPCC-associated adenoma has a high specificity and can thereby direct the mutation screening to the gene affected. However, loss of immunostaining for these proteins occurs at a lower frequency in adenomas than in carcinomas, and depends on the size and location of adenoma. From a clinical diagnostic point of view, retained immunostaining for the mismatch repair proteins in small (<5 mm) and left-sided adenomas cannot be used to exclude HNPCC, whereas loss of staining in a patient suspected of having HNPCC may be used to direct mutation analysis.

Acknowledgements

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