ELECTRONIC LETTER

Mismatch repair gene analysis in Catalanian families with colorectal cancer

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Colorectal cancer (CRC) is one of the most common malignant neoplasms in western countries.¹ Hereditary non-polyposis colorectal cancer (HNPCC) is thought to represent the most common form of familial colorectal carcinoma and may account for approximately 1-6% of all colorectal cancers.² The clinical phenotype of HNPCC is variable, and there is no distinctive clinical hallmark, such as the presence of more than 100 adenomatous polyps in familial adenomatous polyposis (FAP). Therefore, stringent diagnostic criteria, essentially based on personal and family cancer history, have been adopted for the purpose of identifying HNPCC families. The Amsterdam Criteria for HNPCC diagnosis include: (1) the presence of three or more patients affected with CRC or HNPCC related tumours (mainly endometrial cancer), two of whom must be first degree relatives (sib, parent, or offspring) to the other one; (2) vertical transmission of CRC in two successive generations, indicative of autosomal dominant inheritance; (3) early onset (<50 years) of at least one CRC or HNPCC related tumour in the family; and (4) exclusion of FAP.³⁻⁴ However, Amsterdam criteria can be too restrictive when applied to small kindreds, abundant in western countries.⁵ In addition, those cases likely to represent the first mutation in a family, usually single affected members diagnosed before the age of 45, may be missed. Finally, difficulty in eliciting a complete and accurate cancer family history may preclude widespread use of these clinical criteria.^{6,7}

hMSH2 and *hMLH1* are considered to be the two major genes responsible for HNPCC.⁸ Initially, germline mutations in these genes were detected in up to 90% of HNPCC families suggesting that the Amsterdam criteria were useful for selecting kindreds which were candidates for genetic analyses. Nowadays, there is general agreement that approximately 40-50% of families meeting the Amsterdam criteria have detectable *hMSH2* or *hMLH1* mutations.² Furthermore, some recent studies suggest that the mutation detection rate may be even lower (25%).^{9,10} On the other hand, a significant number of germline mutations in the mismatch repair (MMR) genes have been detected in kindreds with clustering of CRC not meeting Amsterdam criteria.¹¹ The prevalence of these mutations in these families is usually lower than that reported for families meeting these criteria. Therefore, both the sensitivity and specificity of the Amsterdam criteria for selecting those patients who are candidates for MMR genetic analysis have been challenged.

The aim of this study was to evaluate the prevalence of germline *hMSH2* and *hMLH1* mutations in 11 families meeting the Amsterdam criteria and 21 HNPCC-like Spanish families (11 of them lacking one AC), and to characterise the mutation spectrum in north eastern Spain. We only detected mutations in the *hMLH1* gene, and the majority of mutations were in families lacking one Amsterdam criterion.

MATERIAL AND METHODS

Patients and families

A total of 60 kindreds with clustering of colorectal cancer were recruited from the Genetic Counselling Unit in Hospital Sant Pau, Barcelona, between September 1996 and November 1998. Most families were referred from other Catalanian hospitals. Genetic analysis was indicated when at least one Amsterdam criterion was present. Thirty-two families were included in a study to analyse the prevalence of *hMSH2* and *hMLH1* gene mutations in HNPCC and HNPCC-like families. Eleven of these families fulfilled the Amsterdam criteria⁴ and the remaining 21 kindreds were defined as HNPCC-like: 11 lacked one Amsterdam criterion and the remaining 10 families lacked two Amsterdam criteria. Pedigrees were constructed up to three generations and data from affected subjects (type, number, and localisation of tumours, age at diagnosis, and pathological features) were collected if available. Data confirmation was obtained after reviewing medical or pathological records or death certificates. All patients eligible for genetic analysis received genetic counselling before and after the completion of the analysis, and informed consent was obtained before the study. The ethics review committee of Sant Pau Hospital approved this study.

Detection of germline mutations in *hMSH2* and *hMLH1* genes

Peripheral blood cells were stored at -20°C until DNA or RNA extraction. High molecular weight DNA was extracted following standard procedures. RNA extraction was performed using the single step method.¹² DNA from at least two healthy controls was analysed as negative controls in each experiment.

hMSH2 analyses

cDNA analyses

In the first 22 cases, mutation detection was performed by PCR/SSCP analyses of cDNA sequences. cDNA was obtained after RT using standard protocols.¹³ The whole coding sequence of the gene was amplified in nine overlapping fragments using primers based upon the published cDNA sequence.¹⁴ Primers used and PCR conditions are available from the authors upon request. SSCP analyses were performed in an ALF-Express apparatus (AmershamPharmacia-biotech) with at least two running conditions: (1) 12% PAGE at 30°C and 30 W for two hours, and (2) 6% PAGE in 10% glycerol at 30°C and 7 W for six hours. For exon 5 deletion analyses,¹⁵ fragments 3 and 4 were amplified in a single PCR reaction (amplicon size 635 bp) and the presence of a second fragment of smaller size (485 bp) was searched for.

Abbreviations: CRC, colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; FAP, familial adenomatous polyposis; MMR, mismatch repair; AC, Amsterdam criteria; MSI, microsatellite instability

DNA analysis

During the period of study of *hMSH2*, the material was changed from RNA to DNA in order to be able to analyse samples referred from other hospitals. Therefore, the whole coding exon sequences with corresponding intron boundaries of the remaining 10 samples were amplified using intronic primers (sequence available upon request to authors). SSCP analyses were run under the same two conditions described above. In this case, unlabelled primers and gel silver staining were used. Whenever an abnormal SSCP pattern was observed, direct sequencing of the amplified fragment was performed using the AmpliCycle Sequencing Kit (Perkin Elmer, Branchburg, NJ) following the manufacturer's instructions. Sequence analyses were performed with either a PE ABIPrism 670 or ALFExpress (AmershamPharmaciaBiotech) sequencer. More than 60% of the fragments amplified showing a normal SSCP pattern were randomly selected for sequencing in order to reinforce the reliability of our negative results.

hMLH1 analyses

hMLH1 gene mutation detection was performed on DNA because of the presence of alternate splicing that could make interpretation of results difficult.¹⁶ All exons of the *hMLH1* gene with their corresponding exon-intron boundaries were analysed by PCR/SSCP following essentially the same protocol as described for *hMSH2*. Primers used and PCR conditions are available from the authors upon request. Whenever an abnormal SSCP pattern was observed, direct sequencing of the amplified fragment was performed as described above. Owing to the detection of a significant number of mutations in the *hMLH1* gene, no sequencing was performed when normal SSCP patterns were observed.

Microsatellite (MSI) analysis

Attempts were made to collect paraffin blocks of affected members from all families studied. Blocks were available from 22 of the 32 families analysed (five of 11 HNPCC families and 16 of 21 HNPCC-like families). Enrichment for tumour cells was performed in all cases. Paired results from tumour and corresponding normal mucosa were always assessed. The strategy for MSI diagnosis has been previously described.¹⁷ Briefly, as a first step, two markers (one containing mono-runs of As BAT26 and one CA repeat D12S95) were assessed. If both markers were stable (no additional bands seen), the tumour was classified MSI(−). If two markers were unstable, the tumour was classified MSI(+) (sensitivity 97%, specificity 100%). Whenever only one of the two markers was unstable (20% of samples), an additional four markers were studied. Tumours were classified as MSI(+) when at least two of the six markers were unstable. Tumours exclusively displaying instability at BAT26 were considered highly suggestive of displaying MSI. Inconclusive results were reported whenever amplification was achieved in less than four markers.

RESULTS

Clinical and pathological features

As expected, age at diagnosis of CRC was lower in HNPCC than in HNPCC-like families. Right sided tumours represented 31% of all CRC in HNPCC families and only 18% of HNPCC-like ones, but the difference was not significant (table 1). Colorectal tumours were detected at similar stages (Dukes A and B) in both groups. No differences were observed regarding the incidence of synchronous and metachronous CRC between the two groups (data not shown).

Germline analyses

Three of the 11 (27%) HNPCC families harboured a MMR gene mutation, exclusively in the *hMLH1* gene (table 2). One of these three *hMLH1* mutations is a previously reported deletion AAG at 1846 (exon 16, codon 616).^{18,19} The remaining two

Table 1 Clinical features of HNPCC and HNPCC-like families

	HNPCC (n=11)	HNPCC-like (n=21)	p
No of CRC	38	54	
Age at diagnosis	48 (22–80)	56 (24–89)	0.089
Localisation			
Right	12 (31.6%)	10 (18.5%)	NS
Left	15 (39.5%)	19 (35.2%)	
Rectum	6 (15.8%)	12 (22.2%)	
Unknown	5	13	
Stage (Dukes)			
A/B	12 (31.6%)	21 (38.9%)	NS
C/D	16 (42.1%)	11 (20.4%)	
Unknown	10	22	
Differentiation*			
W/M	27 (75.1%)	32 (59.3%)	NS
P	2 (5.3%)	4 (7.4%)	
Unknown	5	15	
Synchronous CRC	2 (5%)	3 (5.5%)	NS

*W: well differentiated; M: moderately differentiated; P: poorly differentiated.

mutations are novel and of unknown pathogenicity. Family J/8 harbours a mutation located at position +1 of intron 8 resulting in a disrupted splicing donor site according to Spliceview software (<http://125.itba.mi.cnr.it/genebin/wwwspliceview>). The third mutation is a Leu to His substitution at codon 622 in exon 16 that was detected in two affected members of family J/35. Unfortunately, we have not been able to collect tumour tissue from affected members of the family. We are currently trying to contact additional relatives of the latter families to complete segregation analysis. It is of note that no exon 5 deletion, probably the most frequently reported mutation, was detected despite using a sensitive method to detect this mutation.

A similar proportion of HNPCC-like families (four of 21, 19%) harboured *hMLH1* gene mutations. Interestingly, all these mutations were identified in families that lacked only one Amsterdam criterion resulting in a four of 11 (36%) mutation yield in this subgroup. Two of the four *hMLH1* mutations, both single base substitutions, have been previously described as deleterious (table 2). Accordingly, analysed tumours were MSI(+). A novel mutation, one 4 bp insertion generating a stop codon at nt 1664, is most likely to be deleterious and is associated with a MSI(+) tumour. Finally, a 5 bp deletion located intronically, which apparently does not affect splicing, was detected in family J/39. The fact that the tumour sample analysed was classified as MSI(−) further raises doubts about its pathogenicity.

Since mutations were similarly distributed among HNPCC and HNPCC-like families, the usefulness of individual criteria for predicting the presence of a germline mutations was analysed. No significant differences were observed between (1) vertical transmission, (2) the presence of three or more affected members, (3) age at diagnosis under 50 in at least one affected subject, and (4) the presence of detectable mutations (data not shown). When families were divided according to the presence or absence of detected mutations, no differences were observed regarding clinical and pathological parameters evaluated, including age of onset and the presence of endometrial cancer (data not shown).

Variant alleles

A total of six polymorphisms (three in the *hMSH2* and three in the *hMLH1* genes) were detected (table 3), all of them previously reported. It is of note that an *hMSH2* intron 1 polymorphism was detected in 12 of the 33 (36%) families. Three other polymorphisms at intron 12 of *hMSH2* and introns 8 and 13 of *hMLH1* were detected in three families each.

Table 2 Mutations in the *hMSH2* and *hMLH1* genes in HNPCC and HNPCC-like families

Family	Gene	Location	Nature	Consequence	Pathogenicity	References
HNPCC						
J/8	<i>hMLH1</i>	Intron 8	G>A; nt 677+1	Affects splice donor site	Unknown	Novel
J/42	<i>hMLH1</i>	Exon 16, codon 616	Del AAG; nt 1846	Frameshift	Deleterious	Hamilton <i>et al</i> ¹⁸
J/35	<i>hMLH1</i>	Exon 16, codon 622	T>A; nt 1865	Single AA substitution Leu>Hys	Unknown	Miyaki <i>et al</i> ¹⁹
HNPCC-like						
J/39	<i>hMLH1</i>	Intron 1	del AGTAG; nt 207; -44	Does not affect splicing	Unknown	Novel
J/9	<i>hMLH1</i>	Exon 2, codon 67	G>A; nt 199;	Single AA substitution Gly>Arg	Deleterious	Tannergard <i>et al</i> ²⁰
J/38	<i>hMLH1</i>	Exon 14, codon 555	Ins AAGT; nt 1664	Stop codon	Probably deleterious	Novel
J/56	<i>hMLH1</i>	Exon 19, codon 714	G>A; nt 2141	Stop codon	Deleterious	Froggatt <i>et al</i> ²⁰ Hutter <i>et al</i> ²¹

Table 3 Intragenic variant alleles in the *hMSH2* and *hMLH1* genes in HNPCC and HNPCC-like families

Exon	Location	Nature	Affected families (No)	References
<i>hMLH1</i> gene				
Exon 2	Codon 66	C>T nt198; Thr>Thr	1	Wehner <i>et al</i> ²²
Exon 8	Codon 219	A>G nt 655; Ile>Val	3	Liu <i>et al</i> ²⁵
Intron 13	3' exon 13	G>A nt1558 +14	3	Tannergard <i>et al</i> ²⁰
<i>hMSH2</i> gene				
Intron 1	3' exon 1	C>G nt 211 +9	12	Bubb <i>et al</i> ²³
Exon 2	Codon 113	G>A nt; Lys>Lys	1	Liu <i>et al</i> ²⁵
Intron 12	5' exon 13	T>C nt 2006 -6	3	Kolodner <i>et al</i> ²⁴ Leach <i>et al</i> ¹⁵

Concordance between MSI status and the presence of germline mutations

Tumours from patients belonging to 21 families were available for study (table 4). Six of the 21 (29%) tumours analysed were MSI(+), five of 16 belonging to HNPCC-like families and one of five HNPCC families, and one tumour was classified as highly suspicious of being MSI(+). As previously described, a strong correlation was observed between MSI status and detectable mutations. Mutations were detected in four of six MSI(+) tumours and only one substitution, of unknown pathogenicity, was detected in the 11 MSI(-) cases. If only MSI(+) or inconclusive cases (eight cases) had been analysed for germline mutation, the yield would have been 50% (four of eight).

DISCUSSION

In the present study, we have shown that more than 50% of *hMLH1* putative germline mutations identified in our kindreds were detected in HNPCC-like families lacking one Amsterdam criterion. It has been reported that MMR gene mutations, although not restricted to HNPCC families, occurred at a higher frequency than in HNPCC-like families.²⁰ In our series, the mutation rate in families lacking one criterion is slightly higher than in HNPCC families, in line with observations by other authors.^{10, 21} The mutation rate in *hMSH2* and *hMLH1* genes in HNPCC families is usually around 40-60% (ranging from 22% to 86%). The observed frequency (27%) of mismatch repair gene mutations in our Mediterranean area is among the lowest reported. Several factors may account for the wide variations observed in distinct series. A first possibility is that significant differences may be present in different geographical areas.^{5, 22} We have previously shown that MSI(+) tumours represent a small proportion (7.5%) of Spanish CRC tumours¹⁷ when compared to the USA (up to 15-20%). However, the same low frequency of MMR gene mutations was observed in German HNPCC families.¹⁰ Secondly, differences in the methodology may also be relevant. However, most studies, including those obtaining the most discordant results,

Table 4 Correlation between MSI status and *hMLH1* gene mutations in available tumours

Family		MSI status	<i>MLH1</i> gene
HNPCC	HNPCC-like		
J/6		Negative	WT
J/40		Negative	WT
J/41		Negative	WT
J/42		Positive	del AAG; nt 1846
J/43		Negative	WT
	J/9	Positive	G>A; nt 199
	J/11	Negative	WT
	J/12-13	Negative	WT
	J/27	Highly suggestive	WT
	J/29	Positive	WT
	J/34	Inconclusive	WT
	J/36	Negative	WT
	J/38	Positive	Ins AAGT; nt 1664
	J/39	Negative	del AGTAG; nt 207
	J/39	Negative	WT
	J/44	Negative	WT
	J/46	Inconclusive	WT
	J/53	Negative	WT
	J/55	Negative	WT
	J/56	Positive	G>A; nt 2141
	J/57	Positive	WT

used SSCP as the initial screening technique.^{11, 23, 24} While this technique may overlook some mutations, it is a well accepted method for point mutation screening. It is of note that a recent report has suggested that large deletions, as assessed by Southern blotting, may account for up to 10% of all *hMSH2* mutations.²⁵ However, since none of the previously mentioned reports used this methodology, all of them, including ours, may have underestimated the total number of MMR gene mutations in a similar manner. The incidence of MSI(+) tumours (31%) in our families is consistent with a good sensitivity of the techniques used. Large deletions in *hMSH2* or

hMLH1 and/or mutations in other genes (*hMSH6*) are likely to account for those MSI(+) tumours not showing detectable germline mutations. Finally, a consistent trend towards a lower proportion in MMR(+) cases in later publications may reflect the presence of some positive case bias in early reports probably associated with a larger number of affected subjects per family in the initially selected families. In this regard, recent results may offer a more realistic estimation of the impact of MMR gene analysis in the genetic counselling of these patients.

In our series, like others,¹¹ fulfilment of Amsterdam criteria has shown limited value in the identification of MMR gene mutation carriers. Our results favour the use of two Amsterdam criteria other than exclusion of FAP as a good cut off value for the selection of kindreds that are candidates for genetic analysis. If these clinical criteria are combined with MSI analysis, then the results of MMR analysis are even better, further supporting the combined use of clinical and molecular criteria to select patients for germline analysis.²⁶

The spectrum of mutations shows that *hMLH1* mutations account for the majority of HNPCC germline alterations in this population. Since it was first described, an increasing role for *hMLH1* mutations in HNPCC has been observed.² In our series we only found *hMLH1* mutations, either deleterious or of unknown significance. No *hMSH2* gene mutations were detected in spite of intensive efforts aimed at minimising the rate of false negative results. Neither extensive use of sequencing nor the development of specific strategies for detection of exon 5 deletions has increased the total number of *hMSH2* mutations detected. Altogether our observations suggest that in the Spanish population *hMLH1* should be screened first for mutations and then *hMSH2* should be searched for alterations. Analysis of more families will most likely result in the occasional identification of *hMSH2* germline mutations in our setting.

Neither a founder effect nor hot spots nor recurrent mutations were observed, contrary to what occurs in Finland.⁵ Heterogeneity of mutations has been high with a significant proportion (four of seven) of novel mutations. As usual, the significance of some novel alterations is equivocal especially when they are located in intronic regions or they are single base substitutions. In this situation, segregation analysis may be of help but the difficulty in obtaining biological material of other relatives has precluded it. MSI analysis may also be helpful in interpretation of results: the deletion at intron 1 is associated with a MSI(−) tumour disregarding a putative pathogenetic role. Regarding variant alleles, it is of note that *hMSH2* intron 1 polymorphism has been detected in 12 of the 33 (36%) families, further supporting the possibility that this variant allele confers an increased risk for developing familial CRC. Further studies will be needed to rule out this possibility. Finally, it is of note that, in our series, the prevalence of mutations in families meeting classical and revised Amsterdam criteria (that give extracolonic HNPPC tumours the same diagnostic rank as CRC) was identical, reinforcing the adequacy of modifying the criteria.

The total low number of MMR gene mutations detected strongly suggests that other genes²⁷ will be responsible for the increased CRC predisposition observed in those HNPCC families not harbouring MMR mutations.^{2,5} Whether a family fulfils the clinical criteria or not, a definitive diagnosis of HNPCC can only be established by showing a germline mutation.⁵ However, it is very important to remember that failure to identify a clearly pathogenic mutation in *hMSH2* or *hMLH1* in a person or family meeting a set of clinical criteria for HNPCC should not result in changes in clinical management decisions for at risk family members.²⁰ Some mutations in known genes may escape detection by the testing methods used, or an alteration in a different gene may later be found in the kindred.

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