Detection of Exon Deletions and Duplications of the Mismatch Repair Genes in Hereditary Nonpolyposis Colorectal Cancer Families Using Multiplex Polymerase Chain Reaction of Short Fluorescent Fragments

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Abstract

Large genomic deletions within the mismatch repair MLH1 and MSH2 genes have been identified in families with the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, and their detection represents a technical problem. To facilitate their detection, we developed a simple semiquantitative procedure based on the multiplex PCR of short fluorescent fragments. This method allowed us to confirm in HNPCC families three known deletions of MLH1 or MSH2 and to detect in 19 HNPCC families, in which analysis of mismatch repair genes using classical methods had revealed no alteration, a deletion of exon 5 and a duplication of MSH2 involving exons 9 and 10. The presence of such duplications, the frequency of which is probably underestimated, must be considered in HNPCC families in which conventional screening methods have failed to detect mutations.

Introduction

Identification of the molecular basis of HNPCC or Lynch Syndrome (1) represents one of the most significant achievements in cancer genetics because HNPCC is probably the most common form of inherited colorectal cancer. HNPCC represents a genetic predisposition for a wide spectrum of neoplasia including adenocarcinomas of the endometrium, stomach, ovary, small bowel, and hepatobiliary tract and transitional cell carcinomas of the urinary tract. In mutation carriers, the lifetime risk of colorectal cancer has been estimated to be 68–75% (2). The risk of endometrial cancers has been estimated to be 42% (2). HNPCC results from germ-line mutations of the human homologues of the bacterial MutS and MutL MMR genes (for review, see Ref. 3). Germ-line mutations have been reported within the MSH2 [2p22-p21 (4, 5)] and MSH6 [2p16 (6, 7)] genes, which are homologues of MutS, and within the MLH1 [3p21 (8, 9)], PMS1 (2q31-q33), and PMS2 [7p22 (10)] genes, which are homologues of MutL.

In HNPCC families, detection of a germ-line mutation of a MMR gene allows the identification of relatives who require appropriate surveillance based on colonoscopy and transvaginal sonography every 1–2 years (11) and, in contrast, prevents useless surveillance in noncarrier relatives.

Mutation reports have shown that the majority of the HNPCC cases are due to germ-line MLH1 or MSH2 alterations that are mostly frameshift, nonsense, and missense mutations (12). Nevertheless, large genomic deletions of MLH1 and MSH2 have also been described in HNPCC kindreds: Nyström-Lahti et al. (13) identified in Finnish HNPCC families a 3.5-kb Alu-mediated deletion of exon 16 of MLH1, which was associated with a founder effect. We then reported in a French HNPCC family a 22-kb Alu-mediated genomic deletion of MLH1 involving exons 13–16 (14). Subsequently, MSH2 deletions were shown to be a frequent cause of HNPCC in Dutch families (15).

Detection of genomic rearrangements is technically difficult and requires Southern blot analysis, which is DNA- and time-consuming, therefore limiting the efficiency of molecular screening in HNPCC families. To facilitate the detection of such large rearrangements, we developed a simple semiquantitative procedure based on multiplex PCR of short fluorescent fragments, and this method allowed us not only to detect genomic deletions but to report for the first time duplications of the MMR genes.

Materials and Methods

Multiplex PCR. Short exon fragments corresponding to the 19 MLH1 exons and the 16 MSH2 exons were PCR-amplified from 100 ng of genomic DNA extracted from peripheral blood or lymphocytes by a standard proteinase K/SDS protocol, using primers labeled with the fluorescein dye 6-FAM (Table 1). Exons 1–10 and exons 10–19 of MLH1 and exons 2, 3, 5, 8–10, 12, 14, and 15 and exons 1, 4, 6, 7, 8, 11, 13, and 16 of MSH2 were PCR-amplified in four separate tubes. PCR was performed in a final volume of 50 µl containing between 0.2 and 1 µM of each pair of primers and 1 unit of Taq DNA polymerase (Eurobio, Les Ulis, France). After a 3-min denaturation at 95°C, the PCR consisted of: (a) nine cycles of 10 s at 94°C, 10 s at 60°C (with a decrease of 1°C/cycle), and 10 s at 72°C; (b) 12 cycles of 10 s at 94°C, 10 s at 48°C, and 10 s at 72°C; and (c) a final 7-min extension at 72°C. After purification using the Qiagen Gel Extraction Kit, half of the PCR product was loaded on a 4.25% denaturing polyacrylamide gel. Electrophoresis was performed for 3 h on an Applied Biosystems model 377 automated sequencer (PE Applied Biosystems, Perkin-Elmer), and data were analyzed using the gene scanner model 672 fluorescent fragment analyzer (PE Applied Biosystems, Perkin-Elmer).

Long-range PCR. Long-range PCR was performed using the Expand Long Template PCR system (Boehringer Mannheim) according to the manufacturer’s protocol.

Results

To detect genomic deletions of MLH1 and MSH2, we analyzed all of the exons using a semiquantitative PCR assay based on the simultaneous amplification of 8–10 short exon fragments, using a limited number of cycles and comparing the pattern of amplification between different samples. Each multiplex PCR yielded a pattern composed of 8–10 fluorescent peaks, with each peak corresponding to a specific

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The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair.
Table 1  Primers used for the multiplex PCR of MLH1 and MSH2

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Size of the amplicon (bp)</th>
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<tr>
<td>1</td>
<td>5′-TTCGCGGCTTGAAGAGCAC-3′</td>
<td>5′-CTGTAGCCCTTTAAGTGAC-3′</td>
<td>134</td>
</tr>
<tr>
<td>2</td>
<td>5′-TACATGAGAAAGTCTGAG-3′</td>
<td>5′-AGGAGAGCTCTGAGCTT-3′</td>
<td>207</td>
</tr>
<tr>
<td>3</td>
<td>5′-TAACAGAAGGAAGATCGTGG-3′</td>
<td>5′-ACAATGACTCACTCAGGG-3′</td>
<td>157</td>
</tr>
<tr>
<td>4</td>
<td>5′-CTTCCTTTCTTGGTGAGAAGC-3′</td>
<td>5′-ATTATCTGCGAGACTTGG-3′</td>
<td>222</td>
</tr>
<tr>
<td>5</td>
<td>5′-GATTTTTCTTTCTCTCTTTG-3′</td>
<td>5′-CAAAAGTCCTTACATTCTC-3′</td>
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</tr>
<tr>
<td>6</td>
<td>5′-TTCGCTGTGAGAGGACG-3′</td>
<td>5′-TGAGCTGACTCTGCCAACAA-3′</td>
<td>112</td>
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<td>5′-AGCGGTCCTCAGATCTG-3′</td>
<td>5′-ATGGGTGATGAGATCTAC-3′</td>
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<tr>
<td>8</td>
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<td>5′-ACATGATGACGCCCAAG-3′</td>
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<td>9</td>
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<td>5′-CACATTATTCCCTTGGG-3′</td>
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<td>10</td>
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<tr>
<td>14</td>
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<td>5′-AATCTACACGGTCTGCTGCTG-3′</td>
<td>5′-TACCCCTTTCTTGTTGACAC-3′</td>
<td>296</td>
</tr>
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</table>

* The numbers correspond to the exons. Some of the primers correspond to intronic sequences.

* Primer labeled with 6-FAM.

**exon, and we compared the electropherograms generated from different samples (Fig. 1). Multiplex PCR of control samples generated similar patterns, as illustrated by the superimposition of electropherograms (Fig. 1, a and c). To validate the method, we used as controls three genomic deletions of MLH1 and MSH2 that we had previously detected in French HNPCC families by reverse transcription-PCR and/or sequencing: (a) a 22-kb Alu-mediated deletion of MLH1 involving exons 13–16, which we have described in detail (14); (b) a 4-kb deletion of MLH1 involving exon 2 and caused by a recombination of two Alu repeats located in introns 1 and 2; and (c) a 2-kb deletion involving exon 3 of MSH2. These heterozygote deletions were easily detected by an approximately 0.5 reduction of the area of the peaks corresponding to the deleted exons, as illustrated by the detection of the MLH1 genomic deletion involving exons 13–16 (Fig. 1b) or the MSH2 genomic deletion involving exon 3 (Fig. 1d). Using this method, we then investigated 19 HNPCC families (13 families fulfilling the Amsterdam criteria and 6 families partially meeting these criteria) in which DGGE, heteroduplex, or direct sequencing analysis of MSH2 and MLH1 exons from genomic DNA had revealed no alteration. In one family, we observed a reduction of the peak corresponding to exon 5 of MSH2 (Fig. 2a). Amplification of exons 4–6 of MSH2 from genomic DNA revealed, in addition to the expected normal band of 4 kb, an abnormal shorter fragment in the proband (Fig. 3, a and b) that was shown by sequencing to contain exon 4, 6 bp of intron 4, 450 bp of the 3′ end of intron 5, and exon 6. In another family, we observed an unexpected aberrant profile (Fig. 2b), with a 1.5 increase of the area of the peaks corresponding to exons 9 and 10 of MSH2, suggestive of a partial duplication. Long-range PCR, using a forward primer corresponding to exon 10 and a reverse primer corresponding to intron 9 (Fig. 3e) allowed amplification of an abnormal 7-kb fragment (Fig. 3c). Long-range PCR with primers corresponding to exon 9 also confirmed the partial duplication because, in addition to the expected band at 0.227-kb, an aberrant 10-kb band was detected in the index case (Fig. 3d). The same type of abnormal profile of multiplex PCR, with a relative increase of the peak corresponding to exon 16 of MSH2, was observed in another HNPCC family (data not shown).

**Discussion**

To screen for genomic rearrangements of MSH2 and MLH1, we developed a multiplex PCR based on: (a) the amplification of short fragments to reduce the difference in efficiency between each amplification; (b) a limited number of cycles to allow an exponential amplification; (c) the simultaneous amplification of numerous fragments, allowing an accurate comparison of electropherograms generated from different samples; and (d) the comparison of fluorescence not between different peaks generated from the same sample but between the same peak generated from different samples. The short size of the fragments and the decrease in the annealing temperature during the first cycles allowed us to simultaneously amplify 8–10 fragments with ease. To attenuate the difference in efficiency between each PCR, which is explained in part by the difference in the Tm between each of the primers pairs used in the same multiplex reaction, we adjusted the concentration of each pair. We observed that the addition of GG at the 5′ end of the sense primers, which are dye-labeled, may improve the pattern of the multiplex PCR. Because this
The method is based on the comparison of electropherograms and is not quantitative, the critical point is the quality of the DNA, which was clearly demonstrated by the fact that we were sometimes unable to superimpose electropherograms generated from DNA extracted according different methods.

Using this method, we detected one deletion and two duplications of MSH2 within 19 HNPCC families previously analyzed using conventional methods. Deletion of exon 5 and duplication of exon 9 were confirmed by long-range PCR. We could not confirm the duplication of exon 16 by long-range PCR using primers corresponding to exon 16. Like genomic deletions, these duplications probably result from Alu-mediated recombination during the meiotic prophase, although the breakpoints within the Alu repeats have been characterized at the molecular level only for MLH1 (13, 14). Genomic duplications are probably the most difficult molecular alterations to detect: they cannot be detected by conventional methods based on PCR amplification of single exons from genomic DNA; reverse transcription-PCR is also inappropriate to detect large duplications that generate longer transcripts that are usually unstable because they can contain premature stop codons, resulting in mRNA decay. Large duplications of MMR genes may therefore be an underestimated cause of HNPCC, and the presence of such duplications must be considered in families in which conventional screening methods have failed to detect mutations.

It was recently shown that haploid conversion of human lymphocytes, using the cell fusion strategy, facilitates the detection of certain types of germ-line alterations, which are masked by the presence of the wild-type allele (16). This method, which may be interesting for the detection of alterations within the promoters, requires living cells and the identification of hybrids containing the desired chromosomes.

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**Fig. 1.** Multiplex PCR of MLH1 and MSH2 performed on control samples. Electrophoresis was performed using an automated sequencer (model 377; PE Applied Biosystems, Perkin-Elmer), data were analyzed using the Gene scanner model 672 fluorescent fragment Analyzer (PE Applied Biosystems, Perkin-Elmer), and electropherograms from two individuals were superimposed. The Y axis displays fluorescence in arbitrary units, and the X axis indicates the size in bp. a, multiplex PCR of 10 MLH1 exons in two controls (indicated in blue and red). b, detection of a genomic MLH1 deletion (in red) involving exons 13–16. c, multiplex PCR of nine MSH2 exons in two controls (indicated in blue and red). d, detection of a genomic MSH2 deletion involving exon 3 (in red).

**Fig. 2.** Detection of MSH2 genomic rearrangements using multiplex PCR. a, detection of a genomic deletion involving exon 5 (in red). b, detection of a duplication involving exons 9 and 10 (in blue).
Note Added in Proof

Additional multiplex PCR, including an exon fragment from another gene, revealed that the abnormal profile, which suggested a duplication of the last exon of MSH2 (exon 16), corresponds in fact to a large deletion probably involving exons 1–15.

References

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