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Molecular pathology of mismatch repair deficient tumours with emphasis on immune escape mechanisms

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Conventional and tissue microarray immunohistochemical expression analysis of mismatch repair in hereditary colorectal tumors

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1. ABSTRACT

Immunohistochemistry (IHC) of mismatch repair (MMR) proteins in colorectal tumors together with microsatellite analysis (MSI) can be helpful in identifying families eligible for mutation analysis. The aims were to determine sensitivity of IHC for *MLH1*, *MSH2*, and *MSH6* and MSI analysis in tumors from known MMR gene mutation carriers; and to evaluate the use of tissue microarrays for IHC (IHC-TMA) of colon tumors in its ability to identify potential carriers of MMR gene mutations, and compare it with IHC on whole slides. IHC on whole slides was performed in colorectal tumors from 45 carriers of a germline mutation in one of the MMR genes. The TMA cohort consisted of 129 colon tumors from (suspected) hereditary nonpolyposis colorectal cancer (HNPCC) patients. Whole slide IHC analysis had a sensitivity of 89% in detecting MMR deficiency in carriers of a pathogenic MMR mutation. Sensitivity by MSI analysis was 93%. IHC can also be used to predict which gene is expected to harbor the mutation: for *MLH1*, *MSH2*, and *MSH6*, IHC on whole slides would have correctly predicted the mutation in 48%, 92%, and 75% of the cases, respectively. We propose a scheme for the diagnostic approach of families with (suspected) HNPCC. Comparison of the IHC results based on whole slides versus TMA, showed a concordance of 85%, 95%, and 75% for *MLH1*, *MSH2*, and *MSH6*, respectively. This study therefore shows that IHC-TMA can be reliably used to simultaneously screen a large number of tumors from (suspected) HNPCC patients, at first in a research setting.

2. INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of death because of malignancy in the Western world. The cause of CRC is multifactorial, involving hereditary and environmental factors and somatic genetic changes during tumor progression [1]. A family history of CRC is a clinically significant risk factor and may be found in up to 15% of all patients with CRC [2]. The most common hereditary CRC syndromes are familial adenomatous polyposis coli (FAP), accounting for <1% of CRC cases and HNPCC (hereditary nonpolyposis colorectal cancer), accounting for 1 to 6% of the cases [3]. HNPCC is an autosomal dominantly inherited disorder that is clinically defined by the Amsterdam Criteria [4,5]. In HNPCC, germline mutations have been identified in four DNA mismatch repair (MMR) genes, *MSH2* [6], *MLH1* [7], *PMS2* [8], and *MSH6* [9-14]. In 50 to 70% of the families fulfilling the Amsterdam criteria a germline mutation

is detected in *MLH1* or *MSH2* [15,16]. Germline mutations have been found in *MSH6* in families with atypical HNPCC, ie not entirely fulfilling the Amsterdam criteria [11-14].

Microsatellite instability (MSI) in colorectal tumors, first reported in 1993 [17-19], is caused by a failure of the DNA MMR machinery to repair errors occurring during DNA replication and leading to length alterations in simple, repetitive microsatellite sequences distributed throughout the genome [20]. According to international guidelines, a panel of five specific microsatellite markers has been recommended for MSI evaluation [20]. If at least two markers show instability, the tumor is referred to as MSI-high (MSI-H), if only one marker is unstable, the tumor is considered MSI-low (MSI-L). MSI is reported in 85 to 92% of CRC associated with HNPCC and in 10 to 15% of sporadic CRC [17,21-23].

In 1996, Leach and colleagues [24] and Thibodeau and colleagues [25] reported the use of monoclonal antibodies directed against

MSH2 and MLH1 in the immunohistochemical analysis of CRCs. Subsequent reports described immunohistochemistry (IHC) of MLH1, MSH2, and MSH6 in sporadic and HNPCC tumors with varying results [26-35]. Some authors suggested that IHC can be used as a prescreening method for the actual mutation analysis of the MMR genes [32,36,37]. Others concluded, however, that IHC cannot replace MSI analysis as a prescreening method, because of a lower sensitivity [35,38,39]. The studies on the value of IHC published so far are hampered by small numbers of tumors associated with a known MMR gene mutation. In addition, most studies focused on tumors associated with *MLH1* and *MSH2* mutations and not *MSH6* mutations.

IHC for diagnostic purposes is usually performed on whole slides. A novel approach that allows high-throughput IHC is provided by the so-called tissue microarrays (TMAs) composed by large numbers of small punched-out tissue cores from different tumors [40]. With this technique up to 1000 different samples can be analyzed in a single immunohistochemical staining experiment [41]. Previous reports concluded that binary immunophenotypes can be reliably investigated on TMAs using two to three representative cores per tumor sample [42]. However, validation of data generated by TMA is needed to determine the minimal amount of tissue cores/tumor required in one TMA and to inventory possible problems of TMA that might influence staining results including technical artifacts such as differences in fixation of archived material or loss of tissue [43].

The aim of the present study was to evaluate whether IHC analysis of colorectal tumors could predict the presence of a MMR mutation in tumors in a large series of HNPCC patients with a known mutation, and to compare these results with the outcome of MSI. In addition, we compared the results of IHC performed on

whole slides with the TMA technique. Validation of TMA for IHC has not yet been performed for colorectal tumors.

3. PATIENTS AND METHODS

3.1 Patients

A total of 45 patients (25 males and 20 females) with a known germline mutation in *MLH1*, *MSH2*, or *MSH6*, were selected from 35 HNPCC families. Information on cancer site, age at diagnosis, and location of the colon tumors were collected for all patients (Table 1). The paraffin-embedded tissue blocks from these patients dated back from 1976 until 1999. One tumor from each patient was used for the analysis. In total, 44 CRCs and a single duodenal carcinoma were analyzed.

Among the 35 families with a known MMR defect, 27 different germline mutations have been identified by denaturing gradient-gel electrophoresis or Southern blotting [44-47]: 14 in *MLH1*, 11 of which were pathogenic (nonsense, frameshift, or splice site mutants) and 3 unspecified variants; 8 in *MSH2* (seven pathogenic, one unspecified variant); five in *MSH6* (three pathogenic, two unspecified variants) (Table 1). Twenty-four of the 45 patients in our cohort, originating from 20 families, carry an *MLH1* mutation. The same mutation was identified in six different families (1852_1854del, K618del). Thirteen of the 45 patients, originating from 10 families, were carriers of a mutation in *MSH2*. Two common mutations have been identified in two different families. Seven patients from five families were carriers of five different *MSH6* mutations.

The average age at cancer diagnosis of the carriers of *MLH1* ($n = 21$), *MSH2* ($n = 12$), and *MSH6* ($n = 4$) pathogenic mutations was 44 years (range, 28 to 68 years), 41 years (range, 23 to 61 years), and 54 years (range, 26 to 84 years), respectively.

Gene	Mutation	Exon	Patho- genic?	Number of families	Number of patients	Sex	Age of diagnosis	Site of tumor	IHC MLH1	IHC MSH2	IHC MSH6	MSI	Family diagnosis
a. <i>MLH1</i>	18_34del17, G6fsX25	1	Yes	1	2	M	35	Colon	0	0	0	H	1
	102_103delGA, E34fsX36	1	Yes	1	1	M	39	Ascendens	0	+	0	na	1
	445C>A, Q149X	5	Yes	1	1	F	30	Coecum	0	+	+	H 2/2+	1
	545+3A>G (splice donor)	6	Yes	1	1	F	43	Coecum	0	+	+	H	1
	545+3A>G (splice donor)	6	Yes	1	1	M	31	Transversum	0	+	0	H 4/4+	1
	677+1delG (splice donor)	8	Yes	1	1	F	28	Colon	+	+	+	na	1
	677G>A, R226Q (splice donor)	8	Yes	1	3	M	55	Coecum	0	+	+	S	1
	806C>G, S269X	10	Yes	2	1	F	65	Ascendens	0	+	+	H 4/5	
						M	46	Ascendens	0	+	0	H 4/5+	
					1	F	52	Coecum	0	+	+	H 2/3+	1
	1731+15G>A (splicedonor)	15	Yes	1	1	M	45	Coecum	0	+	0	H 5/5+	1
	1852_1854del, K618del	16	Yes	6	2	M	36	Colon	0	+	+	na	1
						F	57	Flexura hepatica	0	+	+	L 1/4++	1
						M	39	Transversum	+	+	+	H 3/4	
					1	F	45	Colon	+	+	+	H 3/4+	1
					1	M	57	Colon	0	+	+	na	1
					1	F	29	Transversum	0	0	0	H 2/2+	1
					1	M	50	Descendens	0	+	0	H 5/5+	1
					1	F	68	Coecum	0	+	+	H 5/5+	1
	EX16del	16	Yes	1	1	F	44	Colon	0	+	+	na	1
	2103+1G>A (splice donor)	18	Yes	1	1	M	31	Coecum	0	0	0	na	1
	277A>G, S93G	3	?	1	2	F	90	Transversum	+	+	+	H 2/3+	3

793C>T, R265C 1744C>T, L582F	10	?	1	1	M	53	Transversum	0	+	0	H 4/4+	
	16	?	1	1	M	39	Coecum	+	+	0	L 1/1+	1
					M	37	Flexura hepatica	0	+	+	H 3/4+	4
b. <i>MSH2</i> Ex 3del (in frame)	3	Yes	2	2	F	29	Flexura lienalis	+	+	0	H 3/5	2
					F	23	Duodenum	+	0	0	H 3/4++	
				1	M	34	Colon	+	0	0	H 2/4+	1
	5	Yes	1	1	M	45	Sigmoid	+	0	0	na	1
	5	Yes	1	1	M	46	Colon	+	0	0	na	1
	6	Yes	1	1	F	37	Colon	+	0	0	na	1
	1-6	Yes	2	1	M	31	Coecum	+	0	0	na	1
				2	M	28	Colon	+	0	0	na	1
					M	57	Colon	+	0	0	na	
	7	Yes	1	1	F	54	Transversum	+	0	0	H 2/3+	1
	13	Yes	1	2	F	44	Colon	+	0	0	H 4/5+	1
					M	61	Transversum	+	0	0	H	
	11	?	1	1	F	36	Sigmoid	+	+	0	L 1/3+	2
					M	26	Coecum	+	+	0	H 2/4+	1
				2	M	84	Coecum	+	+	0	H 4/5+	2
c. <i>MSH6</i> 742C>T, R248X 1784delT, L595fsX609 4001G>A (splice donor) 2008G>A, G670R 642C>T, Y214Y	4	Yes	1	1	M	26	Coecum	+	+	0	H 2/4+	1
	4	Yes	1	2	M	84	Coecum	+	+	0	H 4/5+	2
					F	49	Transversum	+	+	0	S 0/5+	
	9	Yes	1	1	F	65	Coecum	+	+	+	H 5/5+++	1
	4	?	1	1	F	55	Sigmoid	+	+	0	H 2/4+	3
	4	?	1	2	M	79	Sigmoid	+	+	na	H 2/4+	3
					M	73	Rectum	+	0	na	H 2/4+	

Table 1. MLH1, MSH2, and MSH6 Mutations

M, Male; F, female. IHC: 0, no nuclear staining; +, nuclear staining; na, not analysed. MSI: H, MSI-H; L, MSI-L; S, MSS; eg, 3/5: 3 of the 5 Bethesda markers tested instable; +, ++, +++: respectively 1, 2, or 3 of the 3 additional (BAT40, MSH3, and MSH6) markers are instable; na, no MSI analysis performed. Family diagnosis 1, Amsterdam II+; 2, suspected HNPCC; 3, late onset; 4, sporadic young age.

We defined four categories of clinical diagnoses (Table 1). The first category includes families that fulfilled the revised Amsterdam criteria (AII+) [5]. The second category includes suspected HNPCC families, ie, familial cases fulfilling the Bethesda criteria (B+) [48]. The third category encompasses late onset families consisting of three CRC patients within two or three generations, with no diagnosis made at younger than the age of 50 years. The fourth category includes sporadic patients diagnosed at younger than the age of 40 years.

3.2. MSI

MSI analysis was performed on paired tumor-normal tissue DNA samples using the Bethesda panel of microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26) [20]. This panel was extended with the additional markers BAT 40, *MSH3*, and *MSH6*, as previously reported [49]. Tumors were scored as MSI-H (high) if at least two of the five Bethesda markers showed instability, MSI-L (low) if only one of these markers showed instability, or MSS (stable) if none of the Bethesda markers showed any shift in mobility.

The annotations +, ++, or +++ were used to indicate if, respectively, one, two, or three of the additional (BAT40, *MSH3*, and *MSH6*) mononucleotide markers showed instability.

3.3. Tissue Microarray (TMA)

TMAs were assembled from formalin-fixed, paraffin-embedded tissues as previously described [40] using a 0.6-mm-diameter punch (Beecher Instruments, Silver Spring, MD). The arrays encompass 362 tissue cores from colorectal tumors derived from 129 (suspected) HNPCC patients, including the 45 tumors from MMR gene mutation carriers. These tumor samples dated back from 1974 until 2000. Also, we included three tissue cores from normal colonic

mucosa and one core of lung tissue (for orientation purposes).

Using a tape-transfer system (Instrumedics, Hackensack, NJ), 4 μ m sections were transferred to glass slides. We were unable to analyze, because of tissue loss during processing, 52 (14%), 41 (11%), and 56 (15%) of the punches for MLH1, MSH2, and MSH6, respectively. Because two to three punches were taken per tumor this meant that for six (5%), five (4%), and five (4%) of the tumors, respectively, we were unable to analyze the staining of these proteins.

3.4. IHC

Conventional IHC on whole tumor sections was performed for all tumor samples. Immunohistochemical staining was performed on 4 μ m sections of formalin-fixed, paraffin-embedded tissues. Whole tissue slides and TMA slides were stained with antibodies against MLH1 (clone 14; Calbiochem, Cambridge, MA), MSH2 (clone GB12) and MSH6 (clone 44; Transduction Laboratories/Becton Dickinson, Lexington, KY) in a DAKO Techmate 500+ (Glostrup, Denmark) automated tissue stainer using standard protocols [49] and procedures as indicated by the manufacturer. We initially tested the influence of different fixation intervals on the results of IHC for MLH1, MSH2, and MSH6. Therefore tumor parts of two control cases (one colon carcinoma and one rectal carcinoma) were fixated in buffered formalin for 1, 7, and 40 days, respectively. Overall the results were comparable. When fixation was performed with ethanol (for 1 and 4 days, respectively) in comparison with fixation in buffered formalin (also for 1 and 4 days) staining results were extremely poor after fixation with ethanol. For two initially frozen tissues, subsequent fixation in buffered formalin for 1 and 4 days seems to give less strong staining than what we normally experience after immediate fixation in formalin. Furthermore, when testing

tissue blocks from laboratories that use a pre-treatment step with acetone for tissues such as colon resections we seem to encounter a negative influence on the quality of the stainings of the MMR proteins.

Staining patterns of MMR proteins were evaluated using normal epithelial, stromal, or inflammatory cells, or the centers of lymphoid follicles as internal controls. The pathologist and technician who reviewed the immunostaining of the tissue samples were blinded to the germline mutation status of the patients.

Stained slides and individual cores were scored as either positive (showing nuclear staining in at least some tumor cells) or negative. To validate TMA, patients were considered positive if at least one tissue core showed nuclear staining and negative if none of the tissue cores showed nuclear staining of the protein.

4. RESULTS

4.1. MSI Analysis

MSI analysis was performed in 33 of the 45 tumors derived from HNPCC mutation carriers (Table 1). Although the majority of the cases showed a high frequency of instability (28 MSI-H), 3 MSI-L and 2 MSS tumors (one of which was MSS+), were found. One of the MSI-L tumors was found in a carrier of a pathogenic *MLH1* mutation (1852_1854del, K618del, exon 16). In this tumor, too, the BAT40 and MSH6 markers showed instability. IHC showed positive staining for MSH2 and MSH6 and absent staining for MLH1. Moreover, a CRC from an additional family member was MSI-H. The other two MSI-L tumors occurred in carriers of an unclassified variant. The MSS tumor occurred in a carrier of a pathogenic *MLH1* mutation (677G>A, R266Q, splice donor, exon 8) and could not be tested for the three additional markers BAT40, *MSH3*, and

MSH6. IHC indicated a mutation in *MLH1*. Furthermore, two tumors from additional carriers of the same mutation were scored as MSI-H. In the MSS+ tumor, found in a carrier of a pathogenic *MSH6* mutation (1784delT, L595fsX609, exon 4), the BAT40 marker additionally showed instability. IHC showed absent staining for MSH6 while staining was positive for MLH1 and MSH2. Again, another tumor from an affected family member was MSI-H.

The BAT40 marker showed instability in 26 of the 29 tumors in which it was tested. MSI analysis, if both patients with MSI-H and with MSI-L tumors are considered candidates for mutation analysis, gives a sensitivity of 93% (25 of 27) in predicting an MMR pathogenic mutation if the five standard Bethesda markers are used and a sensitivity of 96% (26 of 27) using the BAT40, *MSH3*, and *MSH6* markers in addition to the standard markers. The increase of sensitivity is mainly because of the use of the BAT40 marker.

In addition, 71 of 84 colorectal lesions from (suspected) HNPCC patients without known mutations were tested for MSI. The vast majority (53 of 71, 74%) of these tumors were classified as MSI-H, whereas 6% (4 of 71) were MSI-L and 20% (14 of 71) MSS.

4.2. Whole Slide Immunohistochemical Analysis of Tumors from Mutation Carriers

4.2.1 Individual Staining of the MMR Proteins

Twenty of the 25 (80%) tumors derived from *MLH1* mutation carriers did not stain for the MLH1 protein. When unspecified variants are excluded, this figure rises to 18 of 21 (86%) (Table 2). The remaining three (14%) MLH1-positive tumors were found in carriers of small in-frame deletions or splice mutations. Notably, two of the MLH1-positive tumors were part of a series of seven tumors from carriers of the 1852_1854del

IHC	MMR	<i>MLH1</i>	<i>MSH2</i>	<i>MSH6</i>	Total
No nuclear staining		18 (86%)	11 (92%)	3 (75%)	
Nuclear staining		3 (14%)	1 (8%)	1 (25%)	
Total		21	12	4	37

Table 2. IHC in Carriers of a Pathogenic Mutation

mutation in exon 16. The remaining five stained negative. Contrasting *MLH1*-staining patterns were also obtained with tumor samples from different carriers of the 277A>G, S93G missense mutation. The third *MLH1*-positive case was found in a carrier of a splice donor (677 + 1delG) mutation (according to the splice site prediction program, BDGP splice site prediction by Neural Network, the value decreased from 0.98 to 0.14).

Eleven of 13 tumors (85%) from *MSH2* mutation carriers show no *MSH2* staining. Again, this percentage increases when unspecified variants are excluded (11 of 12, 92%) (Table 2). *MSH2*-positive staining was observed in only one of three tumors from carriers of an in-frame exon 3 deletion.

Seventy-five percent (three of four) of the tumors from carriers of a pathogenic *MSH6* mutation show no staining for the corresponding protein. Of the three tumors from patients with an *MSH6* unspecified variant, only one (G670R) tumor could be analyzed: no *MSH6* staining was found, thus indicating, but not proving, pathogenicity of this mutation. When unspecified variants are included 80% (four of

five) of the tumors from *MSH6* mutation carriers show absent staining of the corresponding protein.

To determine sensitivity of IHC in detecting MSI in general we considered all tumor samples that showed abrogation of at least one of the three proteins tested to be positive for MMR deficiency. In 86% (18 of 21), 100% (12 of 12), and 75% (3 of 4) of tumors from carriers of a *MLH1*, *MSH2*, or *MSH6* pathogenic mutation, respectively, absent staining for at least one of the three proteins was shown. MMR deficiency would thus have been detected in 89% (33 of 37) of the cases.

4.2.2 Staining Patterns

MMR-IHC analysis in carriers of pathogenic *MLH1* mutations revealed that in only 48% of the cases was an *MLH1*-negative staining accompanied by normal *MSH2* and *MSH6* staining patterns (Table 3). In these cases, the IHC results clearly direct the mutation analysis to a single gene, namely *MLH1*. However, in another subset (24%) of the *MLH1*-mutant tumors, an *MSH6*-negative staining pattern accompanied the loss of *MLH1*

IHC	MMR	<i>MLH1</i>	<i>MSH2</i>	<i>MSH6</i>
1+/2+/6+		3 (14%)		1 (25%)
1-/2+/6+		10 (48%)		
1+/2-/6+				
1+/2+/6-			1 (8%)	3 (75%)
1-/2+/6-		5 (24%)		
1+/2-/6-			11 (92%)	
1-/2-/6+				
1-/2-/6-		3 (14%)		
Total		21	12	4

Table 3. IHC Staining Pattern in Carriers of a Pathogenic Mutation

1, *MLH1*; 2, *MSH2*; 6, *MSH6*; +, nuclear staining; -, no nuclear staining.

signal, thus providing a more ambiguous indication for the subsequent mutation analysis. In three tumors (14%) positive staining for all three proteins was found. All three were scored as MSI-H. Therefore, these patients would not have been considered candidates for mutation analysis if IHC alone had been performed. Another three tumors (14%) showed no staining for all three proteins. Notably, negative staining patterns for all three MMR proteins were found exclusively in combination with a germline *MLH1* mutation (Table 1 and 3).

In the vast majority of tumors from pathogenic *MSH2* mutation carriers (11 of 12, 92%), loss of the *MSH2* signal is accompanied by *MSH6*-negative and *MLH1*-positive staining patterns. Only in one case (exon 3 deletion) were the corresponding *MSH2* and *MLH1* signals positive while *MSH6* staining was lost. The latter would have unjustly indicated mutation analysis of the *MSH6* gene. However, IHC analysis of a tumor from an additional patient from the same family clearly showed both *MSH2*- and *MSH6*-negative staining patterns.

In the three of four cases with a pathogenic *MSH6* mutation, the expected *MSH6*-negative staining is accompanied by normal *MLH1* and *MSH2* patterns. In the fourth case, in which it was predicted that a missense mutation would affect RNA splicing (4001G>A, splice donor), positive staining for all three MMR proteins was found. The tumor was MSI-H (Table 1c).

4.3. Tissue Microarray Immunohistochemical Analysis (TMA-IHC)

A TMA encompassing the total cohort of 129 colorectal tumors was generated. We evaluated TMA-IHC staining for the presence or absence of the three main MMR proteins in the (suspected) HNPCC tumors and compared these with the results obtained by whole tumor section IHC when available (Table 4). An example of the staining pattern in a tumor from an *MLH1* mutation carrier (1744 C>T, L582F; Table 1a) is shown in Figure 1 for the *MLH1* (Figure 1A), *MSH2* (Figure 1B), and *MSH6* (Figure 1C) protein, respectively; *MLH1* is abrogated, whereas *MSH2* and *MSH6* are present in the nuclei of the tumor cells.

Staining was concordant in 71 of 84 (85%) cases tested for *MLH1*, and in 77 of the 81 (95%) for *MSH2*. A somewhat lower level of concordance was found for *MSH6*: only 49 of 65 (75%) tumors showed similar results, mainly because of a high number [13] of positive staining results in TMA, scored as negative on whole slides. Of the latter samples six belonged to *MLH1* mutation carriers (all All+), two to *MSH2* mutation carriers (all All+), one to an *MSH6* mutation carrier and four samples belonged to individuals in whom no mutation was identified (2 times All+, 2 times All-, B+).

	ws +, tma +	ws +, tma -	ws -, tma -	ws -, tma +	Total	Concordance	Sensitivity	Specificity
MLH1	52	10	19	3	84	85%	84%	86%
MSH2	55	2	22	2	81	95%	96%	92%
MSH6	23	3	26	13	65	75%	88%	67%

Table 4. Validation of TMA for Mismatch Repair Proteins

Staining results of the array compared to results of staining of whole slides from the same patients for *MLH1*, *MSH2*, and *MSH6*. Staining was scored as either positive or negative, as described above. Tma, tissue microarray; ws, whole slide; +, positive nuclear staining; -, negative nuclear staining; conc, percentage concordance; sensitivity (percentage of true positives), specificity (percentage of true negatives).

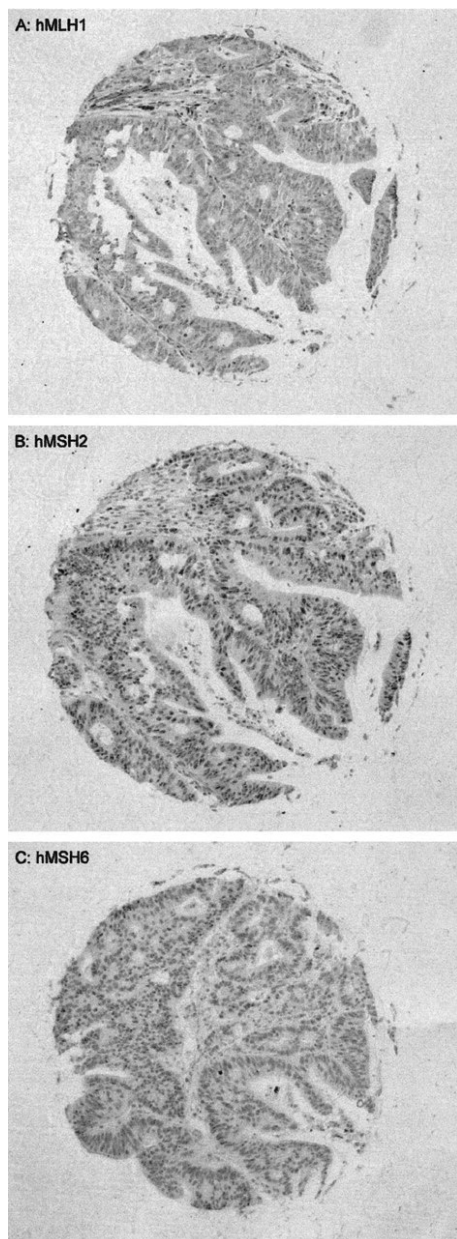


Figure 1. Immunoreactivity in a TMA of mainly (suspected) HNPCC patients. A tissue core with a colon carcinoma from a patient with a germline hMLH1 missense mutation (1744C>T, L582F) is shown, stained for MLH1 (A), MSH2 (B), and MSH6 (C). MLH1 is abrogated, whereas MSH2 and MSH6 are present in the nuclei of the tumor cells. Slides were stained with antibodies against MLH1 (clone 14, Calbiochem, Cambridge, MA), MSH2 (clone GB12, Calbiochem), and MSH6 (clone 44, Transduction Laboratories/Becton Dickinson, Lexington, KY). Original magnifications, x100.

5. DISCUSSION

The identification of MMR gene mutations in suspected HNPCC families is of great relevance for allowing the identification of mutation carriers for whom surveillance of the colon is required and has been proven to lower the risk to develop and to die of colorectal carcinoma [50]. A potential problem in the everyday clinical practice is that MMR genetic testing is expensive and time-consuming. In this study, we first evaluated the sensitivity of conventional whole section IHC analysis of MLH1, MSH2, and MSH6 in colon tumors from 45 established carriers of a MMR gene mutation and compared it with MSI analysis.

The sensitivity of IHC in predicting a pathogenic mutation was 89% (33 of 37), only slightly lower than that of MSI analysis using the Bethesda panel of five markers (93%, 25 of 27), or using the additional three markers (96%, 26 of 27). For IHC these results are remarkable because the paraffin blocks dated back from 1976 until 1999, with 35% of the samples older than 10 years, and fixation until now not fully standardized. We argue that intratumor heterogeneity will not be a problem in hereditary cases because of the fact that loss of MMR, and consequently often abrogation of MMR protein expression, is such an early event that it is present in all tumor cells. Should IHC become a standard of care in unselected cases heterogeneity is an issue that still needs further investigation, although in colorectal tumors with a MMR defect because of somatic abrogation of *MLH1*, this feature seems to be a dominant characteristic, as

can be interpreted from a study on such heterogeneity [51].

An important advantage of IHC compared to MSI analysis is represented by the prediction of the specific MMR gene mutated in the germline of the corresponding patient. In tumors from most *MLH1* mutation carriers (80%), staining of the MLH1 protein was absent, as expected. However, in only half (48%) of the tumors associated with a MLH1 mutation, the staining pattern (MLH1-, MSH2+, MSH6+) would have predicted unequivocally a pathogenic mutation in the *MLH1* gene. In the future, the inclusion of PMS2 staining, which is often negative in tumors associated with *MLH1* germline mutations [52], will most likely lead to a further increase of IHC sensitivity.

Negative staining for both MSH2 and MSH6 was found in tumors from *MSH2* mutation carriers. Tumors from *MSH6* mutation carriers, showed a lack of MSH6 staining only. These findings are most likely because of the failure of MSH6 to form a stable heterodimer in the absence of MSH2 [53]. On the other hand, if MSH6 is absent, a heterodimer can still be formed between MSH2 and MSH3, thus resulting in stabilization and positive staining of the MSH2 protein [49,54]. In our study, the specific staining pattern (MLH1+, MSH2-, MSH6-) of *MSH2* mutated tumors would have correctly predicted the mutated MMR gene in all but one (92%) of the cases, while the specific staining pattern of tumors from *MSH6* mutation carriers (MLH1+, MSH2+, MSH6-) would have predicted the presence of a *MSH6* mutation in 75% of the cases.

In previous smaller studies, the sensitivity of IHC and MSI analysis has been evaluated in colorectal tumors of carriers of specified *MLH1*, *MSH2* [25,30,32,35,55], and *MSH6* [27,54,56] mutations. A problem we cannot solve is the possibility that variable outcomes of IHC analyses

might be because of differences in staining protocols and antibodies used. Furthermore, a considerable number of mutations included in these studies are unclassified variants in which pathogenicity is by definition uncertain. In the present study, only pathogenic mutations were included in the determination of the sensitivity of IHC on whole slides.

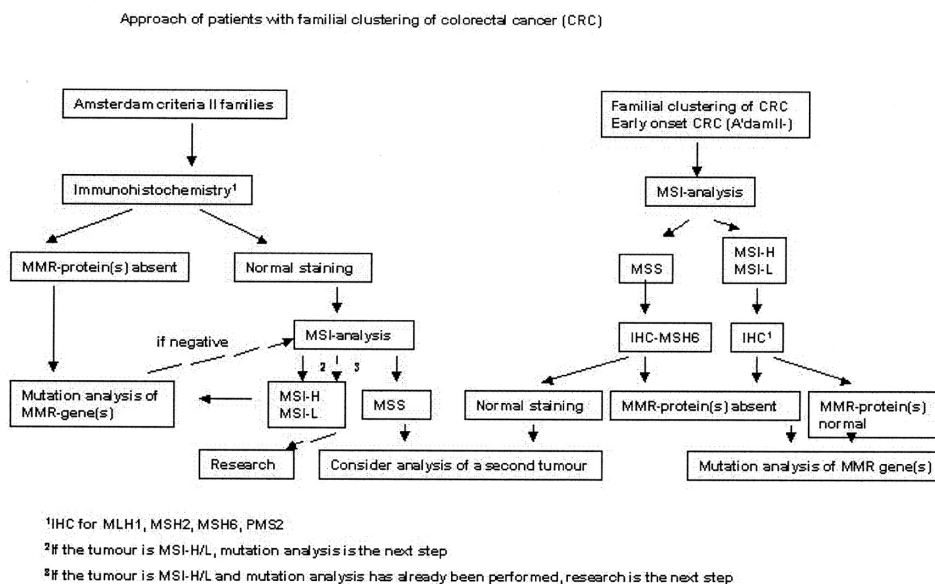
The results of our study show that both IHC and MSI are sensitive prescreening methods to identify patients for mutation analysis. At present, IHC cannot completely replace MSI analysis until the sensitivity of MLH1 staining is improved, as recently discussed by de la Chapelle [57], and as long as the role of other putative MMR genes in hereditary CRC has not been elucidated. Because of its gene predictive value, and because of its speed and low cost, we would recommend IHC as a first diagnostic step in families fulfilling the revised Amsterdam criteria in which the probability of detecting a MMR gene mutation is relatively high [58] and MSI analysis is likely to give superfluous information. If a negative staining pattern is found, mutation analysis of the respective gene(s) is the next step. In case of doubtful interpretation or positive staining of all MMR proteins, MSI analysis should be performed. In the case of the absence of microsatellite instability (MSS), the analysis of a second tumor from the same family is recommended, to exclude intrafamilial variability in MSI analysis and IHC results, as shown in this study, and/or the presence of phenocopies.

In families not fulfilling the Amsterdam criteria, we would recommend MSI analysis as the first step. In these cases, the probability of detecting a *MLH1* or *MSH2* mutation is low [58] and IHC is less likely to be informative. *MSH6* families, predominantly found not to comply with the Amsterdam criteria, represent exceptions. In the total group of Amsterdam-negative families MSI analysis is expected to provide

global information on loss of MMR function, including pathogenic missense mutations and alterations in MMR genes other than the known ones. In MSI-H or MSI-L (if the unstable marker is a mononucleotide marker) cases, IHC of all four MMR proteins should be performed as second step. In the case of MSS, IHC for MSH6 is recommended as it was shown that tumors from MSH6 mutation carriers are characterized by a variable MSI phenotype [54,59]. If no IHC abnormality is found, examination of a second tumor could be considered depending on the family history and age of the patient already tested. A scheme for clinical use, summarizing our current approach to patients from families with suspected HNPCC, is given in Figure 2.

The generation of a tissue array encompassing microsatellite unstable tumors has provided us with a powerful tool to quickly characterize the immunohistochemical staining patterns of

MMR proteins in hereditary colorectal tumors for research purposes. We found a high level of concordance for MLH1 and MSH2 (85% and 95%, respectively). A somewhat lower concordance level was found for MSH6 (75%), primarily because of positive staining within the TMA and negative staining with the whole slide IHC. Six of the 13 tumors with discordant results for MSH6 originated from patients in whom an *MLH1* mutation has been identified, where positive staining for MSH6 is expected. Two and one samples originated from carriers of a MSH2 and MSH6 mutation, respectively, where negative staining for MSH6 is expected. The other four samples originate from individuals in whom to date no mutation is identified and therefore no golden standard is available. Our first goal is to rapidly characterize the staining of other candidate MMR proteins particularly in tumors from (suspected) HNPCC patients in whom to date



MSI: Microsatellite instability, MMR: Mismatch repair, MSS: MSI-stable, MSI-L: MSI-low, MSI-H: MSI-high, IHC: Immunohistochemistry

Figure 2. Approach of patients with familial clustering of CRC. 1: IHC for MLH1, MSH2, MSH6, PMS2. 2: If the tumor is MSI-H, mutation analysis is the next step. 3: If the tumor is MSI-H and mutation has already been performed, research is the next step.

no mutation was detected, to direct mutation analysis. Problems relative to differences in fixation standardization, age of tissues, punching outside the tumor area, and loss of tissue still represent serious obstacles. For fixation standardization we tend to suggest fixation for 1 day in buffered formalin. Use of ethanol fixation and acetone pretreatment should be avoided (see Patients and Methods). However, our study shows the general validity of this approach in the molecular diagnosis of familial CRC. Accordingly, recent IHC-TMA analysis has enabled us to identify a number of patients with abrogated staining of PMS2 with or without MLH1 staining, thus providing direction to mutation analysis of PMS2.

In conclusion, we have demonstrated the value of IHC using both whole slides and TMA as prescreening tools in selecting patients eligible for mutation analysis of MMR genes, in diagnostic and research settings respectively.

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7. REFERENCES

1. Kinzler KW, Vogelstein B: **Lessons from hereditary colorectal cancer.** *Cell* 1996, **87**:159-170.
2. Lynch HT, Smyrk TC, Watson P, Lanspa SJ, Lynch JF, Lynch PM, Cavalieri RJ, Boland CR: **Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review.** *Gastroenterology* 1993, **104**: 1535-1549.
3. Aaltonen LA, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomaki P, Chadwick RB, Kaariainen H, Eskelinen M, Jarvinen H, Mecklin JP, de

- la Chapelle A: **Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease.** *N Engl J Med* 1998, **338**:1481-1487.
4. Vasen HF, Mecklin JP, Khan PM, Lynch HT: **The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC).** *Dis Colon Rectum* 1991, **34**:424-425.
5. Vasen HF, Watson P, Mecklin JP, Lynch HT: **New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC.** *Gastroenterology* 1999, **116**: 1453-1456.
6. Fishel R, Lescoe MK, Rao MR, Copeland NG, Jenkins NA, Garber J, Kane M, Kolodner R: **The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer.** *Cell* 1993, **75**:1027-1038.
7. Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, Kane M, Earabino C, Lipford J, Lindblom A, Tannergard P, Bollag RJ, Gadwin AR, Ward DL, Nordenskold M, Fishel R, Kolodner R, Liskay RM: **Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer.** *Nature* 1994, **368**:258-261.
8. Nicolaides NC, Papadopoulos N, Liu B, Wei YF, Carter KC, Ruben SM, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JC, Dunlop MG, Hamilton SR, Petersen GM, de la Chapelle A, Vogelstein B, Kinzler KW: **Mutations of two PMS homologues in hereditary nonpolyposis colon cancer.** *Nature* 1994, **371**:75-80.
9. Miyaki M, Konishi M, Tanaka K, Kikuchi-Yanoshita R, Muraoka M, Yasuno M, Igari T, Koike M, Chiba M, Mori T: **Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer.** *Nat Genet* 1997, **17**:271-272.
10. Akiyama Y, Sato H, Yamada T, Nagasaki H, Tsuchiya A, Abe R, Yuasa Y: **Germ-line mutation of the hMSH6/GTBP gene in an atypical hereditary**

- nonpolyposis colorectal cancer kindred.** *Cancer Res* 1997, **57**:3920-3923.
11. Wijnen J, de Leeuw W, Vasen H, van der KH, Moller P, Stormorken A, Meijers-Heijboer H, Lindhout D, Menko F, Vossen S, Moslein G, Tops C, Brocker-Vriends A, Wu Y, Hofstra R, Sijmons R, Cornelisse C, Morreau H, Fodde R: **Familial endometrial cancer in female carriers of MSH6 germline mutations.** *Nat Genet* 1999, **23**:142-144.
 12. Wu Y, Berends MJ, Mensink RG, Kempinga C, Sijmons RH, Der Zee AG, Hollema H, Kleibeuker JH, Buys CH, Hofstra RM: **Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations.** *Am J Hum Genet* 1999, **65**:1291-1298.
 13. Kolodner RD, Tytell JD, Schmeits JL, Kane MF, Gupta RD, Weger J, Wahlberg S, Fox EA, Peel D, Ziogas A, Garber JE, Syngal A, Anton-Culver H, Li FP: **Germ-line msh6 mutations in colorectal cancer families.** *Cancer Res* 1999, **59**:5068-5074.
 14. Verma L, Kane MF, Brassett C, Schmeits J, Evans DG, Kolodner RD, Maher ER: **Mononucleotide microsatellite instability and germline MSH6 mutation analysis in early onset colorectal cancer.** *J Med Genet* 1999, **36**:678-682.
 15. Wijnen J, Khan PM, Vasen H, van der KH, Mulder A, Leeuwen-Cornelisse I, Bakker B, Losekoot M, Moller P, Fodde R: **Hereditary nonpolyposis colorectal cancer families not complying with the Amsterdam criteria show extremely low frequency of mismatch-repair-gene mutations.** *Am J Hum Genet* 1997, **61**:329-335.
 16. Liu B, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, Watson P, Jass JR, Dunlop M, Wyllie A, Peltomaki P, de la Chapelle A, Hamilton SR, Vogelstein B, Kinzler KW: **Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients.** *Nat Med* 1996, **2**:169-174.
 17. Thibodeau SN, Bren G, Schaid D: **Microsatellite instability in cancer of the proximal colon.** *Science* 1993, **260**:816-819.
 18. Peltomaki P, Aaltonen LA, Sistonen P, Pylkkanen L, Mecklin JP, Jarvinen H, Green JS, Jass JR, Weber JL, Leach FS, Petersen GM, Hamilton SR, de la Chapelle A, Vogelstein B: **Genetic mapping of a locus predisposing to human colorectal cancer.** *Science* 1993, **260**:810-812.
 19. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M: **Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis.** *Nature* 1993, **363**:558-561.
 20. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S: **A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer.** *Cancer Res* 1998, **58**:5248-5257.
 21. Lothe RA, Peltomaki P, Meling GI, Aaltonen LA, Nystrom-Lahti M, Pylkkanen L, Heimdal K, Andersen TI, Moller P, Rognum TO, Fossa SD, Halderson T, Langmarle F, Bragger A, de la Chapelle A, Borresen LA: **Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history.** *Cancer Res* 1993, **53**:5849-5852.
 22. Aaltonen LA, Peltomaki P, Mecklin JP, Jarvinen H, Jass JR, Green JS, Lynch HT, Watson P, Tallqvist G, Juhola M, Sistonen P, Hamilton SR, Kinzler KW, Vogelstein B, de la Chapelle A: **Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients.** *Cancer Res* 1994, **54**:1645-1648.
 23. Moslein G, Tester DJ, Lindor NM, Honchel R, Cunningham JM, French AJ, Halling KC, Schwab M, Goretzki P, Thibodeau SN: **Microsatellite instability and mutation analysis of hMSH2 and hMLH1 in patients with sporadic, familial and hereditary colorectal cancer.** *Hum Mol Genet* 1996, **5**:1245-1252.

24. Leach FS, Polyak K, Burrell M, Johnson KA, Hill D, Dunlop MG, Wyllie AH, Peltomaki P, de la Chapelle A, Hamilton SR, Kinzler KW, Vogelstein B: **Expression of the human mismatch repair gene hMSH2 in normal and neoplastic tissues.** *Cancer Res* 1996, **56**:235-240.
25. Thibodeau SN, French AJ, Roche PC, Cunningham JM, Tester DJ, Lindor NM, Moslein G, Baker SM, Liskay RM, Burgart LJ, Honchel R, Halling KC: **Altered expression of hMSH2 and hMLH1 in tumors with microsatellite instability and genetic alterations in mismatch repair genes.** *Cancer Res* 1996, **56**:4836-4840.
26. Fink D, Nebel S, Aebi S, Zheng H, Kim HK, Christen RD, Howell SB: **Expression of the DNA mismatch repair proteins hMLH1 and hPMS2 in normal human tissues.** *Br J Cancer* 1997, **76**:890-893.
27. Schweizer P, Moisis AL, Kuismanen SA, Truninger K, Vierumaki R, Salovaara R, Arola J, Butzow R, Jiricny J, Peltomaki P, Nystrom-Lahti M: **Lack of MSH2 and MSH6 characterizes endometrial but not colon carcinomas in hereditary nonpolyposis colorectal cancer.** *Cancer Res* 2001, **61**:2813-2815.
28. Kim H, Piao Z, Kim JW, Choi JS, Kim NK, Lee JM, Park JH: **Expression of hMSH2 and hMLH1 in colorectal carcinomas with microsatellite instability.** *Pathol Res Pract* 1998, **194**:3-9.
29. Maeda K, Nishiguchi Y, Onoda N, Otani H, Nakata B, Yamada S, Okuno M, Sowa M, Chung KH: **Expression of the mismatch repair gene hMSH2 in sporadic colorectal cancer.** *Int J Oncol* 1998, **13**:1147-1151.
30. Curia MC, Palmirotta R, Aceto G, Messerini L, Veri MC, Crognale S, Valanzano R, Ficari F, Fracasso P, Stigliano V, Tonelli F, Casale V, Guadagni F, Battista P, Mariani-Costantini R, Cama A: **Unbalanced germ-line expression of hMLH1 and hMSH2 alleles in hereditary nonpolyposis colorectal cancer.** *Cancer Res* 1999, **59**:3570-3575.
31. Cawkwell L, Gray S, Murgatroyd H, Sutherland F, Haine L, Longfellow M, O'Loughlin S, Cross D, Kronborg O, Fenger C, Mapstone N, Dixon M, Quirke P: **Choice of management strategy for colorectal cancer based on a diagnostic immunohistochemical test for defective mismatch repair.** *Gut* 1999, **45**:409-415.
32. Debnjak T, Kurzawski G, Gorski B, Kladny J, Domagala W, Lubinski J: **Value of pedigree/clinical data, immunohistochemistry and microsatellite instability analyses in reducing the cost of determining hMLH1 and hMSH2 gene mutations in patients with colorectal cancer.** *Eur J Cancer* 2000, **36**:49-54.
33. Chaves P, Cruz C, Lage P, Claro I, Cravo M, Leitao CN, Soares J: **Immunohistochemical detection of mismatch repair gene proteins as a useful tool for the identification of colorectal carcinoma with the mutator phenotype.** *J Pathol* 2000, **191**:355-360.
34. Chiaravalli AM, Furlan D, Facco C, Tibiletti MG, Dionigi A, Casati B, Albarello L, Riva C, Capella C: **Immunohistochemical pattern of hMSH2/hMLH1 in familial and sporadic colorectal, gastric, endometrial and ovarian carcinomas with instability in microsatellite sequences.** *Virchows Arch* 2001, **438**:39-48.
35. Salahshor S, Koelble K, Rubio C, Lindblom A: **Microsatellite instability and hMLH1 and hMSH2 expression analysis in familial and sporadic colorectal cancer.** *Lab Invest* 2001, **81**:535-541.
36. Marcus VA, Madlensky L, Gryfe R, Kim H, So K, Millar A, Temple LK, Hsieh E, Hiruki T, Narod S, Bapat BV, Gallinger S, Redston M: **Immunohistochemistry for hMLH1 and hMSH2: a practical test for DNA mismatch repair-deficient tumors.** *Am J Surg Pathol* 1999, **23**:1248-1255.
37. Paraf F, Gilquin M, Longy M, Gilbert B, Gorry P, Petit B, Labrousse F: **MLH1 and MSH2 protein immunohistochemistry is useful for detection of hereditary non-polyposis colorectal cancer in young patients.** *Histopathology* 2001, **39**:250-258.
38. Wahlberg SS, Schmeits J, Thomas G, Loda M, Garber J, Syngal S, Kolodner RD, Fox E: **Evaluation**

- of microsatellite instability and immunohistochemistry for the prediction of germ-line MSH2 and MLH1 mutations in hereditary nonpolyposis colon cancer families. *Cancer Res* 2002, **62**: 3485-3492.
39. Stone JG, Robertson D, Houlston RS: **Immunohistochemistry for MSH2 and MHL1: a method for identifying mismatch repair deficient colorectal cancer.** *J Clin Pathol* 2001, **54**:484-487.
 40. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP: **Tissue microarrays for high-throughput molecular profiling of tumor specimens.** *Nat Med* 1998, **4**:844-847.
 41. Bubendorf L, Nocito A, Moch H, Sauter G: **Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies.** *J Pathol* 2001, **195**:72-79.
 42. Hoos A, Cordon-Cardo C: **Tissue microarray profiling of cancer specimens and cell lines: opportunities and limitations.** *Lab Invest* 2001, **81**: 1331-1338.
 43. Hoos A, Urist MJ, Stojadinovic A, Mastorides S, Dudas ME, Leung DH, Kuo D, Brennan MF, Lewis JJ, Cordon-Cardo C: **Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors.** *Am J Pathol* 2001, **158**:1245-1251.
 44. Wignen J, Vassen H, Meera Khan P, Menko FH, van der Klift H, van Leeuwen C, van den Broek M, Van Leeuwen-Cornelisse I, Nagengast F, Meijers-Heijboer A, Lindhout D, Griffioen G, Cats A, Kleibeuker J, Varasco L, Bertario L, Bisgaard M-L, Mohr J, Fodde R: **Seven new mutations in hMSH2, an HNPCC gene, identified by denaturing gradient-gel electrophoresis.** *Am J Hum Genet* 1995, **56**:1060-1066.
 45. Wijnen J, Khan PM, Vasen H, Menko F, van der Klift H, van den Broek M, Van Leeuwen-Cornelisse I, Nagengast F, Meijers-Heijboer EJ, Lindhout D, Griffioen G, Cats A, Kleibeuker J, Varesco L, Bertario L, Bisgaard ML, Mohr J, Kolodner R, Fodde R: **Majority of hMLH1 mutations responsible for hereditary nonpolyposis colorectal cancer cluster at the exonic region 15-16.** *Am J Hum Genet* 1996, **58**:300-307.
 46. Wijnen J, van der Klift H, Vasen H, Khan PM, Menko F, Tops C, Meijers-Heijboer H, Lindhout D, Moller P, Fodde R: **MSH2 genomic deletions are a frequent cause of HNPCC.** *Nat Genet* 1998, **20**: 326-328.
 47. Wijnen J, de Leeuw W, Vasen H, van der KH, Moller P, Stormorken A, Meijers-Heijboer H, Lindhout D, Menko F, Vossen S, Moslein G, Tops C, Brocker-Vriends A, Wu Y, Hofstra R, Sijmons R, Cornelisse C, Morreau H, Fodde R: **Familial endometrial cancer in female carriers of MSH6 germline mutations.** *Nat Genet* 1999, **23**:142-144.
 48. Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, Lynch H, Perucho M, Smyrk T, Sobin L, Srivastava S: **A National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome: meeting highlights and Bethesda guidelines.** *J Natl Cancer Inst* 1997, **89**:1758-1762.
 49. Leeuw de WJ, Dierssen J, Vasen HF, Wijnen JT, Kenter GG, Meijers-Heijboer H, Brocker-Vriends A, Stormorken A, Moller P, Menko F, Cornelisse CJ, Morreau H: **Prediction of a mismatch repair gene defect by microsatellite instability and immunohistochemical analysis in endometrial tumours from HNPCC patients.** *J Pathol* 2000, **192**:328-335.
 50. Jarvinen HJ, Aarnio M, Mustonen H, Aktan-Collan K, Aaltonen LA, Peltomaki P, de la Chapelle A, Mecklin JP: **Controlled 15-year trial on screening for colorectal cancer in families with hereditary nonpolyposis colorectal cancer.** *Gastroenterology* 2000, **118**:829-834.
 51. Samowitz WS, Slattery ML: **Regional reproducibility of microsatellite instability in sporadic colorectal cancer.** *Genes Chromosom Cancer* 1999, **26**:106-114.

52. Young J, Simms LA, Biden KG, Wynter C, Whitehall V, Karamatic R, George J, Goldblatt J, Walpole I, Robin SA, Borten MM, Stitz R, Searle J, McKeone D, Fraser L, Purdie DR, Podger K, Price R, Butten-shaw R, Walsh MD, Barker M, Leggett BA, Jass JR: **Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis.** *Am J Pathol* 2001, **159**:2107-2116.
53. Fishel R: **Signaling mismatch repair in cancer.** *Nat Med* 1999, **5**:1239-1241.
54. Berends MJ, Wu Y, Sijmons RH, Mensink RG, van Der ST, Hordijk-Hos JM, de Vries EG, Hollema H, Karrenbeld A, Buys CH, Der Zee AG, Hofstra RM, Kleibeuker JH: **Molecular and clinical characteristics of MSH6 variants: an analysis of 25 index carriers of a germline variant.** *Am J Hum Genet* 2002, **70**:26-37.
55. Dieumegard B, Grandjouan S, Sabourin JC, Le Bihan ML, Lefrere I, Bellefqih, Pignon JP, Rougier P, Lasser P, Benard J, Couturier D, Bressac-de Paillerets B: **Extensive molecular screening for hereditary non-polyposis colorectal cancer.** *Br J Cancer* 2000, **82**:871-880.
56. Plaschke J, Kruger S, Pistorius S, Theissig F, Saeger HD, Schackert HK: **Involvement of hMSH6 in the development of hereditary and sporadic colorectal cancer revealed by immunostaining is based on germline mutations, but rarely on somatic inactivation.** *Int J Cancer* 2002, **97**:643-648.
57. de la Chapelle A: **Microsatellite instability phenotype of tumors: genotyping or immunohistochemistry? The jury is still out.** *J Clin Oncol* 2002, **20**:897-899.
58. Wijnen J, Khan PM, Vasen H, van der KH, Mulder A, Leeuwen-Cornelisse I, Bakker B, Losekoot M, Moller P, Fodde R: **Hereditary nonpolyposis colorectal cancer families not complying with the Amsterdam criteria show extremely low frequency of mismatch-repair-gene mutations.** *Am J Hum Genet* 1997, **61**:329-335.
59. Wu Y, Berends MJ, Mensink RG, Kempinga C, Sijmons RH, Der Zee AG, Hollema H, Kleibeuker JH, Buys CH, Hofstra RM: **Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations.** *Am J Hum Genet* 1999, **65**:1291-1298.

