Microsatellite instability—a useful diagnostic tool to select patients at high risk for hereditary non-polyposis colorectal cancer: a study in different groups of patients with colorectal cancer

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Abstract

***Background*—Clinical diagnosis of hereditary non-polyposis colorectal cancer (HNPCC) is based on a typical family history. As molecular genetic testing is predominantly restricted to these families, gene carriers not meeting the clinical criteria may be missed.**

***Aims*—To examine the value of microsatellite instability (MSI) as a tool to increase the likelihood for uncovering a mismatch repair germline mutation in patients with colorectal cancer and to identify a genotype-phenotype relation in families with verified mutations.**

***Methods*—Systematic search for germline mutations (hMSH2 and hMLH1 genes) was performed in 96 patients: 57 fulfilled the Amsterdam criteria (group 1) and 12 the looser HNPCC criteria (group 2). Seventeen patients showed familial clustering of cancers (group 3) and 10 patients under 50 years had sporadic cancer (group 4), the latter of whom all exhibited MSI$^+$ tumours.**

***Results*—A similar proportion of germline mutations was found in patients who fulfilled the clinical criteria of HNPCC and had MSI$^+$ tumours (groups 1 and 2; 15/39) compared with patients who did not meet these clinical criteria but who had MSI$^+$ tumours (groups 3 and 4; 8/27 patients). Affected relatives of patients with hMLH1 mutations showed a significantly higher frequency of colorectal cancer but a lower frequency of endometrium cancer than those with hMSH2 mutations.**

***Conclusions*—MSI in tumour tissue is a useful criterion for selecting patients who should be tested for germline mutations in the mismatch repair genes hMSH2 and hMLH1 irrespective of their family history. Among carriers of hMSH2 mutations the tumour spectrum was broader than among carriers of hMLH1 mutations.**

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Hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome) accounts for approximately 1–6% of all colorectal cancers. The syndrome is characterised by an autosomal dominant predisposition to early onset colorectal cancer and tumours of the endometrium, the upper gastrointestinal tract, and the urinary tract.[1–4] In order to standardise the recruitment of families for collaborative studies the International Collaborative Group on HNPCC proposed the so called Amsterdam criteria: at least three relatives in two successive generations with colorectal cancer, one of whom is a first-degree relative of the other two; one of the relatives is diagnosed for colorectal cancer before age 50; and familial adenomatous polyposis is excluded.[5] Nevertheless, the clinical diagnosis of HNPCC remains poorly defined.

The discovery that mutations in DNA mismatch repair genes (hMSH2, hMLH1, hMSH6/GTBP, hPMS1, hPMS2) are involved in the tumourigenesis of HNPCC was a breakthrough in the delineation of HNPCC.[6–13] Most of the studies intending to find germline mutations in these genes included large families that were selected on the basis of the Amsterdam criteria. Only a few mutations could be identified in patients without a typical family history of colorectal cancer (for review see Peltomaki and Vasen[14]). While persons at risk from families who meet the Amsterdam criteria are usually included in a special surveillance programme, the risk estimate and the decision for surveillance needs a validated basis in those relatives of patients who are apparently non-familial. This is particularly true for sporadic cases with an early onset of colorectal cancer and for patients with different malignancies that show some familial accumulation but do not meet the clinical criteria of HNPCC. In addition, it is often impossible to obtain the complete family history of patients. Thus, the limitation of a search for germline mutations in patients meeting the strict HNPCC criteria means that a large proportion of individuals at risk of malignancies of the HNPCC tumour spectrum would be excluded from surveillance.

Tumorigenesis in HNPCC may be initiated, or promoted, by the deficiency in DNA mismatch repair which results when the corre-

Abbreviations used in this paper: HNPCC, hereditary non-polyposis colorectal cancer; MSI, microsatellite instability; SSCA, single strand conformation analysis; HA, heteroduplex analysis; PCR, polymerase chain reaction.

sponding wild type allele of the DNA mismatch repair gene (mutated in the germline) is either mutated or lost in a tumour cell; this may be related to the resulting increase in mutation rate, but other mechanisms cannot be excluded. The genetic instability characteristic of HNPCC tumours can be shown by an examination of short repeat sequences known as microsatellites.[12 15–19] In this study, we investigated the value of microsatellite instability (MSI) in increasing the likelihood of uncovering a mismatch repair germline mutation in patients with colorectal cancer. For this purpose we examined patients who met the clinical criteria for HNPCC as well as patients either with colorectal cancer before the age of 50 or with multiple synchronous or metachronous tumours at any age.

An appreciable proportion of germline mutations could be identified in patients who exhibit a high degree of MSI irrespective of their family history. Thus, the MSI phenotype is a useful marker to select patients at a high risk for HNPCC.

In addition, our mutation analysis in a large sample of patients supports and extends the present knowledge on the genotype-phenotype relation in HNPCC.

Patients and methods

A total of 160 index patients who had given their informed consent to the study were classified into four subgroups (table 1): patients with colorectal cancer who met the strict Amsterdam criteria, group 1 (n=57); patients with colorectal cancer who fulfilled the looser criteria of HNPCC—that is, they had a family history with extracolonic malignancies (cancer of the endometrium, stomach, duodenum, urinary tract, bile duct, or ovary) that were considered equal to colorectal cancer within the Amsterdam criteria, group 2 (n=12); patients with colorectal cancer before the age of 50 and some relatives affected by different malignancies of the HNPCC tumour spectrum without meeting the HNPCC criteria, group 3 (n= 45); and patients with apparently sporadic colorectal cancer aged below 50, or with multiple synchronous or metachronous colorectal tumours without any age limitation, group 4 (n=46).

Blood samples and tumour tissues were obtained from the index patients. The diagnostic procedure depended on the family history of the patients obtained through interviews and verified by the available medical records. Patients of groups 1 and 2 were screened for germline mutations in the hMSH2 and hMLH1 genes irrespective of MSI status, whereas patients of groups 3 and 4 were only examined for germline mutations if the tumours were MSI^+.

MICROSATELLITE INSTABILITY

Formalin fixed, paraffin wax embedded tumours were cut into 10 µm sections. Reference sections were stained with haematoxylin and eosin, areas of tumour growth were marked by a pathologist, and tumour DNA was extracted by use of the QiaAmp Tissue Kit (Qiagen) according to the manufacturer's instructions. Mono-, di-, and tetranucleotide repeat sequences (D2S123, D2S136, D3S1298, D3S1611, D5S346, D6S470, D18S35, D18S37, HBAP1, BAT25) were used and microsatellite analysis was performed by polymerase chain reaction in the presence of α-^{32}P-dCTP and autoradiography. Matched samples of tumour and normal DNA were examined with up to 10 microsatellite markers. The evaluation of microsatellite instability was as follows: MSI^+ = sequence length alterations with at least two microsatellite markers, tumours showing microsatellite instability with more than 40% of the examined markers were classified as highly instable; MSI^- = no alteration with 10 microsatellite markers; $MSI^?$ = only one unstable marker out of 10 examined markers.

MUTATION ANALYSIS

Genomic DNA isolated from peripheral blood samples was subjected to polymerase chain reaction (PCR) by use of primers described by Kolodner and colleagues[10 20] and examined for mutations in the hMSH2 and hMLH1 genes by non-radioactive single strand conformation analysis (SSCA) and heteroduplex analysis (HA).[21] In addition, the protein truncation test[22] was applied to samples where mRNA was available. All fragments showing aberrant bands were sequenced with sequenase 2.0 (Amersham) according to the manufacturer's instructions.

Results

There was no significant difference between the four groups of patients with regard to their

Table 1 Results of the examinations for microsatellite instability in the tumours and screening for germline mutations in the hMSH2 and hMLH1 genes in the index patients of the four different groups

	Group 1	*Group 2*	*Group 3*	*Group 4*
Number of unrelated patients	57	12	45	46
Mean age at diagnosis of the first colorectal cancer (years)	44 (11.8)	37 (10.8)	42 (8.3)	39 (10.2)
Number of tumour tissues examined for MSI	49*	8*	45	46
MSI^+	35 (71.4%)	4 (50%)	26 (57.8%)	16 (34.8%)
MSI^-	12	1	18	26
$MSI^?$	2	3	1	4
Examined for germline mutations	57	12	17	10
Total number of identified mutations	15 (26%)	2 (17%)	5	3
Proportion of identified mutations in patients with MSI^+ tumours	13/35† (37%)	2/4 (50%)	5/17 (29%)	3/10 (30%)

Group 1, Amsterdam criteria; group 2, looser criteria including extracolonic cancers; group 3, some familial clustering of tumours; group 4, no family history (age at diagnosis <50 years).
*In eight patients of group 1 and four patients of group 2 no tumour tissue was available.
†In two patients with identified germline mutations no tumour tissue was available.

Table 2 Germline mutations in the hMSH2 and hMLH1 genes

Gene	Exon	Codon	Mutation	Consequence	Family	Patient group	Method		
							SSCA	HA	PTT
hMSH2	2	97	289 ins 22	Frameshift	130†	1	+	+	+
	3	173	518 del T	Frameshift	24*	1	+	+	NE
	Intron 5		942+3 a→t	In frame deletion of exon 5	119	2	NE	−	+
	7	383	1147 C→T	Arg→stop	177	2	+	−	NE
	7	409	1226 del AG	Frameshift	225	1	+	+	NE
	11	561	1683 del A	Frameshift	27*	4	−	+	NE
	11	567	1699 del 5	Frameshift	167†	3	+	+	NE
	13	697	2090 G→Phe	Cys→Phe	62*	1	+	−	−
hMLH1	1	1	2 T→A	Initiation codon	72*	1	+	+	NE
	1	25	73 del A	Frameshift	110*	1	+	+	NE
	1	28	83 C→T	Pro→Leu	52*	3	+	+	NE
	2	62	184 C→T	Gln→stop	91*	3	+	−	NE
	2	62	184 C→T	Gln→stop	127	4	+	−	+
	2	62	184 C→T	Gln→stop	234	1	+	−	+
	3	84	250 A→G	Lys→Glu	171	3	+	−	−
	8	226	676 C→T	Arg→stop	12*	1	+	−	NE
	Intron 9		790+4 a→g	Splice variation	67	1	+	NE	−
	10	264	791 del 4	Frameshift	143	1	+	+	NE
	11	329	986 A→C	His→Pro	96‡	1	+	−	−
	12	356	1068 del 8	Frameshift	259	3	+	+	NE
	13	497	1489 ins C	Frameshift	144	1	NE	+	+
	14	541	1622 del C	Frameshift	113	1	+	+	NE
	14	547	1640 T→A	Leu→stop	5	1	+	+	NE
	Intron 17		1989+1 g→t	In frame deletion of exon 17	156	1	NE	NE	+
	19	722	2166 ins 6	In frame insertion	154	4	NE	+	NE

*Included in Wehner and colleagues[21]; †included in Kruse and colleagues[27] ‡included in Wang *et al.*[28]
SSCA, single strand conformation analysis; HA, heteroduplex analysis; PTT, protein truncation test.
+, mutation detected; −, mutation not detected; NE, not examined.

mean age at the time they were diagnosed with their first colorectal cancer (table 1).

MICROSATELLITE INSTABILITY

Genomic instability could be examined in 148 tumours of 160 index patients; in 12 patients from groups 1 or 2 no tumour tissue was available. In group 1, 71.4% of the tumours were found to be MSI$^+$. Four of eight tumours from patients meeting the looser criteria (group 2) had a positive MSI phenotype. The results of group 2 were similar to the results of group 3 (57.8% unstable tumours). In groups 1–3 the majority of the MSI$^+$ tumours showed instability in more than 40% of markers examined and were judged to be highly unstable (94%, 75%, or 96%, respectively). In group 4, 34.8% of the tumours were MSI$^+$, 69% of which could be classified as highly unstable.

GERMLINE MUTATIONS

A total of 25 germline mutations in the hMSH2 or hMLH1 genes were identified (table 2). Mutation analysis was completed in 57 patients from group 1 and 12 patients from group 2 irrespective of the results of microsatellite analysis. Seventeen germline mutations (25%) were identified. In 57 of the 69 patients from groups 1 and 2, tumour tissue was available and examined for microsatellite instability (table 1). In 15/39 (38%) of those patients with a definite MSI$^+$, mutations could be identified.

An appreciable proportion of germline mutations was uncovered in those patients of groups 3 and 4 who exhibited an MSI$^+$ phenotype (5/17 in group 3 and 3/10 in group 4, table 1). We also attempted to study the familial segregation of the identified germline mutation in the families of the three patients of group 4. A de novo mutation could be excluded in patient 127 who developed colorectal carcinoma at the age of 23. The mutation was also identified in his father (still healthy at age 52). In the remaining two apparently sporadic cases, the parents had either died from diseases other than cancer or refused genetic testing.

A non-sense mutation was found in exon 2 of the hMLH1 gene (CAA→TAA at codon 62) in three apparently unrelated patients. Each of the remaining 22 mutations were restricted to single families (table 2).

Table 3 Tumour spectrum of the affected relatives in the 22 families with identified germline mutations (the index patients are not included)

	Germline mutation			
	hMSH2 (n=7)		hMLH1 (n=15)	
	Male	Female	Male	Female
Total number of relatives affected by a malignancy*	18	17	32	29
Colorectal	13 (72%)	9 (53%)	28 (85%)	19 (59%)
Stomach	2	1	1	1
Pancreas	1		2	
Duodenum	1		1	
Endometrium		7 (41%)		2 (6%)
Breast				4
Urothelium	2	1	1	1
Others		3		5

*Some patients had multiple malignant tumours.

GENOTYPE–PHENOTYPE RELATION

Eleven of 17 families with hMLH1 mutations and four of eight families with hMSH2 mutations met the strict Amsterdam criteria. When the tumour spectrum in families, excluding the index patients with either hMSH2 or hMLH1 mutations was compared, some differences were conspicuous. The frequency of colorectal cancer was significantly higher in affected relatives of patients with hMLH1 mutations (77%) compared with relatives of patients with hMSH2 mutations (63%) (relative risk 1.3; 95% confidence interval 0.98–7.4, Cochran-Mantel-Haenszel statistics). On the other hand, the frequency of

endometrium cancer was significantly higher in families with hMSH2 mutations (relative risk 1.6; 95% confidence interval 1.6–2.3; table 3).

Discussion

The goal of the study was to examine the effectiveness of a positive MSI test in selecting patients at a high risk for HNPCC. To this end patients with colorectal cancer who belonged to different groups according to their family history of tumour disease were included in the study.

Germline mutations in the hMSH2 or hMLH1 genes could be identified in 26% of the patients who met the strict Amsterdam criteria. When only patients with a positive MSI phenotype are considered, the detection rate of germline mutations increases to 37%. A similar proportion of germline mutations (30%) could be uncovered in patients with apparently sporadic colorectal cancer but who had highly unstable tumours (table 1). In contrast, no mutation was found in 11 patients with sporadic colorectal cancer and a negative MSI phenotype who had been included in the search for germline mutations before the MSI status was determined (data not shown).

Irrespective of microsatellite instability of the tumours, Wijnen and colleagues[23] found germline mutations in either the hMSH2 and hMLH1 genes in three of 39 unrelated patients (8%) in whom at least one of the conditions of the Amsterdam criteria was unfulfilled. Our results show that the effectiveness of mutation detection rises considerably even in patients with apparently sporadic colorectal cancers when the search for germline mutations is confined to patients with a positive MSI phenomenon in their tumours. This conclusion is particularly valid for highly unstable tumours, as all tumours in patients with identified germline mutations exhibited microsatellite instability in more than 40% of examined markers. Liu and colleagues[12] succeeded in finding germline mutations in 42% of the patients with MSI$^+$ sporadic colorectal cancer, but these authors included only cases in whom the tumours had occurred before age 35. We calculated an average age of 42 (7) years for patients with colorectal cancer before the age of 50 either with a family history of tumours not meeting the HNPCC criteria (group 3) or with apparently sporadic colorectal cancer (group 4) in whom we uncovered germline mutations. Thus, a major proportion of patients with identifiable germline mutations are missed when the cut off age of colorectal cancer is too low. Because the size of the families is shrinking only part of the families are potentially able to meet the clinical criteria of HNPCC. However, given the high prevalence of CRC in the general population worldwide it could prove important to preselect patients at high risk for mismatch repair gene mutations. Our results clearly show that the test for genomic instability in the tumour is an efficient tool to increase the likelihood of uncovering a germline mutation in the hMLH1 and hMSH2 genes.

Although our findings reveal no clear relation between mutations in the involved mismatch repair genes and the tumour spectrum of the affected relatives, at least some interesting trends emerge. More colorectal cancers occurred in carriers of germline mutations in the hMLH1 than in the hMSH2 gene, while carriers of hMSH2 mutations tended to develop more extracolonic malignancies such as tumours of the endometrium. Similar findings were reported by Vasen and colleagues.[24] These authors found a higher relative risk for endometrium and urothelium cancers, but not for colorectal cancers, in carriers of hMSH2 as compared with carriers of hMLH1 germline mutations. A similar tendency was observed in a group of index patients who were selected on the basis of MSI$^+$ sebaceous skin tumours and colorectal carcinoma. In these patients germline mutations in the hMSH2 gene proved to be much more common than in the hMLH1 gene.[25] Thus, evidence is increasing that in patients with hMSH2 mutations the tumour spectrum is more variable than in patients with hMLH1 mutations.

Taken together, our results show that a positive MSI phenotype is an efficient indicator of a germline mutation in the mismatch repair genes hMSH2 and hMLH1, both in patients meeting the clinical criteria for HNPCC and patients with sporadic colorectal cancer. It is a matter of debate whether a cut off age should be used at all for the MSI test. Patients with colorectal cancer before 50 years should be examined in any case, even if the family history is negative. It has been shown that some microsatellite markers such as BAT26 seem to be very sensitive in detecting microsatellite instability.[26] Thus, in the future the MSI test could be limited to a small panel of microsatellite markers and could become a standard examination for every colorectal cancer.

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