Immunohistochemical detection of the hMLH1 and hMSH2 proteins in hereditary non-polyposis colon cancer and sporadic colon cancer*

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Defects in DNA mismatch repair system are involved in carcinogenesis of sporadic and inherited human cancers. We assessed the feasibility of using immunohistochemistry to detect tumors with DNA mismatch repair deficiency.

We analyzed 81 samples (74 colon cancers (CC), 1 colon dysplasia and 6 extracolonic cancers) for hMLH1 and hMSH2 protein expression, microsatellite instability (MSI) and/or mutational analysis. A metaanalysis of the published data on immunohistochemistry of hMLH1/hMSH2 proteins was performed. Sensitivity and specificity of the method was calculated. Twenty four of 29 tumors from *hMLH1/hMSH2* mutation carriers and 10 of 13 sporadic high frequency MSI tumors lost one of the proteins. None of the 42 tumors with stable microsatellites or low frequency MSI lost the proteins. Based on literature review of 49 publications on colorectal cancer, hMLH1 immunohistochemistry was able to detect 136 of 154 tumors from *hMLH1* germline mutation carriers (the sensitivity of 88.3% [95% CI, 85.8–90.8%]), hMSH2 immunohistochemistry detected 99 of 109 tumors from *hMSH2* mutation carriers (the sensitivity of 90.8% [95% CI, 88.5–93.1%]), and hMLH1/hMSH2 immunohistochemistry identified 1262 of 1382 tumors with high-frequency microsatellite instability not correlated with mutational analysis (the sensitivity of 91.3% [95% CI, 90.4–92.2%]). The specificity of the method was 99.4% (95% CI, 99.2–99.6%). In conclusion, immunohistochemistry of hMLH1 and hMSH2 proteins is a useful method to predict the presence of mismatch repair deficiency, although its sensitivity is lower than that of MSI analysis.

Key words: colon cancer – hereditary nonpolyposis, hMLH1 gene – mutations, hMSH2 gene – mutations, hMLH1/hMSH2 protein – detection, microsatellite instability

Defects in DNA mismatch repair (MMR) system are involved in carcinogenesis and tumor progression of sporadic and inherited human cancers [22]. In humans, MMR is mediated by at least six genes, including *hMLH1, hMSH2, hMSH3, hSMH6, hPMS1,* and *hPMS2* [2]. MMR deficiency leads to the accumulation of base-base mismatches and short insertion/deletion mispairs, generated as a consequence of DNA replication errors and homologous recombinations. Most cells deficient in *hMLH1* and *hMSH2* genes often display a high level of genomic instability, characterized by changes in repeat numbers of simple repetitive sequences, microsatellite instability (MSI) [5, 32]. Mutations in genes such as *hMSH6* results in only a partial deficiency of MMR and low levels of MSI (the MSI-L phenotype) [75]. MSI can be detected in 90% of tumors with a germ-line MMR defect [1].

Inherited mutations of *hMLH1* and *hMSH2* genes have been demonstrated as the cause of a majority of hereditary non-polyposis colorectal cancers (HNPCC). By contrast, germline *hMSH3, hMSH6, hPMS1*, and *hPMS2* mutations

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have been rarely identified in HNPCC patients [44, 49]. MMR genes are involved in the development of 10–15% of sporadic colorectal carcinomas [32, 42]. The MSI phenotype in these cases, is consistent with a somatic MMR defect [29].

Patients with mutation of *hMLH1* and *hMLH2* genes have a lifetime risk of 80% for developing colorectal carcinoma which usually occurs in the fourth and fifth decade. Women having these mutations have a 40–60% life-time risk even of endometrial cancer [69]. Cancer of the stomach, small intestine, ovary, hepatobiliary tract, and urothelium also have increased incidence ratios in these patients [55, 74]. In HNPCC families, genetic counselling should be made and a program of screening and prophylactic procedures should be offered to mutation carriers [13]. Endoscopic surveillance of patients with HNPCC results in reduced mortality due to colorectal cancer [33]. Family history is considered the most useful indicator of HNPCC [13, 78]. MSI is used as a screeening method to diagnose a MMR defect in the patient's tumor. Immunohistochemical detection of the MMR protein expression is an alternative method of identifying this defect in the tumor [57]. We analyzed expression of the hMLH1 and hMSH2 proteins in a series of hereditary and sporadic colorectal carcinomas and HNPCC-associated tumors and compared the results with those of MSI and mutational analyses.

Patients and methods

Patients. A total of 81 neoplasms from 78 patients were studied. There were 25 samples from 22 patients in 13 families with a germline mutation of the *hMLH1* gene: 18 samples of colorectal carcinomas, 1 colon dysplasia, 1 stomach cancer, 1 kidney cancer, 1 endometrial cancer, 1 small bowel cancer, 1 ovary cancer, and 1 breast cancer. There were 4 colon cancers from 4 unrelated patients with a germline mutation of the *hMSH2* gene. There were 38 colorectal cancers from patients in whom mutational analysis excluded a germline mutation in *hMLH1* or *hMSH2* and 14 colorectal cancer samples from patients on whom mutational analysis was not performed. Informed consent was obtained from the patients.

Immunohistochemical analysis. Indirect immunohistochemical technique was used to detect the proteins [57]. Before immunostaining, heat-induced antigen retrieval was performed by treatment in a microwave oven (4 times 10 minutes at 900 W with a 1 minute break between every 10 minute interval), tissue sections being immersed in 10 mM citrate buffer, pH 6.0. Mouse monoclonal antibodies against hMLH1 protein (clone G168-15, PharMingen, San Diego, Ca; prepared against full-length protein) and hMSH2 protein (clone G219-1129, PharMingen, San Diego, Ca; prepared against full-length protein) and amplification system EnVision™ (DakoCytomation, Denmark) were used. The activity of peroxidase was visualized by DAB.

The normal staining pattern for hMLH1 and hMSH2 was nuclear and a case was considered positive in the presence of nuclear staining of neoplastic cells. A case was considered negative for expression of hMLH1 or hMSH2 protein only when there was a complete absence of nuclear staining of neoplastic cells in the presence of an unquestionable internal positive control represented by normal epithelial cells, stromal cells, or lymphocytes.

DNA isolation. Genomic DNA was isolated from peripheral blood lymphocytes using a standard desalting method. DNA from paraffin-embedded tumor tissue was extracted using Nucleospin C+T kit (Macherey-Nagel, Düren, Germany).

Mutational screening. All 19 exons of *hMLH1* gene, 16 exons of *hMSH2* gene, and 10 exons of *hMSH6* gene were amplified by polymerase chain reaction (PCR) by using the primers described by WU et al [76]. After formation of heteroduplexes, PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE). Samples with abnormal mobility in DGGE were re-amplified and sequenced. Sequencing was performed with Sequenase ver.2.0 DNA Sequencing kit and ^{35}S-dATP, or with BigDyeTerminator DNA Sequencing kit on ABI Prism 310 Genetic Analyser.

Microsatellite instability analysis. Paired normal and tumor DNA were used to amplify sequences (using PCR) of the following 4 mononucleotide and 5 dinucleotide microsatellite loci: BAT-RII, BAT-25, BAT-26, BAT-40, D2S123, D3S1029, D5S346, D17S250, D18S58 (Applera, Czech Republic).

PCR conditions were 94 °C 1' (94 °C 40'' 58 °C 40'' 72 °C 1')$_{40}$ 72 °C 7' for D2S123 and 94 °C 1'(94 °C 30'' 50 °C 30'' 72 °C 30'')$_{40}$ 72 °C 7' for the other markers; 1x PCR buffer with $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 M dNTP, 1 μM of each primer, 2 u/100 μl Taq DNA polymerase (Fermentas Inc., Hannover, USA). Fluorescent primers (upstream strand) were labeled on the 5' end with a fluorescent mark.

Fluorescent labeled fragments were analyzed at ABI Prism 310 Genetic Analyzer. In most cases, multiplex analysis was used; labeled fluorescent fragments from the same tissue sample were mixed together and analyzed in one cycle. Both tumor and nontumor fragments were compared and analyzed for MSI and/or loss of heterozygosity (LOH).

The presence of additional bands in the PCR product from tumor DNA, not observed in DNA from normal tissue from the same patient was scored as instability at that particular locus. Any pair of samples of normal DNA and tumor DNA that displayed instability in at least two of five loci (or at least 30–40%) was scored as having high-frequency MSI (MSI-H), whereas a sample pair with no instability was scored as having microsatellite stability (MSS). Any sample pair observed to have instability at one microsatellite locus was scored as having MSI-L.

Table 1. Tumors from *hMLH1/hMLH2* germline mutation carriers – results of mutational, MSI and immunohistochemical analyses

Exon/ Intron	*hMLH1* germline mutation (type of mutation)	Pt. No.	Type of tumor	MSI	IHC hMLH1	IHC hMSH2	hMSH2 expression in tumor compared to normal epithelium
ex.1	c.73delA (frameshift, premature termination)[b,c]	1	CC	H	–	+	i
		2	kidney ca	H	–	+	
		3	C dysplasia	–	–	+	
		4	ovarian ca	S	+	+	
ex. 1	c.85_86delGCinsTG (missense)[a]			S/LOH			
ex. 12	+c.1360G>C (missense)[a]	5	CC	D5S346	+(d)	+	i
ex. 2	c.199G>A (missense)[c]	6	CC	H	–	+	i
ex. 4	350C>T (missense)[b,c]	7	CC	H	–	+	
		7	CC metachr.	H	–	+	
		8	CC	H	–	+	
		8	stomach ca	–	–	+	i
ex. 10	c.860delA (frameshift,premature termination)[a,b]	9	CC	H	–	+	i
ex. 10	c.793C>T (missense)[c]	10	CC	H	–	+	i
ex. 11	c.1026_1027insG (frameshift,premature termination)[a,b]	11	CC	H	–	+	d
ex. 13	c.1489_1490insC (frameshift,premature termination)[b,c]	12	CC	H	–	+	
ex. 13	c.1411_1414delAAGA (frameshift, premature termination)[c]	13	CC	H	–	+	
ivs 7	c.588+5G>A (splice defect)[a,b]	14	CC	H	–	+	
		15	CC	H	–	+	
ivs 12	c.1409+1_2insG(splice defect)[a]	16	CC	H	–	+	i
		17	CC	–	–	+	i
		18	CC	H	–	+	i
		19	endometrial ca	H	–	+	i
ivs 12	c.1409+1_2insG (splice defect)[a]	20	CC	H	–	+	i
ivs 17	c.1897-3C>G (splice defect)[a,b]	21	small bowel ca	H	–	+	d
				S/LOH			
		21	breast ca	D3S1029	+(d)	+	
		22	CC	H	–	+	d
	hMSH2 germline mutation (type of mutation)						
ex. 3	c.435T>G (IIe145Met)(missense)[a]	23	CC	H	+	–	
ex. 6	1030C>G (premature termination)[c]	24	CC	H	+	–	
ex. 9	1500_1501insC (frameshift, premature termination)[b]	25	CC	H	+	+	
				S/LOH			
ex. 15	2576_2584delAATCGCAAG (in-frame deletion)[a]	26	CC	D5S346	+	+	

MSI – microsatellite instability, IHC – immunohistochemistry, ex. – exon, ivs – intron. Mutations: [a]a new, previously not identified mutation, [b]a mutation involved in the ICG HNPCC database (http://www.nfdht.nl) as unpublished, [c]a mutation published by other authors and found in other populations. Pt. – patient, CC – colon cancer, metachr. – metachronous, ca – cancer, H – high frequency MSI, S – stable microsatellites, LOH – loss of heterozygozity, +(d) – positive, but decreased expression, d – decreased expression, i – increased expression.

Metaanalysis of the published data on hMLH1/hMSH2 imunohistochemistry in correlation with MSI and/or mutational analyses was performed. The articles were searched using PubMed and reference lists of the appropriate articles. We have divided the data into following categories: tumors from *hMLH1* and *hMSH2* mutation carriers, MSI-H tumors from patients with a negative result of mutational analysis, MSI-H tumors from patients in whom mutational analysis was not performed, tumors with microsatellite stability and low-microsatellite instability tumors.

Statistical analysis. Specificity and sensitivity of the immunohistochemical classification for MSI-H status was calculated. Exact 95% confidence intervals (CIs) were calculated using the binomial distribution. Sensitivity was defined as the absence of hMLH1 and hMSH2 expression on immunohistochemistry in MSI-H tumors. Specificity was defined as intact expression of hMLH1 and hMSH2 on immunohistochemistry in MSS or MSI-L tumors.

Results

Results of mutational, microsatellite instability and immunohistochemical analyses are summarized in Tables 1 and 2.

The underlying mismatch repair gene inactivation was identified by immunohistochemistry in all but three of the 25 tumors from HNPCC patients with a known *hMLH1* germline mutation. All MSI-H tumors in these patients

Table 2. Results of immunohistochemical analysis correlated with results of mutational and/or MSI analyses

	hMLH1 germline mutation group I (n=25)			*hMSH2* germline mutation group II (n=4)		no germline mutation group III (n=38)				no mutational analysis group IV (n=14)	
	MSI-H	MSS	NI	MSI-H	MSS	MSI-H	MSI-L	MSS	NI	MSI-H	MSS
Loss of hMLH1 expression											
colon ca	16/16	0/1	1/1	0/3	0/1	4/8	0/3	0/26	0/1	4/5	0/9
colon dysplasia			1/1								
extracolonic tumors	3/3	0/2	1/1								
Loss of hMSH2 expression											
colon ca	0/16	0/1	0/1	2/3	0/1	2/8	0/3	0/26	0/1	0/5	0/9
colon dysplasia			0/1								
extracolonic tumors	0/3	0/2	0/1								

MSI-H – high-frequency microsatellite instability, MSS – stable microsatellites, MSI-L – low-frequency microsatellite instability, NI – non-informative result or non-tested.

lossed the hMLH1 protein. A colon cancer from a female patient aged 48 years without a positive family history, in whom mutational analysis revealed two missense mutations in exons 1 and 12 of the *hMLH1* gene, had stable microsatellites and expressed the hMLH1 protein at a reduced level. The other tumor retaining hMLH1 expression was a microsatellite stable breast tumor from a 43 years old *hMLH1* mutation carrier who was diagnosed with cancer of the jejunum at the same age. The latter cancer and colon cancer of the patient's brother exhibited high frequency MSI and loss of hMLH1 protein. The family fullfiled Amsterdam criteria for the diagnosis of HNPCC [68]. The third tumor expressing the hMLH1 protein was an ovarian cancer of a 50-year old patient from a family with a germline *hMLH1* mutation fullfilling the Amsterdam criteria [68]. Although cancers from 3 members of this family were MSI-H and showed loss of the protein, the ovarian cancer had stable microsatellites and retained hMLH1 protein expression.

There were 2 tumors with retained hMSH2 expression from *hMSH2* mutation carriers. One of them was from a 54-year old patient from a family fullfilling the Bethesda criteria [54] in whom a germline mutation was detected in exon 15 of *hMSH2* gene. This tumor exhibited stable microsatellites. The other patient (43 years old) carries a germline mutation in exon 9 and his tumor was MSI-H.

As regards the group of cancers with no germline mutation in *hMLH1* and *hMSH2* genes, 4 of 8 MSI-H tumors lossed the hMLH1 protein, 2 lossed the hMSH2 protein and 2 expressed both the proteins. Both patients having tumors with loss of the MSH2 protein were from families that fullfilled the Amsterdam criteria [68]. Tumors with loss of hMLH1 protein were from patients who fullfilled the Bethesda criteria [54].

In the group of patients without mutational analysis, there were 5 MSI-H tumors. Four of them losed the hMLH1 protein and all were sporadic.

hMLH1/hMSH2 immunohistochemistry identified mismatch repair deficiency in our patients with a 88.2% sensitivity (95% confidence interval [CI], 81.2–95.2%) and a 100% specificity.

In all MSH2-positive tumors from patients without a germline *hMLH1/hMSH2* mutation, expression of hMSH2 protein was stronger in the tumor than in adjacent normal epithelial cells. Three of 14 tumors from *hMLH1* germline mutation carriers exhibited weak expression of the hMSH2 protein, weaker than in nuclei of normal epithelial cells (Tab. 2).

We have reviewed 49 articles dealing with immunohistochemical detection of the hMLH1 and hMSH2 proteins in colon cancer. Results of the metaanalysis are summarized in Table 3. hMLH1 immunohistochemistry was able to detect 136 of 154 tumors from *hMLH1* germline mutation (the sensitivity of 88.3% (95% CI, 85.8–90.8%)), and hMSH2 immunohistochemistry detected 99 of 109 tumors from *hMSH2* mutation carriers (the sensitivity of 99.8% (95% CI, 88.5–93.1%)). hMLH1/hMSH2 immunohistochemistry identified 191 of 244 MSI-H tumors from patients without a germline *hMLH1/hMSH2* mutation (the sensitivity of 78.3% [95% CI, 75.1–81.5%]) and 1262 of 1382 tumors with high-frequency microsatellite instability (the sensitivity of 91.3% [95% CI, 90.4–92.2%]). The specificity of the method was 99.4% (95% CI, 99.2–99.6%).

Discussion

Immunohistochemical analysis of hMLH1 and hMSH2 protein expression is a practical method of identifying cancers with mismatch repair deficiency. As both copies of the gene are inactivated in these tumors the protein expression is usually absent. Patients with HNPCC inherit one mutant copy of the mismatch repair genes (usually *hMLH1* or *hMSH2*) from an affected parent, and one wild-type copy

Table 3. Correlation of immunohistochemical detection of hMLH1/hMSH2 proteins with results of mutational and/or MSI analyses

Reference (first author)	Germline mutation in *hMLH1* (loss of protein/ total No)	Germline mutation in *hMSH2* (loss of protein/ total No)	MSI-H;no *hMLH1/hMSH2* germline mut. (loss of the proteins (hMLH1+hMSH2+both)/total No)	MSI-H; mutational status unknown (hMLH1+hMSH2+both)/total No)	MSS (hMLH1+hMSH2+both)/total No)	MSI-L (hMLH1+hMSH2+both)/total No)	Used antibody anti-hMLH1 clone (manufacturer)	Used antibody anti-hMSH2 clone (manufacturer)
Leach [39]		2/2					–	FE11, own prod.
Thibodeau [65]	5/6	2/2	7(5+2+0)/11		0/7		G168-728(PM)	FE11(O)
Fujiwara [23]		12/14	(ND+1)/9*	(ND+1)/26*	(MD+0)/28*		–	FE11(O)
Cunningham [15]	4/5		16(16+0)/16		0/10		G168-728(PM)	FE11(O)
Marcus [45]	3/3	7/7		21(16+4+1)/22	0/34		G168-728(PM)	FE11(O)
Curia [16]	3/4	5/5	3(1+1+1)/9		0/3		14(O)	FE11(O)
Debniak [17]	3/4	2/2	2(2+0+0)/4		0/15		G168-728(PM)	polyc.rabbit(O)
Dieumegard [19]	3/4	2/3	5(4+1+0)/6		0/17		14(O)	FE11(O)
De Leeuw [40]	1/2	2/2					14(C)	GB-12(C)
Terdiman [64]	6/6	5/6	18(14+4+0)/20		0/40		G168-728(PM)	FE11(C)
Berends [4]	6/6	7/7					G168-728(PM)	GB-12(C)
Stone [60]	4/4		18(13+5+0)/19		0/23		(O)	FE11(C)
Salahshor [56]	12/14	7/7		15(15+0+0)19	0/3		G168-15(PM)	FE11(C)
Huang [30]	1/1						G168-728(PM)	–
Schweizer [58]	32/32	3/3					G168-15(PM)	GB-12(C)
Cravo [14]	2/2	2/2			0/5		G168-728(PM)	FE11(O)
Furukawa [24]	3/3	5/5	58(44+10+4)/74				G168-728(PM)	FE11(O)
Scartozzi [57]	2/2	1/2	0/5		1(1+0+0)/11	4(2+2+0)/8	G168-728(PM)	FE11(O)
Wahlberg [71]	1/5	3/4	5(1+4+0)/7		0/1	0/2	G168-728(PM)	FE11(O)
Hartmann [25]		1/1					G168-728(PM)	polyc.rabbit(O)
Hendriks [28]	18/21	11/12					14(C)	GB12(TL/BD)
Rigau [53]	4/4	4/4		21(18+3)/23	0/150	0/23	G168-728(PM)	FE11(C)
Kruger [36]	2/3	3/3					G168-15(PM)	FE11(O)
Stormorken [61]	1/1	4/4					G168-15(PM)	FE11(C)
Christensen [12]	3/4	7/8	3(1+0+2)/6		1/16	0/1	G168-15(PM)	FE11, GB-12(O)
Our results	17/18	2/4	6(4+2+0)/8	4(4+0+0)/5	0/37	0/3	G168-15(PM)	G219-1129(PM)
Kuismanen [37]			37(30+1+6)/46				G168-728(PM)	G219-1129(PM); FE11(O)
Renkonen [52]			13(8+5+0)/13	1(1+0+0)/14		0/2	G168-15(PM)	FE11(C)
Dietmaier [18]				14(6+8+0)/15	0/31	0/12	G168-728(PM)	polyc.rabbit(O)
Kim [35]				5(1+3+1)/6	0/26		G168-728(PM)	G219-1129(PM)
Thibodeau [65]				40(38+2+0)/42	0/129	0/17	G168-728(PM)	FE11(O)
Cawkwell [6]				66(53+13+0)/66	0/6	0/14	G168-728(PM)	FE11(C)
Chaves [10]				6(5+1+0)/12	2(2+0+0)/57	1(1+0+0)/11	G168-728(PM)	FE11(O)
Edmonston [20]				27(11+16+0)/30	0/48	0/19	G168-728(PM)	polyc.rabbit(O)
Cawkwell [7]				1(0+1+0)/1	0/28	0/4	G168-728(PM)	FE11(C)
Iono [31]				45(34+11+0)/48	1(0+1)/81		G168-15(PM)	FE11(O)
Jass [34]				22(21+1)/23	0/41	0/19	NI	NI
Chiaravalli [11]				20(16+4+0)/22	0/50		G168-15(PM)	FE11(O)
Paraf [47]				14(NS)/17	1(NS)/24	1(NS)/2	G168-15(PM)	FE11(O)
Ward [73]				27(24+3+0)/33	1(1+0+0)/275		(PM)	(PM)
Hawkins [26]				29(29+ND)/29	0/29		(BC)	–
Young [78]				145(98+47+0)/152			G168-15(PM)	G219-1129(PM)
Wullenweber [77]				23(12+11+0)/23			G168-15(PM)	FE11(C)
Plaschke [51]				25(15+10+0)/29	0/162	0/22	G168-15(PM)	FE11(O)
Lanza [38]				120(106+14+0)/132	0/150	0/23	G168-728(PM)	FE11(O)
							G168-728(PM)	FE11(O)
Lindor [41]				323(228+95+0)/350	0/680	0/111	G168-15(PM)	G219-1129(PM)
Valentini [67]				11(2+4+5)/15	1(0+1+0)/10		G168-728(PM)	FE11(O)
Planck [50]				16(3+13+0)/22	0/8	0/6	G168-15(PM)	FE11(O)
Ruszkiewicz [55]				167(126+41)/187	1/555		G168-15(PM)	FE11(O)
Ericson [21]				55(23+32)/59	2+F32/89	0/4	G168-15(PM)	FE11(O)
Total	136/154	99/109	191/244	1262/1382	12/2865	6/303		
Overall sensitivity of IHC (%) (95% CI)	88.3 (85.8–90.8)	90.3 (88.5–93.1)	78.3 (75.1–81.5)	91.3 (90.4–92.2)				
Overall specificity of IHC (%) (95% CI)					99.4(99.2–99.6)			

*data not induced into the sensitivity and specificity calculation; own prod – own production; PM – PharmMingen; O – Oncogene, Oncogene Science, Oncogene Research Products; C – Calbiochem; TL/BD – Transduction Laboratories/Beckton Dickinson; NI – not indicated; polyc – polyclonal

from the unaffected parent [9, 49, 72]. In normal cells, mismatch repair activity is maintained by expression of the normal protein product from the wild-type allele [48]. In tumors, the wild-type allele is inactivated resulting in mismatch repair deficiency [27]. In sporadic MSI-H cancers, both alleles are inactivated in the tumor [15, 37].

The weak positivity of hMLH1 protein expression in the tumor from the patient with two missense mutations in exons 1 and 12 of the *hMLH1* gene might be associated with the fact, that these mutations might reduce the level of protein expression, that was still detectable. However the stability of microsatellites in this tumor suggests sufficient function of the MMR system.

It is possible that the breast cancer with stable microsatellites in the patient with *hMLH1* germline mutation may arise without association with her hereditary predisposition. Breast cancer does not belong to the spectrum of HNPCC-associated tumors. Its incidence does not appear to be increased in HNPCC patients. There are HNPCC patients suffering from breast cancer. However, it is not clear if this cancer is based on mismatch repair deficiency or arises coincidentally. A similar case has been described in a patient with a germline mutation in the *hMSH6* gene who developed breast and rectal cancer between the ages of 51 and 54 years. Breast cancer in this patient did not exhibit MSI or loss of hMSH6 expression [51]. On the other hand, two breast cancers from a patient with a germline *hMLH1* mutation and from a patient with Muir-Torre syndrome ith a germline *hMSH2* mutation showing loss of the appropriate protein and exhibiting a high level of MSI have been described [16, 59].

Although ovarian cancer is classified as a HNPCC-associated tumor [12] it is probable that the ovarian cancer with stable microsatellites and retained hMLH1 protein expression in our patient arose sporadically.

There were 2 tumors with retained hMSH2 expression from *hMSH2* mutation carriers. An almost full-length, detectable protein might be produced in the one from the patient with a germline mutation in exon 15. This tumor exhibited stable microsatellites. Tumors from patients harbouring mutations in exon 16 of the *hMLH1* gene lacking MSI have been observed and a different mechanism involved in carcinogenesis has been suggested [14, 43]. The positivity of immunohistochemistry in the other tumor is difficult to explain, since the patient carries a germline mutation in exon 9 of the *hMSH2* gene and the tumor was MSI-H. It is possible that the mutation results in the production of a relatively stable nonfunctional protein fragment that was detected by immunohistochemistry.

As regards the group of cancers from patients with a negative result of mutational analysis, there were 2 of 8 MSI-H tumors that did not have any abnormal staining of the hMLH1 and hMSH2 proteins. This phenomenon might either have been based on deficiency of *hMLH1/hMSH2* genes, impairing function without reducing the level of protein expression to an undetectable level, or a different gene might have been impaired. The same refers to the one of 5 MSI-H tumors from patients without performed mutational analysis.

There were 2 MSI-H tumors that lossed the hMSH2 protein. All were from patients fullfilling the Amsterdam criteria for HNPCC diagnosis and it is probable, that these patients may have germline alterations of MMR genes that escape detection by conventional techniques [52]. All the other tumors losed the hMLH1 protein which corresponds to the fact that MMR deficiency in sporadic tumors is mostly associated with epigenetic modifications in the regulatory regions of the *hMLH1* gene [62].

Concomitant loss of hPMS2 expression in hMLH1-deficient tumors and concomitant loss of hMSH6 in hMSH2-deficient tumors has been repeatedly described and is associated with the heterodimerization-dependent stability of hMSH6 and hPMS2 proteins in the MutSα and MutLα complexes, respectively [8, 50, 51]. In tumors with absence of either hMLH1 or hMSH2 protein expression, more intense staining of the remaining intact mismatch repair protein in tumor nuclei, relative to internal control nuclei, has been described [45]. In the group of *hMLH1* mutation carriers in our study, 3 cancers exhibited reduced expression of the hMSH2 protein along with loss of hMLH1 protein expression. In the other cancers, expression of hMSH2 was more intense in tumor nuclei than in normal epithelial cells, that served as internal control, and was similar to that observed in tumors of patients without a germline *hMLH1/hMSH2* mutation. Reduced expression of hMSH2 in *hMLH1* mutation carriers and of hMLH1 in *hMSH2* mutation carriers has been observed in some cases of endometrial cancer [40].

In most published articles, loss of hMLH1/hMSH2 expression correlated with the MSI-H phenotype. The only striking exception are the results of SCARTOZZI et al [57] who found no correlation between MSI and immunohistochemistry, since tumors from 2 patients with *hMLH1* and 1 patient with *hMSH2* missense mutations exhibited a low frequency MSI and showed loss of the appropriate protein.

Results of the metaanalysis show that immunohistochemistry of hMLH1 and hMSH2 proteins was able to detect tumors from *hMLH1* and *hMSH2* germline mutation carriers and generally MSI-H tumors with approximately 90% sensitivity. Its sensitivity for detecting MSI-H tumors from patients without *hMLH1/hMSH2* mutation is lower and this reflects the higher rate of mutations in other MMR genes in this group. Specificity of the method reaches nearly 100%. There may be some difficulties with staining of hMLH1 protein in some laboratories associated with usage of different technical protocols [46]. However, a significant majority of tested laboratories demonstrated excellent results including high discriminatory power with both antibodies in a study [46].

There are a number of clinical situations in which immunohistochemical analysis of MMR deficiency can be used. Families who meet the Amsterdam criteria [68], the most restrictive criteria for HNPCC diagnosis, should be offered genetic testing directly, as these criteria have a relatively high sensitivity and specificity for finding a pathogenic germline mutation [13, 63, 70]. In these patients, immunohistochemistry can indicate a missed mutation in case of a negative result of mutational analysis. Analysis of HNPCC cases identified by less strict criteria such as the Modified Amsterdam [3] and Bethesda criteria [54], led to an increased sensitivity and a decreased specificity for the indentification of germline *hMLH1* and *hMSH2* mutations. These criteria identify almost all cases with a germline mutation but include many cases without a germline mutation [70]. In this group of patients, screening by MSI analysis or immunohistochemistry that indentifies the presence of a MMR defect in the tumor selects patients with HNPCC in whom mutational analysis should be performed [13]. In this setting, immunohistochemical analysis complements microsatellite analysis of tumor DNA. It is able to identify either *hMLH1* or *hMSH2* as the underlying inactivated gene. However, it cannot fully replace MSI analysis owing to possible false negative results in immunohistochemical analysis. MSI analysis identifies also tumors due to germline mutations in other genes of the mismatch repair complex [51]. It has been recommended to use immunohistochemistry as the first screening. If positive (no nuclear staining of hMLH1 or hMSH2 proteins), sequencing should be performed; if negative, MSI analysis should be performed [12].

Immunohistochemistry may also be applied to unselected tumors without knowledge of family history. Like microsatellite testing, immunohistochemistry does not discriminate between germline and somatic alterations. It is estimated that only 10% to 20% of unselected MSI-H colorectal cancers have germline *hMLH1* or *hMSH2* mutations [15, 29, 37]. The unique carcinogenic mechanisms underlying MMR-deficient cancers, whether germline or somatic in origin, suggests biological differences that may be of importance in predicting prognosis and in tailoring specific therapies [13].

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Note added in proof: Breast cancer occurrence in HNPCC families might be associated with interactions between MMR and BRCA proteins [2].

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