Microsatellite Instability, Immunohistochemistry, and Additional PMS2 Staining in Suspected Hereditary Nonpolyposis Colorectal Cancer

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ABSTRACT

Purpose: **Immunohistochemistry (IHC) and microsatellite instability (MSI) analysis can be used to identify patients with a possible DNA mismatch repair defect [hereditary nonpolyposis colorectal carcinoma (HNPCC)]. The Bethesda criteria have been proposed to select families for determination of MSI. The aims of this study were to assess the yield of MSI analysis in families suspected for HNPCC, to compare the results of immunohistochemical staining and MSI analysis, and to assess the additional value of PMS2 staining.**

Experimental Design: **Clinical data and tumors were collected from 725 individuals from 631 families with suspected HNPCC. MSI analysis was performed using eight markers including the 5 National Cancer Institute markers. Four immunohistochemical staining antibodies were used (MLH1, MSH2, MSH6 and PMS2).**

Results: **A MSI-H (tumors with instability for >30% of the markers) phenotype in colorectal cancers (CRCs) was observed in 21–49% of families that met the various Bethesda criteria. In families with three cases of CRC diagnosed at age > 50 years, families with a solitary case of CRC diagnosed between ages 45 and 50 years, and families with one CRC case and a first-degree relative with a HNPCC-related cancer, one diagnosed between ages 45 and 50 years (all Bethesda-negative families), the yield of MSI-H was 10–26%. Immunohistochemical staining confirmed the MSI results in 93% of the cases. With IHC, adding PMS2 staining led to the identification of an additional 23% of subjects with an *hMLH1* germ-line mutation (35 carriers were tested).**

Conclusions: **The Bethesda guidelines for MSI analysis should include families with three or more cases of CRC diagnosed at age > 50 years. The age at diagnosis of CRC in the original guidelines should be raised to 50 years. Routine IHC diagnostics for HNPCC should include PMS2 staining.**

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of death due to malignancy in the Western world. The cause of CRC is multifactorial, involving genetic and environmental factors (1). The most common hereditary colorectal carcinoma syndrome is hereditary nonpolyposis colorectal carcinoma (HNPCC), which accounts for 1–6% of all CRC cases (2). HNPCC is an autosomal dominant disease characterized by an increased risk of early-onset CRC and other cancers, including tumors of the endometrium, stomach, small intestine, hepatobiliary system, kidney, ureter, brain, and ovary (3–6). In up to 15–25% of all cases of CRC, clustering of this type of tumor is observed in the family (7). The role of environmental or genetic factors in these cases is largely unknown.

The increased risk for malignancy in HNPCC is caused by a mutation in one of the following DNA mismatch repair (MMR) genes: *MLH1*; *MSH2*; *MSH6*; *PMS1*; and *PMS2* (8–10). Germ-line mutations of *MLH1* and *MSH2* account for >90% of all known MMR mutations in HNPCC (11), and germ-line mutations of *MSH6* account for 5–10% of all known MMR mutations in HNPCC, whereas mutations of other genes are rare (10, 12).

Mutations in DNA MMR genes result in a failure to repair errors in repetitive sequences that occur during DNA replication. This failure leads to microsatellite instability (MSI) of the tumor, which is the hallmark of HNPCC (13–16).

Due to the heterogeneity of the mutation spectrum of the MMR genes, screening for mutations is both time-consuming and expensive. In addition to family history, MSI analysis and immunohistochemistry (IHC) can both be used to identify families eligible for mutation analysis of the MMR genes (2, 17). In 1997, the Bethesda criteria were proposed to select families for MSI testing. In the present study, we evaluated the yield of MSI analysis in families categorized according to these criteria. We also evaluated MSI in other subsets of families that do not meet these criteria.

Previous studies from numerous groups, including ours

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(Refs. 18, 19 and the references herein) have shown that immunohistochemical analysis using antibodies against the MLH1, MSH2, and MSH6 proteins is another sensitive method to identify carriers of MMR gene mutations. Because the PMS2 protein forms a heterodimer with the MLH1 protein, it might be expected that absence of the MLH1 protein due to a germ-line mutation also leads to loss of the PMS2 protein caused by abrogation of the total protein complex (20). In the present study, we compared the results of immunohistochemical staining with the outcome of MSI analysis and evaluated the additional value of IHC using PMS2 staining.

PATIENTS AND METHODS

Patients. We used the database of colorectal tumors from the unit molecular diagnostics of the pathology department from the Leiden University Medical Center, the Netherlands. This database contains colorectal tumors ($n = 771$) sent to our department for MSI analysis from different medical genetic centers and laboratories in the Netherlands between November 1999 and December 2002. For 46 patients, it was impossible to perform MSI analysis because the obtained formalin-fixed, paraffin-embedded material was not sufficient, or MSI analysis was redundant due to the fact that the segregating mutation was already known in the family. This resulted in MSI analysis of 725 tumors from individuals with CRC of 631 families with clustering of CRC or with a solitary patient with CRC at a young age. Retrospectively, we scored the available complete pedigrees (528 pedigrees were enclosed with the request for MSI analysis from the medical genetic centers; from 103 subjects, only a fragmentary pedigree was sent) according to the Bethesda and additional criteria (Table 1), and we performed immunohistochemical staining (MLH1, MSH2, and, subsequently, MSH6 and PMS2). Finally, we had 528 tumors with complete information from the pedigree and MSI analysis, 330 tumors with results on MLH1 and PMS2 staining (including 35 tumors from patients with a *hMLH1* mutation), and 284 tumors with interpretable results on both MSI and IHC (four proteins). The reason for the major decrease in the number of patients was that in this retrospective series, not all samples were still available for additional staining. From 84 families, we had tumor material of at least two relatives.

DNA Isolation. Genomic DNA of normal and tumor tissue was isolated from the paraffin-embedded material by taking tissue punches (diameter, 0.6 mm) with a tissue microarrayer (Beecher) from tumor and normal areas selected on the basis of a HE-stained slide. Using the Chelex extraction method, DNA was isolated from three punches, resuspended in 96 μl of PK-1 lysis buffer [50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20, and 0.1 mg/ml gelatin] containing 5% Chelex beads (Bio-Rad, Hercules, CA) and 5 μl of proteinase K (10 mg/ml), and incubated for 12 h at 56°C. The suspension was incubated at 100°C for 10 min and centrifuged at 13,000 rpm for 10 min, and the supernatant containing the DNA was carefully transferred to a new tube.

MSI Analysis. Eight microsatellite markers were evaluated [two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, and D17S250) recommended by the National Cancer Institute Workshop on MSI for Cancer Detection and Familial Predisposition (13), supplemented by three mononucleotide repeat markers (BAT40, MSH3, and MSH6)]. BAT40 is a very informative marker. The choice for MSH3 and MSH6 was initially for research purposes. Tumors were classified as (*a*) tumors with instability for >30% of the markers (MSI-H), (*b*) tumors with instability for <30% of the markers (MSI-L), and (*c*) tumors with no instability [microsatellite stability (MSS)]. We distinquished between MSI-L with instability of only a dinucleotide marker (MSI-Ld) and instability of only a mononucleotide marker (MSI-Lm).

IHC. Staining of MMR proteins was performed with anti-MLH1 (initially with clone 14; 1:75; Calbiogen, Cambridge USA, later supplemented and substituted by clone G168-728; 1:50; BD Biosciences, NJ), anti-PMS2 (clone A16-4; 1:50; BD Biosciences), anti-MSH2 (clone GB-12; 1:100; Oncogene Research Products, San Diego, CA), and anti-MSH6 (clone 44; 1:400; BD Biosciences). Immunohistochemical staining was performed on 4-μm-thick, formalin-fixed, paraffin-embedded tissue sections that were prepared on DAKO slides and dried overnight at 37°C. Next, tissue sections were deparaffinized three times in xylene for a total of 15 min and subsequently rehydrated. Antigen retrieval was done by boiling in 10 mM citrate buffer [pH 6.0 (MSH6 and MLH1), clone G168-728] or in 1 mM EDTA (MLH1, clone 14, PMS2 and MSH2) for 10 min using a microwave oven, after which the sections were cooled in this buffer for at least 1 h at room temperature. After rinsing in demiwater, the tissue sections were stained in a DAKO Techmate 500+ automated tissue stainer using the DAKO ChemMate System Kit Peroxidase/DAB K5011 (DAKO, Glostrup, Denmark). Briefly, in this system, slides were incubated with the primary antibody diluted in ChemMate Antibody diluent (DakoCytomation, Glostrup, Denmark) for 8 h at room temperature. Sections were automatically washed and incubated with ready-to-use biotinylated secondary antibody for 30 min and washed. Endogenous peroxidase was then blocked in peroxidase

Table 1 Explanation of used criteria

Bethesda	Criteria	
Positive	1	Fulfilling the Amsterdam II criteria
	2	Solitary patient with CRC[a] and a HNPCC-related cancer
	3	Patient with CRC and a FDR with a HNPCC-related cancer, one of the cancers diagnosed age < 45 yrs
	4	Solitary patient with CRC diagnosed at age < 45 yrs
Negative	5	Solitary patient with CRC diagnosed at age 45–50 yrs
	6	Patient with CRC and a FDR with a HNPCC-related cancer, one of the cancers diagnosed at age 45–50 yrs
	7	Late-onset family: patient with CRC and two FDRs with a HNPCC-related cancer, both cancers diagnosed at age > 50 yrs
	8	Patient with CRC and a FDR with a HNPCC-related cancer, both cancers diagnosed at age > 50 yrs

[a] CRC, colorectal cancer; HNPCC, hereditary nonpolyposis colorectal carcinoma; FDR, first-degree relative.

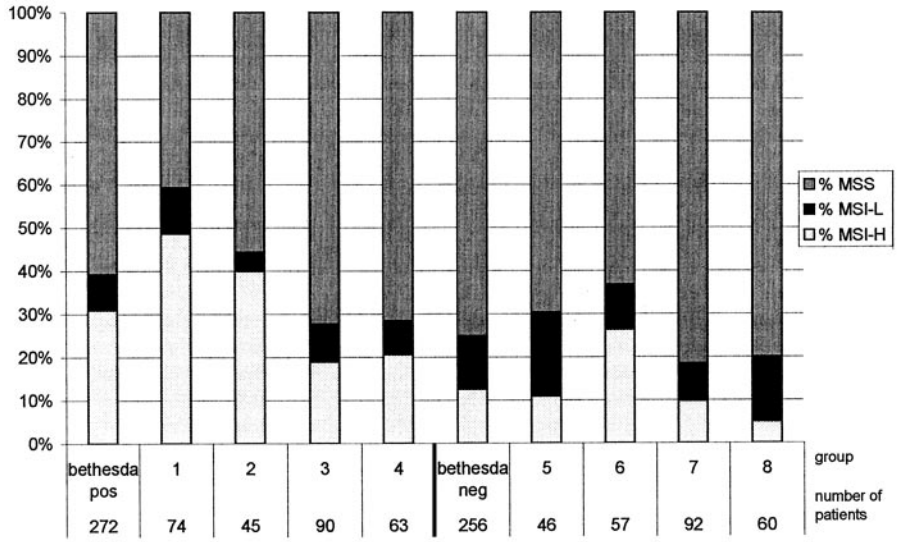

Fig. 1 Yield of MSI in several types of families ($n = 528$). See Table 1 for groups 1–8.

blocking solution for 7.5 min and washed and incubated with ready-to-use streptavidin-conjugated with peroxidase for 30 min. Sections were washed and developed with two-component hydrogen peroxide/diaminobenzidine for 15 min. The sections were then counterstained with hematoxylin for TechMate, dehydrated, cleared in xylene, and mounted with micromount. Microscopic analysis was done by a pathologist (H. M.). Tissue stroma and normal epithelium or lymph follicles served as positive internal controls when analyzing MLH1, PMS2, MSH2, and MSH6 expression. Expression of MLH1, PMS2, MSH2, and MSH6 was scored as positive (+), negative with a positive internal control (0/+), and doubtfully negative [when both tumor and internal control stain negative (0/0)], and when the internal control was stronger than the positive tumor cells, it was scored as +/++.

RESULTS

Yield of MSI in Subjects from Bethesda-Positive and -Negative Families. In the families that met the Bethesda criteria (Bethesda-positive group; $n = 272$), 84 tumors (31%) were MSI-H, 23 tumors (8%) were MSI-L, and 165 tumors (61%) showed MSS (Table 1; Fig. 1). In tumors from subjects from an Amsterdam-positive family ($n = 74$), the yield of MSI-H was 49%; for Bethesda 2 ($n = 45$), it was 40%; for Bethesda 3 ($n = 90$), the yield was 19%; and for Bethesda 4 ($n = 63$), it was 21%. The proportion of MSI-L tumors in these four groups was 11%, 4%, 9%, and 8%, respectively.

In the families that did not meet the criteria (Bethesda-negative group; $n = 256$), 32 colorectal tumors (12.5%) were MSI-H, 32 tumors (12.5%) were MSI-L, and 192 tumors (75%) showed MSS. We subdivided the Bethesda-negative families into four subgroups (Table 1, criteria 5–8). In Fig. 1, the yield of MSI for the different subgroups is shown.

IHC and MSI. Data on MSI analysis as well as immunohistochemical staining (four proteins) were available for 284 tumors. Among these 284 tumors (Table 2), 91 tumors showed MSI-H as well as abnormal staining, and 136 tumors showed MSS and normal protein expression, leading to concordant results in 93% (227 of 245) of the MSI-H and microsatellite stable tumors. In view of the remarks by Perucho (21) on the marker sets used for MSI, we subdivided MSI-L in MSI-Lm (instability of only a mononucleotide marker) and MSI-Ld (instability of only a dinucleotide marker). Although the number of tumors is small, we found a difference between both groups: 35% of tumors in the MSI-Lm group and 13% of tumors in the MSI-Ld group showed absence of at least one MMR protein (Table 2).

Subsequently, we evaluated the results of IHC in the Bethesda-negative groups (Table 3).

PMS2 Staining. To evaluate the additional value of PMS2 staining, we compared the results of MLH1 and PMS2 staining in 330 tumors (see "Patients and Methods"). Among these, 35 tumors were from *hMLH1* mutation carriers (25 tumors had been described previously, without staining for PMS2; Ref. 18). Thirty tumors were from subjects in whom a *hMLH1* mutation could not be detected, and 265 tumors were from subjects with an unknown mutation status of *hMLH1* (from these 265 tumors, 7 tumors were from carriers of a *hMSH2* germ-line mutation, and 10 were from subjects with a *hMSH6* germ-line mutation; Table 4). In Fig. 2, three staining examples are shown. In 292 tumors (88%), both stainings gave the same results. If MLH1 stained negative with a positive internal con-

Table 2 Comparing MSI[a] and IHC staining results

MSI	Normal expression	≥1 MMR protein absent	Concordance
MSI-H	12	91	88%
MSS	136	6	96%
MSI-Lm	20	11	
MSI-Ld	7	1	

[a] MSI, microsatellite instability; IHC, immunohistochemistry; MMR, mismatch repair; MSS, microsatellite stability; MSI-Lm, MSI-L with instability of only a mononucleotide marker; MSI-Ld, MSI-L with instability of only a dinucleotide marker.

Table 3 MSI[a] and IHC results of the Bethesda-negative groups

See Table 1 for explanation of the groups.

	Intact expression of all 4 proteins				Absent expression ≥ 1 protein			
	Group 5	Group 6	Group 7	Group 8	Group 5	Group 6	Group 7	Group 8
Total no. of cases	25	22	46	22	6	9	8	1
No. of MSS	22	20	45	20	0	0	0	0
No. of MSI-L	3	1	1	0	1[b]	1[c]	0	0
No. of MSI-H	0	1	0	2	5[d]	8[e]	8[f]	1[g]

[a] MSI, microsatellite instability; IHC, immunohistochemistry; MSS, microsatellite stability.
[b] Abrogation of MSH6.
[c] Abrogation of MSH6.
[d] Abrogation of MLH1, or PMS2, or MLH1/PMS2 (2 cases), or MSH2/MSH6.
[e] Abrogation of MLH1/MSH6, PMS2, MSH2, or MSH2/MSH6, or MSH6 (4 cases).
[f] Abrogation of MLH1/PMS2 (2 cases), or PMS2, or MSH2, or MSH6 (2 cases), MSH2/MSH6, or MSH2/MSH6/PMS2.
[g] Abrogation of MLH1/PMS2.

trol (0/+), PMS2 also stained 0/+ in 39 cases (93%). If MLH1 stained positive, PMS2 stained positive in 252 (92%) cases. Among these 252 cases is 1 carrier of a *hMLH1* mutation (unclassified variant; Table 5, case 8). In 19 tumors 7% (all MSI-H tumors), there was 0/+ staining of PMS2, whereas staining of MLH1 was positive (+, 16 tumors; +/++, 3 tumors). In the latter scoring (+/++), the internal control clearly stained more positive than the tumor nuclei (Fig. 2). Among these 19 patients were 8 *hMLH1* germ-line mutation carriers (Tables 4 and 5; cases 18, 19, and 26–31). In three cases, a possible *PMS2* defect was present, and in eight cases, the mutation status of *hMLH1* was not yet determined. Two tumors showed 0/+ MLH1 staining and positive staining for PMS2. One subject is a carrier of a *hMLH1* mutation (case 14, Table 5) and the mutation status of the second subject, although tested, is still unknown (case 1, Table 5). In 14 cases, the MLH1 staining was not interpretable (0/0) because of the absence of staining of normal tissue, whereas the PMS2 staining was interpretable in 13 of these 14 cases. In one case, both stainings were not interpretable (0.3%). Therefore, overall, PMS2 staining gave additional value in 32 of 330 tumors (10%).

When only evaluating the 35 tumors of *hMLH1* mutation carriers, 23 tumors showed absence of at least MLH1. In only 17 tumors (49%) was an MLH1-negative staining accompanied by normal MSH2 and MSH6 staining patterns. Eight of the 35 tumors showed absence of only PMS2 (all other three proteins showed positive expression). Therefore, an additional 23% of the subjects with an *hMLH1* germ-line mutation were identified solely with IHC.

Table 4 PMS2 and MLH1 staining

	PMS2			
hMLH1	0/+[a]	+	0/0	Total
hMLH1 mutation				
0/+[a]	21	1	1	23
+	5	1		6
+/++	3			3
0/0	3			3
No *hMLH1* mutation				
0/+	5			5
+	3	18	1	22
0/0		3		3
Mutation status unknown				
0/+	13	1		14
+	8	233	2	243
0/0	2	5	1	8
Total	63	262	5	330

[a] Staining results: +, nuclear staining; +/++, internal control is stronger than the positive tumor cells; 0/+, negative with a positive internal control; 0/0, tumor and internal control stain negative.

Disconcordant Results. When we evaluated all results in the whole database, there were 31 cases with remarkable combinations of results of the (pre-)screening tests (Table 5). Six cases (cases 2–7) are patients with microsatellite stable tumors in combination with abnormal IHC (see also Table 2). Cases 7–12 are patients with a germ-line MMR variant (two of them are considered to be true pathogenic mutations, and four are unclassified variants), but without evidence of instability. Cases 13–21 are patients, all with MSI-H tumors, but with an uncommon combination of absence of proteins. Then we noticed a group of patients (cases 15 and 21–25) without a mutation of *hMLH1*, *hMSH2*, or *hMSH6*, but with a MSI-H or MSI-L tumor and the absence of one or more proteins. The last group (cases 18, 19, and 26–31) has already been described in this article (see also Table 4).

Interfamilial Variety. We identified 84 families in which we assessed MSI in tumors from at least two relatives. We evaluated the phenotype in these tumors. In 69 families, the first tumor was microsatellite stable. The second tumor in these families showed MSI-H in 13 tumors (19%) and MSI-L in 12 tumors (17%). We evaluated whether we could find an explanation by evaluating the pedigree. We did not find a difference in family type between families with two microsatellite stable tumors and families with one microsatellite stable and one MSI-H or MSI-L tumor. Overall, in the 69 families in which the first tumor showed MSS, 24 tumors were located in the rectum. In the 25 families (mutation unknown) with two affected relatives tested, one relative with a microsatellite stable tumor and one with a MSI-H or MSI-L tumor (in total, 50 tumors), eight of the tumors first tested were located in the rectum.

DISCUSSION

Identification of families with HNPCC is extremely important because it makes it possible to target effective preventive measures that lead to a substantial reduction in CRC-related

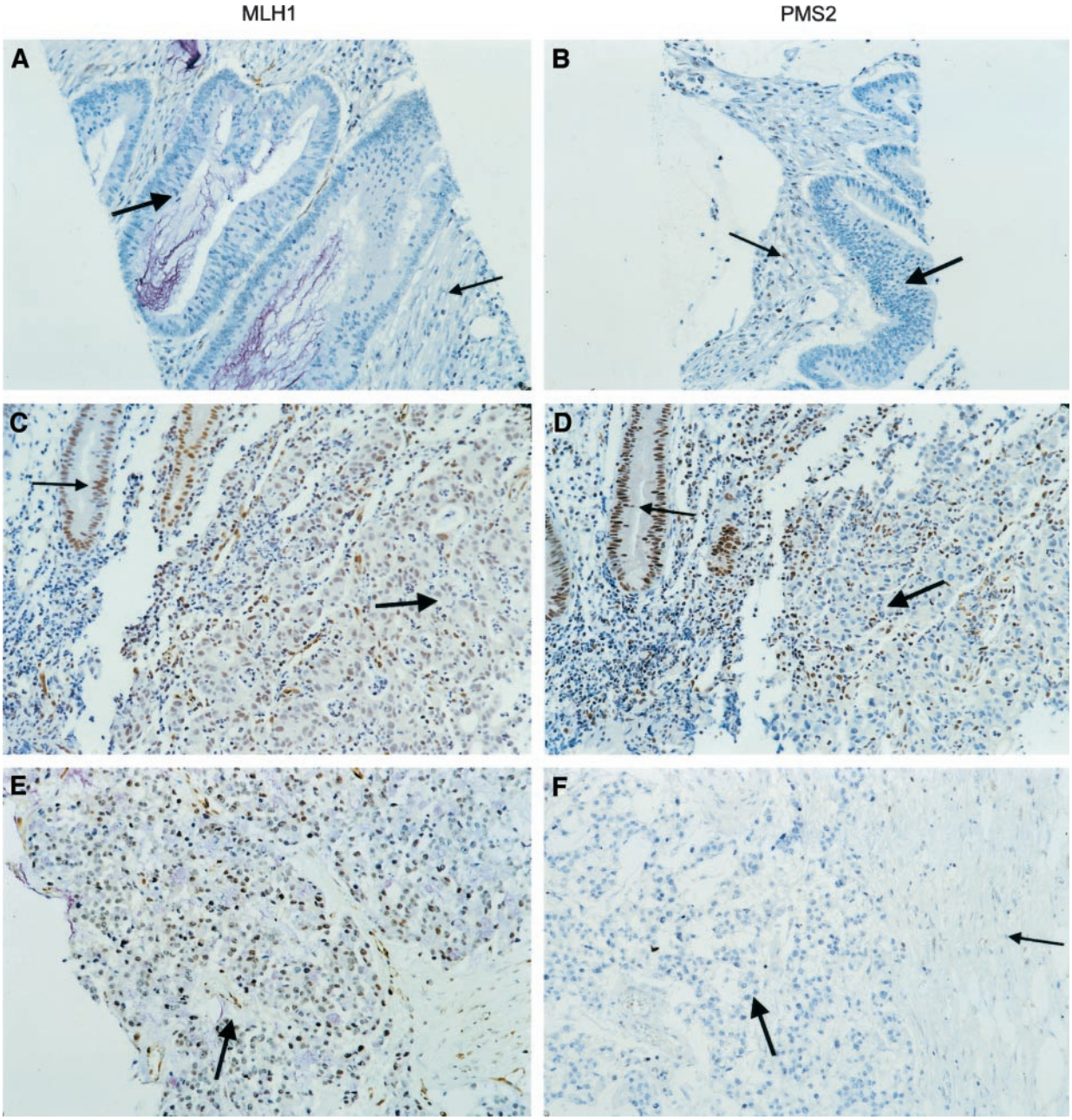

Fig. 2 Immunostaining with antibodies against MLH1 and PMS2. *Thick arrows* indicate tumor cells. *Thin arrows* indicate internal control cells, either stromal or epithelial. *A* and *B*, well-differentiated MSI-H colon carcinoma (*hMLH1* germ-line mutation, exon 16 delK618) with negative MLH1 and PMS2 staining in tumor nuclei, with retained staining of stromal cells. *C* and *D*, poorly differentiated MSI-H colon carcinoma (*hMLH1* germ-line mutation, exon 5 Q149X, 445C>T) with positive MLH1 staining but negative PMS2 staining in tumor nuclei, although there is stronger positivity for MLH1 in normal crypt cells than in tumor cells. *E* and *F*, poorly differentiated MSI-H colon carcinoma (*hMLH1* germ-line mutation exon 1, G6fsX25, 18_34del17) with retained MLH1, MSH2, and MSH6 staining but abrogated PMS2 staining.

mortality (22). In the present study, we evaluated the value of MSI analysis and immunohistochemical staining for the identification of HNPCC in a large series of families. A significant proportion of MSI-H tumors were detected not only in families that complied with the Bethesda criteria but also in families that met other specific criteria. In addition, we found that immunohistochemical staining (including staining for PMS2) and MSI analysis gave concordant results in 93% of the cases. IHC alone, including PMS2 staining, led to the identification of an additional 23% of subjects with an *hMLH1* germ-line mutation.

Several years ago, the Bethesda guidelines were developed for selection of families whose tumors should be tested for MSI. In the present study, we examined the validity of these criteria in relation to MSI status. Another important aim was to assess

Table 5 Special cases (see Table 1 for family diagnosis)

Case	Sex	Age at diagnosis (yrs)	Family type	Site of tumor	MSI[a]	IHC[b] MLH1	IIHC MSH2	IHC MSH6	IHC PMS2	Mutated gene[c]	Exon	Amino acid change	Nucleotide change
1	M	65	7	Rectum	Ld	0/+	+	+	+	?			
2	F	39	4	Sigmoid	S	+	+	0/+	+	?			
3	F	41	4	Cecum	S	+	+	0/+	+	no 6			
4	F	70	3	Colon	S	+	+	0/+	na	no 1/2/6			
5	M	40	4	Right colon	S	+	0/+	0/+	+	?			
6	M	51	7	Left colon	S	+	0/+	na	+	?			
7	M	56	1	Cecum	S	0/+	+	+	0/+	*MLH1*	8	R226Q	677G>A (splice donor)
8	M	42	4	Sigmoid	S	+	na	na	+	*MLH1*, UV		DelIVS13	500 bpdeletion
9	F	51	5	Left colon	S	na	na	na	na	*MSH2*, UV	15	S860L	2579C>T
10	M	65	5	Colon	S	+	+	+	+	*MSH6*, UV	4	S503C	1508C>G
11	M	34	4	Ascendens	S	na	na	na	na	*MSH6*, UV	5	T1102T	3306T>A
12	M	45		Right colon	S	na	na	na	na	*MSH6*	4	V907fsX	2719_2720delGT
13	M	71	1	Ascendens	H	0/+	+	0/+	na	(*MLH1*)	11	Q301X	901C>T
14	M	46		Cecum	H	0/+	+	0/+	+	*MLH1*	16	K618del	1852–1854del
15	M	40	4	Flexura lienalis	H	0/+	+	0/+	0/+	no 1/2/6			
16	F	47	3	Flexura hepatica	H	0/+	+	0/+	0/+	?			
17	M	74		Cecum	H	+	+	0/+	0/+	?			
18	F	38		Colon	H	+	+	0/+	0/+	*MLH1*, UV	10	R264C	
19	M	39	1	Cecum	H	+	+	0/+	0/+	*MLH1*, UV	10	R264C	
20	M	42		Flexura lienalis	H	0/0	+	0/0	0/+	(*MSH6*, UV)	IVS 9		3969_4002+51dup
21	M	74	7	Left colon	H	+	0/+	0/+	0/+	no 1/2/6			
22	M		7	Left colon	H	+	0/+	na	+	no 1/2/6			
23	M	53	1	Cecum	H	+	0/+	na	na	no 1/2/6			
24	F	49		Right colon	H	+	0/+	na	+	no 1/2/6			
25	M	35	4	Transversum	Lm	+	0/+	0/+	+	no 1/2/6			
26	M	39	1	Ascendens	H	+	+	0/+	0/+	*MLH1*	1	G6fsX25	18_34del17
27	M	39	1	Transversum	H	+	+	+	0/+	*MLH1*	16	K618del	1852–1854del
28	F	90	7	Transversum	H	+	+	+	0/+	*MLH1*, UV	3	S93G	277A>C
29	M	34	4	Cecum	H	+/++	+	na	0/+	*MLH1*, UV	2	S44F	131C>T
30	M	46	1	Colon	H	+/++	na	na	0/+	*MLH1*	5	Q149X	445C>T
31	F	43	1	Cecum	H	+/++	+	+	0/+	*MLH1*	5	Q149X	445C>T

[a] MSI, microsatellite instability; H, MSI-H; Lm, MSI-L with instability of only a mononucleotide marker; Ld, MSI-L with instability of only a dinucleotide marker; S, stability; MSS, microsatellite stability.

[b] IHC (immunohistochemistry). 0/0, tumor cell, no nuclear staining, internal control also absent; 0/+, no nuclear staining; +, nuclear staining; +/++, internal control more positive than tumor; na, not analyzed.

[c] Mutated gene: no 1/2/6, no mutation found in *hMLH1/hMSH2* or *hMSH6*; no 6, no mutation found in *hMSH6*; ?, mutation status not fully tested; (*MLH1*), relative is known with a *MLH1* mutation, in this case not tested; UV, unclassified variant.

whether other criteria should be added to identify more families with MSI-positive tumors.

The yield of MSI-H in our series of families that met the different Bethesda criteria varied from 19% to 49%. This is in agreement with the results of previous studies on the yield of MSI in such families (23, 24).

The families in our series who did not meet the Bethesda criteria comprised families with one CRC diagnosed between age 45 and 50 years (category 5); families with one case of CRC and a first-degree relative with a HNPCC-related cancer, one diagnosed between age 45 and 50 years (category 6); families with three or more CRC cases diagnosed at age > 50 years (category 7); and families with one CRC and a first-degree relative with a HNPCC-related cancer, both diagnosed at age > 50 years (category 8). In categories 5, 7, and 8, the yield of MSI-H tumors was relatively low. In categories 5 and 7, all MSI-H tumors showed absence of at least one protein (see earlier). It is remarkable that only 4 of 13 tumors in these two categories might be explained by MLH1 abrogation due to promoter methylation. Theoretically, hypermethylation of the other MMR genes is possible, but not yet known. In the families of category 6, the yield of MSI-H tumors was 26%, which is higher than the percentage of MSI-H tumors (~10%) reported for sporadic CRC (25), often due to MLH1 promoter methylation (16, 26). Based on these results, we suggest the extension of the Bethesda criteria with criteria that can identify these types of families (groups 5–7).

A few studies have shown that immunohistochemical staining of tumors using antibodies against the MMR proteins is a sensitive method to identify families eligible for mutation analysis (27–30). Most studies reported so far used antibodies against MLH1, MSH2, and MSH6. Rigau *et al.* (19) also included PMS2 antibodies. Because the PMS2 protein forms a heterodimer with the MLH1 protein, absence of the MLH1 protein due to a mutation also leads to loss of the PMS2 protein caused by abrogation of the total protein complex (20). Absence of PMS2 staining might therefore suggest the presence of a *hMLH1* or *PMS2* germline mutation or somatic abrogation of *hMLH1*.

When we compare the results of MLH1 and PMS2 staining, concordant results were observed in 88% of the cases. In the 35 tumors associated with a known *hMLH1* mutation, absence

of both MLH1 and PMS2 staining was observed in 21 tumors. In eight other tumors, staining for PMS2 was negative, whereas staining for MLH1 was positive. This finding means that by using staining for PMS2, significantly more *hMLH1* mutation carriers would have been identified. Rigau *et al.* (19) observed four cases with isolated loss of PMS2, and all were microsatellite stable. Rigau *et al.* (19) concluded that there is no need to include PMS2 in the panel of antibodies to be used when looking for MMR-deficient cases by IHC. The majority of their MSI-H tumors, however, most likely consisted of tumors with sporadic abrogation of MLH1, in which PMS2 staining is indeed not necessary. In our studied cases, the type of underlying mutation (missense mutation, in-frame deletion, or unclassified variant) may explain why the MLH1 protein was still intact in the nucleus, whereas the binding of PMS2 was abrogated (*e.g.*, due to conformational changes). Another possibility is that, in the case of an unclassified *MLH1* variant, an unidentified pathogenic mutation in *PMS2* is responsible. We also do not know what exactly happened with the second *MLH1* allele in these tumors, which potentially might influence the staining results. Finally, technical problems with the MLH1 staining in individual cases and perhaps also the type of the MLH1 antibody used might play a role (31, 32). An illustration of the arguments above follows: seven cases in our database with an identical *MLH1* mutation (K618del) were tested (18). Only one of these (case 27, Table 5) was concluded to have retained nuclear MLH1 staining in tumor cells, but with loss of PMS2 staining. We identified three tumors (Table 5), all from *hMLH1* carriers, that stained +/++ for MLH1. In the literature, it is known that in individual cases, abnormally high sensitivity of the IHC can account for false positive interpretation (31). Whatever the explanation, the latter three cases illustrate the additional value of adding the +/++ score to the traditional scoring scheme.

Overall, we found that immunohistochemical staining using four antibodies confirmed the results of MSI analysis in 93% of the cases. This is nearly identical to that reported in the recent literature in studies using only three antibodies [hMLH1, hMSH2, and hMSH6 (19)] or even two antibodies [hMLH1 and hMSH2 (30)]. This discordance might be explained by the consecutive case series used in the study of Rigau *et al.* (19). The majority of their MSI-H tumors (very few HNPCC cases) are most likely due to methylation of hMLH1. The concordance between MSI-H and loss of MLH1 expression in the sporadic cases will be 100%, as expected. In the study of Lindor *et al.* (30), the concordance in the consecutive case series was indeed 100%. In the other three series, included in the same study, all from centers from a Cooperative Family Registry for Colon Cancer Studies, the concordance varied widely, from 84% to 95%. The exact reason for the discordance is unknown (30).

We classified the MSI-L tumors in our series into two groups: tumors with instability of only a mononucleotide marker (MSI-Lm); or tumors with instability of only a dinucleotide marker (MSI-Ld). The MSI-Lm tumors seem more informative for a true MMR deficiency than the MSI-Ld tumors, which seems to be in line with the views of Perucho (21): "The alterations in di-, tri- or tetranucleotide repeats can be also due to spontaneous errors of replication of these highly unstable sequences."

Ninety-five percent of all microsatellite stable tumors showed positive staining for the four MMR proteins, which implies that additional IHC in microsatellite stable tumors is often redundant. However, the value of MSH6 staining in microsatellite stable tumors might although not neglectable (33, 34). In our study, four of six microsatellite stable tumors with abnormal IHC showed an absence of MSH6 (Table 5, cases 2–5), although no mutation has been detected in this set of patients. On the basis of these results and the results of Wahlberg *et al.* (35), we recommend a possible decision scheme for (suspected) HNPCC as suggested previously (18). Rigau *et al.* (19) suggested that MSH6 (and possibly PMS2) can be considered as useful only in second line, when MLH1 and MSH2 show no abnormalities in MSI-H tumors or in suspected HNPCC. At our department, however, the costs for performing two or four stainings at the same time are almost equal, whereas performing them in two sessions is more labor intensive.

We recommend testing a second tumor from another relative in our decision scheme when MSI analysis of a tumor (from a family suspected of HNPCC) shows no evidence of instability because it is possible that we are dealing with a phenocopy within a HNPCC family. In the present series, we analyzed a second colon tumor in 69 families in which the first tumor showed MSS. MSI in the second tumor was found in 36% of the families. Furthermore, on basis of our results and those of others (36), we recommend, if possible, not to test a rectal tumor as first choice.

We noticed several cases in the whole database with discordant results (Table 5). The number of patients (8 patients) with a microsatellite stable tumor with a MMR mutation [five of eight were unclassified variants (in total, 11% of all subjects with a MMR mutation in our database)] falls within the range of about 10% published in the literature (12). In total, there were 70 cases (MSS, MSI-L, or MSI-H) in the whole database in which the search for a mutation in *hMLH1*, *hMSH2*, or *hMSH6* was negative. Five of 70 cases had a MSI-H tumor with absence of one or more proteins. This number (7%) is comparable with that seen in the literature (25).

In sum, on the basis of the present study, we recommend the inclusion of PMS2 staining in the panel of antibodies to identify families eligible for mutation analysis. The addition of PMS2 staining will lead to a marked increase of detection of *hMLH1* mutation carriers. Moreover, we suggest the following revisions to the Bethesda criteria: include late-onset families (three or more cases of CRC diagnosed at age $>$ 50 years) and raise the age at diagnosis of CRC from 45 to 50 years in the original criteria.

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