**MSH2 in Contrast to MLH1 and MSH6 Is Frequently Inactivated by Exonic and Promoter Rearrangements in Hereditary Nonpolyposis Colorectal Cancer**

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**ABSTRACT**

To estimate the relative frequency of mismatch repair genes, rearrangements in hereditary nonpolyposis colorectal cancer (HNPCC) families without detectable mutations in MSH2 or MLH1, we have analyzed by multiplex PCR of short fluorescent fragments MSH2, MLH1, and MSH6 in 61 families, either fulfilling Amsterdam criteria or including cases of multiple primary cancers belonging to the HNPCC spectrum. We detected 13 different genomic rearrangements of MLH1 in 14 families (23%), whereas we found no rearrangement of MSH2 and MSH6. Analysis of 31 other families, partially meeting Amsterdam criteria, revealed no additional rearrangement of MLH1. All of the MLH1 rearrangements, except one, corresponded to genomic deletions involving one or several exons. In 8 of 13 families with a MLH1 genomic deletion, the MLH1 promoter was also deleted, and the 5’ breakpoint was located either within or upstream the MLH1 gene. This study demonstrates the heterogeneity of MLH2 exonic and promoter rearrangements and shows that, in HNPCC families without detectable MSH2 or MLH1 point mutation, one must consider the presence of MSH2 genomic rearrangements before the involvement of other mismatch repair genes. The simplicity and rapidity of their detection, using fluorescent multiplex PCR, led us to recommend to begin the molecular analysis in HNPCC by screening for MSH2 rearrangements.

**INTRODUCTION**

HNPCC is the most common form of inherited colorectal cancer (for review, see Ref. 1). In HNPCC, detection of the causal alteration of the MMR gene is essential for a proper management of the families, because it allows to identify relatives with high risk for colorectal or endometrial cancer, who require the appropriate screening (2, 3) and, conversely, to avoid useless surveillance in noncarrier relatives. The efficiency of the colonoscopy screening, especially in terms of mortality, has been demonstrated recently in HNPCC families (4). Molecular diagnosis of HNPCC is complicated by the genetic heterogeneity of the syndrome because of the involvement of the different MutS and MutL homologues, MSH2 (2p22-p21), MSH6 (2p16), and MLH1 (3p21) and PMS2 (7p22), respectively (5–11). Mutation studies (12) have shown that MSH2 and MLH1 are involved in approximately half of the families fulfilling the Amsterdam criteria I (at least three relatives with CRC, one of whom is a first-degree relative of the other two; at least two successive generations affected; and at least one of the cases of CRC diagnosed before age 50; tumors verified by pathological examination).

The large number of HNPCC families without detectable MSH2 or MLH1 mutations may be explained by: (a) alterations of MSH2 or MLH1 missed by conventional screening methods based on PCR amplification of each exon; (b) the involvement of other MMR genes; or (c) the existence of HNPCC not related to a defect within the MMR pathway. The first case of a MLH1 genomic deletion, involving exon 16, was reported in 1995 by Albert de la Chapelle’s group in Finnish families. This deletion, detected by RT-PCR analysis, was shown to result from an Alu-mediated recombination (13). Using also RT-PCR analysis, we subsequently detected two distinct Alu-mediated deletions removing exons 13–16 and exon 2 of MLH1 in two French HNPCC families (14, 15).

An extensive screening by Southern blot analysis of 137 HNPCC families, without detectable point mutations within the MMR genes, led Wijnen et al. (16) to identify, in 8 families, four distinct genomic deletions of MSH2, removing exon 1, exon 2, exon 3, and exon 6. Two other genomic deletions of MSH2, involving exons 1–6 and exons 1–7, were identified using the conversion approach, based on the cell fusion strategy (17). To facilitate the detection of genomic rearrangements, we recently developed a simple and rapid screening method, based on the multiplex PCR amplification of short fluorescent fragments. This method allowed us to identify two additional deletions of MSH2, removing exon 5 and exons 1–15, and the first case of a partial duplication of this gene affecting exons 9–10 (15). However, the frequency of the rearrangements in the MMR genes has not been estimated thus far, and this information is essential to determine the best strategy of the molecular analyses in HNPCC families. Therefore, we have systematically screened for genomic rearrangements a series of 92 families without detectable point mutations.

**MATERIALS AND METHODS**

**Families.** This study included a total of 92 families corresponding to: (a) 49 families fulfilling the Amsterdam criteria I or II for HNPCC (at least three relatives with CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis, one of whom is a first-degree relative of the other two; at least two successive generations affected; and at least one diagnosed before age 50; Refs. 18 and 19); (b) 12 families, partially fulfilling the Amsterdam criteria, but including cases with MPCs belonging to the HNPCC spectrum (CRC, cancer of the endometrium, stomach, ova, small bowel, ureter, or renal pelvis), the first of which developed before 50 years of age; and (c) 31 families which met only partial Amsterdam criteria and without cases of MPC. For each family, previous screening of MSH2 and MLH1 by Denaturing Gradient Gel Electrophoresis, heteroduplex, and/or direct sequencing analysis, from genomic DNA of the index case, had revealed no point mutation. RT-PCR analysis had also been performed in 43 families, for which high quality mRNA was available, and had revealed no alteration.

**Fluorescent Multiplex PCR.** We used a slightly modified version of the protocol described previously (15). Briefly, short fragments (<300 bp) of the 16 MSH2, 19 MLH1, and 10 MSH6 coding exons (Table 1) or of the MSH2 upstream region (Table 2) were simultaneously PCR amplified, using 6-Fam labeled primers. For MSH2 and MLH1, two multiplex PCRs were necessary to cover all of the exons (Table 1). An additional fragment, corresponding to another gene used as a control, was systematically amplified in each multiplex PCR. PCR was performed in a final volume of 25 μl containing 100 ng of...
the PCR consisted of 21 cycles of 15 s at 94°C polymerase (Eurobio, Les Ulis, France). After a denaturation of 3 min at 95°C followed by a final extension of 7 min at 72°C.

DNA, 0.2–1 μM of each pair of primers, and 1 unit of Taq DNA polymerase (Eurobio, Les Ulis, France). After a denaturation of 3 min at 95°C, the PCR consisted of 21 cycles of 15 s at 94°C, 15 s at 55°C, and 15 s at 72°C, followed by a final extension of 7 min at 72°C.

**Analysis of the Multiplex PCR.** After electrophoresis for 3 h on an automated sequencer (Applied Biosystems), data were analyzed using the Gene scanner Model 672 Fluorescent Fragment Analyzer (Applied Biosystems). Electropherograms were superposed to those generated from control DNAs, and the areas of the corresponding peaks between the different samples were compared. In the fluorescent multiplex PCR assay, a 0.5 reduction of the peak(s) area indicates an heterozygote deletion of the corresponding exon(s), whereas exonic duplications result in a 1.5 increase (15). Each positive result was controlled on a second independent fluorescent multiplex PCR.

**Sequence Analysis.** After purification by electrophoresis on low-melt agarose gel, PCR products were directly sequenced on both strands using the Big Dye Terminator Kit (Applied Biosystems) and a model 377 automated sequencer (Applied Biosystems).

**Long-range PCR.** Long-range PCR was performed using the Expand Long Template PCR system from Boehringer Mannheim according to the protocol of the supplier.

### RESULTS

Using the fluorescent multiplex PCR assay, based on the simultaneous amplification of short genomic fragments (15), we first screened for rearrangements of *MSH2, MLH1, or MSH6* in 61 HNPCC families, either fulfilling the Amsterdam criteria (49 families) or selected for the presence of cases with MPC belonging to the HNPCC spectrum (12 families). In 14 families (23%), a genomic rearrangement of *MSH2* was detected (Fig. 1, Table 3). *MSH2* genomic rearrangements (Table 3) were detected in 10 of the 49 families fulfilling Amsterdam criteria I or II (20%) and in 4 of the 12 families with MPC (33%). In contrast, the fluorescent multiplex PCR assay revealed no *MLH1* or *MSH6* genomic alteration. We then extended the analysis of *MSH2* to 31 other families partially meeting Amsterdam criteria, and we detected no additional rearrangement of *MSH2*.

All of the *MSH2* rearrangements, except one, corresponded to genomic deletions, which involved in 6 families a single exon and in
7 families several exons (Table 3). We also analyzed families R3 and R4 (Fig. 2a), as well as families P5 and L14 (15), using long-range PCR. In each case, the rearrangement was confirmed. In 8 of 13 (62%) families, exon 1 was deleted (Table 3; Fig. 4). In only 2 families (R2 and L7), we detected an aberrant PCR product (Fig. 2, b and c), confirming the deletion. Sequencing analysis revealed that, in the R2 family, the first 3041 bp of the MSH2 promoter region were fused to the last 2132 bp of intron 1, deleting 1297 bp of the promoter region (Fig. 4b).

Alignment and analysis of the nucleotide sequences, using the RepeatMasker program, revealed that the recombination had occurred in Alu repeats and involved a 54-bp homologue sequence between the promoter region and intron 1. In family L7, the breakpoint within the promoter region was mapped to the same position as in family R2, and the promoter sequence was fused to the last 148 bp of intron 4. The breakpoint within intron 4 was located ~210 bp downstream an Alu repeat. In the 6 other families with an exon 1 deletion (R1, Li8, R9, R10, R11, and P12), the absence of an abnormal PCR product detected by long-range PCR (Fig. 2b) suggested that the 5' breakpoint was located upstream the MSH2 promoter region. We then designed a new multiplex PCR assay (Table 2; Fig. 3) to analyze ~24 kb of genomic sequences upstream the transcription initiation site of MSH2 (Fig. 4b). The multiplex PCR assay revealed (Fig. 3) that the 5' breakpoint was located, in 2 families (R9 and R11), downstream the LOC65359 gene and in the 4 other families (R1, Li8, R10, and P12), at least 24 kb upstream the transcription initiation site of MSH2, because the LOC65359 gene was also deleted (Fig. 4b). In family R9, long-range PCR, using a sense primer corresponding to exon 9 (P9) of the LOC65359 gene (Fig. 4a) and an antisense primer corresponding to exon 8 of MSH2, confirmed the presence of a large genomic deletion of ~55 kb (Fig. 2d). Sequence analysis showed that the breakpoint had occurred within an Alu repeat, 529 bp downstream the last exon of LOC65359, removing 16 kb of genomic sequences upstream the transcription initiation site of MSH2 (Fig. 4b). Within intron 7, the breakpoint was located 600 bp upstream the splice acceptor site also within an Alu repeat.

Table 3: Detection of MSH2 exonic rearrangements

<table>
<thead>
<tr>
<th>Family</th>
<th>Genomic rearrangement</th>
<th>Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Del exon 1</td>
<td>AMS I</td>
</tr>
<tr>
<td>R2</td>
<td>Del exon 1</td>
<td>MPC (E, 47; C, 50)</td>
</tr>
<tr>
<td>L7</td>
<td>Del exons 1–4</td>
<td>AMS I</td>
</tr>
<tr>
<td>L8</td>
<td>Del exons 1–6</td>
<td>AMS II</td>
</tr>
<tr>
<td>R9</td>
<td>Del exons 1–7</td>
<td>AMS II</td>
</tr>
<tr>
<td>R10</td>
<td>Del exons 1–8</td>
<td>AMS I</td>
</tr>
<tr>
<td>R11</td>
<td>Del exons 1–8</td>
<td>MPC (CRC, 35; CRC, 35)</td>
</tr>
<tr>
<td>P12</td>
<td>Del exons 1–15</td>
<td>AMS I</td>
</tr>
<tr>
<td>R3</td>
<td>Del exon 3</td>
<td>MPC (CRC, 41; CRC, 44; CRC, 59)</td>
</tr>
<tr>
<td>R4</td>
<td>Del exon 3</td>
<td>MPC (CRC, 32; O, 53)</td>
</tr>
<tr>
<td>P5</td>
<td>Del exon 5</td>
<td>AMS I</td>
</tr>
<tr>
<td>P13</td>
<td>Del exons 5–6</td>
<td>AMS I</td>
</tr>
<tr>
<td>L6</td>
<td>Del exon 7</td>
<td>AMS I</td>
</tr>
<tr>
<td>L14</td>
<td>Dup 9–10</td>
<td>AMS I</td>
</tr>
</tbody>
</table>

* Fulfilling Amsterdam criteria I (19).

+ Including cases of multiple primary cancers belonging to the HNPCC spectrum.

1 E, endometrial cancer; O, cancer of the ovary.

2 Fulfilling Amsterdam criteria II (19).

3 Haplotype analysis, using the microsatellite markers D2S288, D2S2227, and D2S123, had indicated that, in families R10 and R11, the rearrangements had been recurrently generated.

4 Previously reported in Charbonnier et al. (15).
DISCUSSION

This study was designed to estimate the relative frequency of MMR gene rearrangements in HNPCC families without detectable point mutations within the MSH2 and MLH1 genes. Southern blot analysis, which is the method commonly used to detect genomic rearrangements, can miss small deletions or duplications, and restriction site polymorphisms may complicate the interpretation of the restriction patterns. Therefore, we took advantage of a simple semiquantitative method, based on the multiplex PCR of short fluorescent fragments (15). We had initially validated this method on MLH1 and MSH2 rearrangements, detected previously by RT-PCR and confirmed by long-range PCR (15). The fluorescent multiplex PCR assay was subsequently shown to be a powerful method for the detection of heterozygote deletions of SMN1 in spinal muscular atrophy (20) and for the detection of C1NH exonic deletions and duplications (21). In the present study, this method allowed us to show that, in HNPCC families screened previously by conventional methods, genomic rearrangements are frequently found in MSH2 but not in MLH1 and MSH6. We observed that 20% of the HNPCC families fulfilling Amsterdam criteria had a genomic rearrangement of MSH2 (10 of 49), a value higher than the first estimate provided by Wijnen et al. (16) and obtained by Southern blot analysis (6 of 51, 12%). An additional finding of the present study is the high frequency of MSH2 rearrangements in patients with MPCs belonging to the HNPCC spectrum, which confirms that this criterion, independently of the familial history, is highly suggestive of HNPCC. In 31 families, which only partially met Amsterdam criteria and without cases of MPC, we detected no rearrangement of MSH2. Wijnen et al. (16) had described, among 86 HNPCC families not fitting Amsterdam criteria, only 2...
cases of MSH2 rearrangements. In both studies, this low frequency of MSH2 genomic rearrangements in these families might be explained in part by the heterogeneity of the criteria used for the selection of the kindreds. Nevertheless, it is also possible that genomic rearrangements, in contrast to other types of germ-line alterations, such as missense or splice mutations, may result into a complete loss of function and, therefore, in a high penetrance.

Wijnen et al. (16) had identified by Southern blot analysis four types of MSH2 rearrangements in 8 families. The present study highlights the remarkable allelic heterogeneity of MSH2 rearrangements, because 13 distinct rearrangements were detected in 14 HNPCC families (Fig. 4). Moreover, we showed that the MSH2 promoter was also deleted in 8 of 14 families. Furthermore, we demonstrated that the 5′ breakpoint of the deletions removing the promoter is highly heterogeneous (Fig. 4b). The majority of the rearrangements that we have identified (Fig. 4), like those reported by Wijnen et al. (16) and Yan et al. (17), involve the region between the 5′ end of the gene and intron 8. This distribution could be explained by the distribution of the Alu sequences within the MSH2 gene. Indeed, analysis of the genomic sequence, using the RepeatMasker program, revealed that among the 105 Alu sequences detected within the MSH2 gene, the majority (88) was located between the promoter and exon 9. The involvement of Alu-mediated recombination events in the MSH2 rearrangements was confirmed by sequence analysis of the breakpoint in 3 families.

The high frequency of genomic rearrangements of MSH2 contrasts with the absence of genomic rearrangements detected, in this study, within MLH1 and MSH6. At the present time, only three Alu-mediated genomic rearrangements, involving respectively exon 2, exon 16, and exons 13–16, have been reported thus far within MLH1 (13–15). Nevertheless, in certain populations, as reported previously by Nystrom-Lahti et al. (13) for the deletion of exon 16, certain rearrangements of MLH1 might be associated with a founder effect, justifying their specific screening in the corresponding populations. The contrast in the frequencies of MSH2, MLH1, and MSH6 rearrangements documented by this study has important practical implications. In HNPCC families fulfilling Amsterdam criteria or with cases of MPC belonging to the HNPCC spectrum and without detectable MSH2 or MLH1 point mutations, one must consider the presence of MSH2 genomic rearrangements. The frequency of these alterations is probably higher than that of point mutations within the other MMR genes.

In 71 families fulfilling the original Amsterdam criteria and without detectable MSH2 or MLH1 point mutations, Wijnen et al. (23) have detected a pathogenic MSH6 mutation only in 3 families. In a study including 90 HNPCC families, 23 of which meeting Amsterdam criteria, Huang et al. (24) recently identified only one germ-line mutation of MSH6 and no mutation of MSH3, demonstrating that these genes are rarely involved in HNPCC. The percentage of HNPCC families fulfilling Amsterdam criteria and carrying a MSH2 genomic rearrangement can be estimated to ~10%, because the families analyzed in the present study had been selected on the basis of the absence of point mutations in MSH2 or MLH1, a situation observed in approximately half of the kindreds. This significant percentage and the simplicity and rapidity of the fluorescent multiplex PCR assay led us to suggest that it is probably efficient to begin the molecular screening of MMR genes in HNPCC families by searching for genomic rearrangements of MSH2.

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