Deletions Account for 17% of Pathogenic Germline Alterations in MLH1 and MSH2 in Hereditary Nonpolyposis Colorectal Cancer (HNPCC) Families

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ABSTRACT

Hereditary nonpolyposis colorectal cancer (HNPCC) is due to defects in DNA mismatch repair (MMR) genes MSH2, MLH1, MSH6, and to a lesser extent PMS2. Of 466 suspected HNPCC families, we defined 54 index patients with either tumors of high microsatellite instability (MSI-H) and/or loss of expression for either MLH1, MSH2, and/or MSH6, but without a detectable pathogenic point mutation in these genes. This study cohort was augmented to 64 patients by 10 mutation-negative index patients from Amsterdam families where no tumors were available. Deletion/duplication screening using the multiplex ligation-dependent probe amplification (MLPA) revealed 12 deletions in MSH2 and two deletions in MLH1. These deletions constitute 17% of pathogenic germline alterations but elucidate the susceptibility to HNPCC in only 22% of the mutation-negative study cohort, pointing towards other mutation mechanisms for an inherited inactivation of MLH1 or MSH2. We describe here four novel deletions. One novel and one known type of deletion were found for three and two unrelated families, respectively. MLPA analysis proved a reliable method for the detection of genomic deletions in MLH1 and MSH2; however, sequence variations in the ligation-probe binding site can mimic single exon deletions.

INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) is the most frequent autosomal dominant predisposition for early-onset colorectal cancer, often associated with an increased risk of tumors of the endometrium, stomach, small intestine, hepatobiliary system, ureter and renal pelvis, ovary, brain, and skin (MIM no. 1114500) (Lynch, 1999). Clinical diagnosis of HNPCC is made according to the Amsterdam criteria (Vasen et al., 1991, 1999; Vasen and Wijnen, 1999), which require three colorectal cancer patients per family, vertical transmission, and young age at onset. The “Bethesda criteria” follow less stringent inclusion criteria and represent an alternative clinical screening protocol for families or patients, in which clinical data or family history arouses the suspicion of HNPCC (Rodriguez-Bigas et al., 1997).

HNPCC is caused by germline mutations in DNA mismatch repair (MMR) genes leading to genomic instability in tumor cells. Among the several known DNA MMR genes, the majority of HNPCC cases are based on genetic defects in the MSH2
(MIM no. 120435) and MLH1 (MIM no. 120436) genes. More atypical HNPCC cases are caused by mutations in MSH6 (MIM no. 600678) and PMS2 (MIM no. 600259), whilst, for example, EXO1 (MIM no. 606063) and MLH3 (MIM no. 604395) rather seem to act as modifier genes (Hamilton et al., 1995; Kolodner and Marsischky, 1999; Wijnen et al., 1999; Wu et al., 1999, 2001a,b; Wagner et al., 2001; Jagmohan-Changur et al., 2003). The mutation detection rate for MLH1 and MSH2 is 38–45% for families fulfilling the Amsterdam criteria (Girardiello et al., 2001; Wang et al., 2003). The detection rate can be improved if microsatellite analysis of the patients tumor is performed prior to mutation analysis, as mutations are found for 64–70% of the Amsterdam positive patients with MSI-H tumors (Liu et al., 1996; Gille et al., 2002; Wang et al., 2003).

For individuals at risk, predictive genetic testing is indispensable in order to initiate appropriate surveillance programs in a timely manner. In our laboratory, genetic analysis of the “classical” HNPCC families follows a step-wise procedure. First, microsatellite instability (MSI) analysis of five markers recommended by the National Cancer Institute (D2S123, D5S346 [APC], BAT25, D17S250 [Mfd15] and BAT26) (Boland et al., 1998) is performed on tumor DNA. In parallel, paraffin-embedded tumor sections are examined immunohistochemically for the respective probes are amplified by conventional polymerase chain reaction (PCR)-based techniques. Subsequently, the relative copy number of each MLH1 and MSH2 exon is quantified by fragment gel electrophoresis (Charbonnier et al., 2002; Schouten et al., 2002). In the clinical setting, MLPA has successfully and reliably been applied, for example, in the identification of large deletions in the BRCA1 gene (Hogervorst et al., 2003) and in HNPCC testing in the Netherlands, Germany and France (Gille et al., 2002; Wang et al., 2003). We have extended these observations to HNPCC families investigated at the Institute of Human Genetics at the University of Munich and at the Center of Medical Genetics in Munich.

MATERIALS AND METHODS

Patients

A total of 466 consenting colorectal cancer families fulfilling the Amsterdam criteria I or II for HNPCC or the less stringent Bethesda criteria (“suspected HNPCC”) were included in this study. Within this cohort, we found 54 index patients with either MSI-H tumors and/or loss of expression for either MLH1, MSH2, or both MSH2 and MSH6, but without detectable truncating mutations in these genes. Additionally, 10 index patients from Amsterdam families were identified who did not carry truncating mutations and where no tumor material was available for analysis. These 64 patients are highly suspicious for truncating mutations and where no tumor material was available for analysis. These 64 patients are highly suspicious for being mutation carriers in MLH1, MSH2, or both MSH2 and MSH6, and were therefore chosen as a study cohort for deletion analysis in MLH1 and MSH2. In addition, we included 20 index patients from Amsterdam families with microsatellite stable (MSS) tumors and a negative mutation analysis for the DNA-mismatch repair genes, as the MSS phenotype might be the result of a coincidental sporadic tumor. The patients included in this study are summarized in Table 1.

MSI and mutation analysis

Investigation of MSI and immunohistochemistry in tumors was performed according to standard procedures (Dietmaier et

<table>
<thead>
<tr>
<th>Microsatellite status</th>
<th>IHC for MLH1, MSH2, MSH6</th>
<th>Truncating point mutation status in MLH1, MSH2, MSH6</th>
<th>Patients included in MLPA analysis</th>
<th>Deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI-H</td>
<td>Loss</td>
<td>Negative</td>
<td>39</td>
<td>10</td>
</tr>
<tr>
<td>MSI-H</td>
<td>No loss</td>
<td>Negative</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>MSI-H</td>
<td>n.a.</td>
<td>Negative</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>MSI-H</td>
<td>n.a.</td>
<td>Negative</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>n.a.</td>
<td>Loss</td>
<td>Negative</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>n.a.</td>
<td>n.a.</td>
<td>Negative</td>
<td>64</td>
<td>14</td>
</tr>
<tr>
<td>MSS</td>
<td>n.a.</td>
<td>Negative</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>84</td>
<td>14</td>
</tr>
</tbody>
</table>

In 54 patients, the microsatellite status and/or immunohistochemical results indicated the involvement of mismatch repair genes in tumorigenesis. Additionally, 10 mutation-negative Amsterdam-positive index patients with no tumor material available were included in the study cohort. Furthermore, 20 mutation-negative index patients of Amsterdam-families with MSS tumors have been analyzed. Deletions have been identified in 14 families. n.a., not available.
al., 1997; Boland et al., 1998). Mutation screening of MLH1, MSH2, and MSH6 was done by DHPLC analyses as published elsewhere (Kolodner et al., 1999; Holinski-Feder et al., 2001; Cederquist et al., 2004).

**MLPA analysis**

Genomic DNA samples of 84 families were subjected to MLPA analysis (MRC-Holland) according to the supplied protocol and examined for deletions/duplications in MLH1 or MSH2. The multiplex probe mix contained 16 exon probe pairs for MSH2, 19 exon probe pairs for MLH1, and seven control probe pairs specific for DNA sequences outside the MSH2 and MLH1 genes. An additional MLPA kit provided quantitative analyses of the MSH6 gene exons 1, 2, 3, 4, 5, 6, and 10, APC gene exons 1, 3, 7, 14, and 15, MSH3 gene exons 1, 3, and 20, MSH3 gene exons 2, 3, and 10, MUTH gene exons 2 and 16, and PMS2 gene exons 1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 in all mutation- and deletion-negative HNPCC patients. An additional MLPA kit provided quantitative analyses of the MSH6 gene exons 1, 2, 3, 4, 5, 6, and 10, APC gene exons 1, 3, 7, 14, and 15, MSH3 gene exons 1, 3, and 20, MSH6 gene exons 2, 3, and 10, MUTH gene exons 2 and 16, and PMS2 gene exons 1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 in all mutation- and deletion-negative HNPCC patients. Details on probe sequences, see (www.mrc-holland.com). Annealing, ligation, and cycling reactions were performed in a thermocycler with heated lid using 200–500 ng of genomic DNA following the manufacturer’s instructions. The PCR amplification was carried out with 33 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. A final extension was performed at 72°C for 20 min. Fragment analysis was carried out on ABI model 310 capillary sequencer (Applied Biosystems, Forster City, CA) using TAMRA-500 as size standard. Data analysis was performed using Genescan software (Applied Biosystems). To analyze patient samples, peak areas were imported into Excel spreadsheets developed in house and peak fractions were calculated by comparing peak areas of each fragment to those of a control sample to obtain a dosage quotient (DQ) representing the gene dosage of each fragment. In normal individuals this calculation results in a value of 1.0 representing two copies of the target sequence and 0.5 and 1.5 for deleted or duplicated fragments, respectively. The use of Excel spreadsheets enables the accurate calculation of the patient’s data in comparison to one or more control samples. It furthermore enables the comparison of two runs of the same patient or the comparison of the results of family members harboring the same deletion. The data generated by the excel program can be exported into a graphical presentation of the data (Fig. 1).

**Cytogenetics**

Metaphase chromosome spreads (lymphocyte culture from peripheral blood) were prepared according standard procedures. BAC clones RP11-436K12, RP11-295P2 (both MSH2) were obtained from the BACPAC resource center at the Children’s Hospital & Research Center Oakland (CHORI). BAC clone RP11-1N7 (control 2p25.3) was kindly provided by Prof. M. Rocchi (University of Bari, Italy). DNAs were prepared using a standard alkaline lysis procedure. Nick translation labelling with TAMRA-dUTP (RP11-436K12, RP11-295P2) and FITC-dUTP (RP11-1N7) and hybridization of the probes were performed according to standard protocols. Posthybridization washes were at 72°C for 2 min in 0.4 × SSC, 0,3% Tween 20 and at room temperature for 1 min in 2 × SSC, 0,1% Tween 20. DAPI-counterstained metaphase spreads were analyzed using a CCD camera (Sensys) coupled to a Zeiss Imaging II microscope and PathVision software (Applied Imaging, Newcastle, UK).

**RESULTS**

**Deletion/duplication analysis**

DNA samples of the 84 Amsterdam- or Bethesda-positive colorectal cancer patients listed in Table 1 were subjected to MLPA analysis to search for deletions or duplications encompassing one or more exons of MLH1 or MSH2. Using the mul-

![FIG. 1. Graphical presentation of the deletion of MSH2 exon 3–5. The ratio of MSH2 exon 3–5 is reduced to 0.5 representing a deletion of exon 3–5 in the MSH2 gene. The ratio of MSH2 exon 1 is reduced to 0.7 due to a nucleotide exchange in MSH2 (c.114C>G; D38E) in the exon 1 probe target hybridization site 13 bp distant from the ligation site.](image-url)
Deletions at the rear end of the cDNA (MSH2 exon 9–16 and exon 15–16) result in deletions of 500 and 142 amino acids, respectively, and are expected to severely damage DNA and/or protein synthesis. In two cases (MLH1 exon 1–10, MSH2 exon 1), the initiation codon and presumably the promoter region were deleted. The other deletions were out-of-frame deletions generating a premature stop codon and a deletion of the whole MSH2 gene. All these deletions resulted in a loss of expression by immunohistochemical staining in the tumors available (Table 2).

The complete loss of one copy of the MSH2 gene on one allele was confirmed by FISH analysis. The BAC clones RP11-436K12 and RP11-295P2 covering the genomic region of MSH2 hybridized with the homologous region of only one chromosome 2 of the metaphase spread, while the distally positioned BAC clone RP11-1N7 was present on both chromosomes 2 (Fig. 2).

Immunohistochemistry for study cohort patients without truncating mutations or deletions/duplications was pathological in 18 cases for MLH1 (one also for MSH6) and in 13 cases for MSH2 (five also for MSH6). Two patients had unverified mutations in MLH1 (E234E and I565F) but an IHC loss in MSH2 or MSH2 and MSH6, respectively, suggesting that these sequence variants are not disease-causing. In three patients with unclassified sequence alterations (MLH1, L653L; MSH2, V161D; and MSH6, T1102T), IHC revealed a loss of expression in the corresponding genes (Table 3).

To search for deletions or duplications in further genes involved in DNA repair pathways, we quantified selected exons of the genes MSH6, APC, MSH3, MLH3, MUTYH, and PMS2. All 50 mutation- and deletion-negative patients of our cohort have been tested, but no pathogenic results have been obtained for these additional genes.

### Method evaluation

The MLPA assay proved a reliable method for the detection of genomic deletions in MLH1 and MSH2. Small amounts of genomic DNA resulted in poor amplification of larger fragments leading to high standard deviations in the dosage quotient (DQ) estimates. This, however, also resulted in aberrant control peaks, so these samples were easily recognized as abnormal results.

However, sequence variants detected in the mutation screening have to be considered in the evaluation of the ratios in the deletion screening. We underscore the necessity of exon sequencing in the case of assumed single exon deletions. A yet unpublished nucleotide substitution in MSH2 exon 1 (c.114C>G; D38E) resulted in a constant reduction of the exon intensity in independent experiments to ratios between 0.7 and 0.8, but not to a DQ of 0.5 as expected for deletions. This nucleotide substitution is covered by one of the ligation probes and is located 13 bp from the ligation site. The same patient presented a deletion of MSH2 exon 3–5 in addition to the reduced DQ in MSH2 exon 1. The same constellation of deletion and missense mutation was found for the sibling. In another sample an unspecified sequence variant in MLH1 exon 15 (c.1693A>T; I565F) was located 1 bp next to the ligation site and reduced the amplification signal of exon 15 to a ratio of 0.5, mimicking a deletion of this exon (Fig. 3). Therefore, in patients with a deletion of one exon in MLPA analysis, we recommend resequencing the exon with intronic primers to exclude sequence alterations that affect probe ligation.

### Table 2. Deletions in the DNA Mismatch Repair Genes MLH1 and MSH2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Deleted exons</th>
<th>MSA</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLH1</td>
<td>1–10</td>
<td>MSI-H</td>
<td>Loss in MLH1, MSH6</td>
</tr>
<tr>
<td>MLH1</td>
<td>3–9</td>
<td>MSI-H</td>
<td>n.a.</td>
</tr>
<tr>
<td>MSH2</td>
<td>1</td>
<td>MSI-H</td>
<td>Loss in MSH2</td>
</tr>
<tr>
<td>MSH2</td>
<td>3</td>
<td>MSI-H</td>
<td>Loss in MSH2</td>
</tr>
<tr>
<td>MSH2</td>
<td>3–5</td>
<td>MSI-H</td>
<td>Normal</td>
</tr>
<tr>
<td>MSH2</td>
<td>3–6</td>
<td>MSI-H</td>
<td>Loss in MSH2, MSH6</td>
</tr>
<tr>
<td>MSH2</td>
<td>7</td>
<td>MSI-H</td>
<td>Loss in MSH2, MSH6</td>
</tr>
<tr>
<td>MSH2</td>
<td>7</td>
<td>MSI-H</td>
<td>Loss in MSH2</td>
</tr>
<tr>
<td>MSH2</td>
<td>9–16</td>
<td>MSI-H</td>
<td>Loss in MSH2</td>
</tr>
<tr>
<td>MSH2</td>
<td>10–12</td>
<td>MSI-H</td>
<td>n.a.</td>
</tr>
<tr>
<td>MSH2</td>
<td>15–16</td>
<td>MSI-H</td>
<td>Loss in MSH2, MSH6</td>
</tr>
<tr>
<td>MSH2</td>
<td>15–16</td>
<td>MSI-H</td>
<td>Loss in MSH2, MSH6</td>
</tr>
<tr>
<td>MSH2</td>
<td>1–16</td>
<td>MSI-H</td>
<td>Loss in MSH2, MSH6</td>
</tr>
</tbody>
</table>

In MLH1, two deletions have been found. In MLH2, 12 unrelated families showed nine different subtypes deletions. Results of microsatellite analysis of five markers (MSA) and immunohistochemistry (IHC) are displayed; otherwise, data were not available (n.a.).

n.a., not available.
DISCUSSION

Mutations in DNA mismatch repair genes are the leading cause of HNPCC, an otherwise clinical diagnosis defined by the fulfilment of the Amsterdam criteria. For up to 60–70% of the index patients with either microsatellite instability and/or loss of expression of the DNA mismatch repair genes MLH1, MSH2, or MSH6 in the corresponding tumors, disease-causing point mutations can be found in one of these genes (Liu et al., 1996). For the remaining 30–40%, no disease-causing mutations are detectable by the most frequently used methods (i.e., DHPLC and direct sequencing).

### Table 3. Results of 31 Patients with Abnormal Results in Immunohistochemistry But Without Truncating Point Mutations or Deletions/Duplications

<table>
<thead>
<tr>
<th>Patients</th>
<th>Truncating point mutation in MLH1, MSH2, MSH6</th>
<th>Deletion/duplication</th>
<th>Unclassified variant in MLH1, MSH2, MSH6</th>
<th>IHC for MLH1</th>
<th>IHC for MSH2</th>
<th>IHC for MSH6</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Negative</td>
<td>Negative</td>
<td>None</td>
<td>Loss</td>
<td>No loss</td>
<td>No loss</td>
</tr>
<tr>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>MSH6 T1102T</td>
<td>Loss</td>
<td>No loss</td>
<td>Loss</td>
</tr>
<tr>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>MLH1 L653L</td>
<td>Loss</td>
<td>No loss</td>
<td>No loss</td>
</tr>
<tr>
<td>6</td>
<td>Negative</td>
<td>Negative</td>
<td>None</td>
<td>No loss</td>
<td>Loss</td>
<td>No loss</td>
</tr>
<tr>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>(MLH1 E234E)</td>
<td>No loss</td>
<td>Loss</td>
<td>No loss</td>
</tr>
<tr>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>MSH2 V161D (and MSH2 D133D)</td>
<td>No loss</td>
<td>Loss</td>
<td>No loss</td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
<td>Negative</td>
<td>None</td>
<td>No loss</td>
<td>Loss</td>
<td>Loss</td>
</tr>
<tr>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>(MLH1 I565F)</td>
<td>No loss</td>
<td>Loss</td>
<td>Loss</td>
</tr>
</tbody>
</table>

Whole gene deletions in the genes MSH6, APC, MSH3, MLH3, MUTYH, and PMS2 have been excluded by MLPA. Tumors of 18 patients showed a loss of expression for MLH1 (one also for MSH6) and of 13 patients a loss of expression for MSH2 (five also for MSH6). Three unclassified sequence variants were found in combination with a loss in IHC of the corresponding gene. The two sequence variants of MLH1 in parentheses (I565F and E234E) coincide with an IHC loss of MSH2. MSH2 D133D is in parentheses and classified as non-pathogenic (Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff).
In this study, a total of 466 suspected HNPCC patients underwent classical mutation analysis. Of these, we were able to define a cohort of 54 patients with either MSI-H and/or loss of expression of MLH1, MSH2, or both MSH2 and MSH6 in their corresponding tumors, but without a detectable truncating mutation in one of the DNA mismatch repair genes MLH1 or MSH2. This study cohort was augmented to 64 patients by 10 mutation-negative index patients from Amsterdam families where no tumors were available. Using the MLPA-kit, we found genomic deletions in 14 obviously unrelated HNPCC families, 12 in MSH2 and two in MLH1, which equals 22% of the patients of the study cohort and 3% of the whole HNPCC cohort.

These results imply that, at least for the remaining patients with MSI-H tumors and pathological IHC results, there must be other disease causing mutation mechanisms for MLH1, MSH2, and MSH6. IHC results (Table 3) reveal that, in almost 60% of these cases, MLH1 might harbor the disease-causing defect. However, MSH2 and MSH6 seem to contribute disease-causing mutations, suggesting that no major mutation mechanism affecting only one gene on the basis of a founder mutation can be expected for the so far unresolved cases.

The deletions found here include 11 different deletion subtypes, of which four — MLH1 del exon 3–9, MSH2 del exon 3–5, 10–12, and 15–16 — are novel (Table 2). Two MSH2 deletions, the deletion of MSH2 exon 7 and the novel deletion of MSH2 exon 15–16, were found for three and two unrelated families, respectively.

In the cohort of 466 HNPCC index patients, we found 177 germline alterations in MLH1 and MSH2 (38%), with a predicted pathogenic effect in 70 mutation cases (15%), 93 unclassified variants (20%), and 14 deletion cases (3%). Deletions for one or several exons in the MMR genes MSH2 and to lesser extent in MLH1 account for 17% of pathogenic germline alterations. Similar results have been reported in the literature. In HNPCC collections of 59, 126, and 368 patients, deletions represent 21–28% of pathogenic germline deviations (Gille et al., 2002; Wagner et al., 2003; Wang et al., 2003).

Further genes play only a minor part in germline mutations predisposing to hereditary colorectal cancer. Pathogenic mutations in MSH6 amount to a further 2% of unclassified variants and 0.6% of predicted pathogenic mutations in our cohort, and mutations and deletions are generally reported to be rare (Peterlongo et al., 2003; Plaschke et al., 2003). We could not find whole gene deletions in the genes MSH6 or APC, MSH3, MLH3, MUTYH, and PMS2 in our cohort.

As it has been shown that the distribution of breakpoints is highly heterogeneous (Charbonnier et al., 2002), we did not not assess the breakpoints via long-range PCR. Many of the deletions seem to be caused by recombination between Alu repeats as these are particularly numerous in the MSH2 locus and might frequently be involved in MMR gene inactivation (Wijnen et al., 1998; Charbonnier et al., 2002; Wang et al., 2003) (Fig. 4). The frequent deletions beginning in MSH2 intron 2 (deletion of exon 3, exon 3–5, and exon 3–6) and the deletion of MSH2 exon 7 in three kindreds are assumed to originate from Alu repeats. However, as other hypothesized breakpoints (such as MLH1 intron 9, MSH2 intron 5, 14, and 15) lack Alu repeats completely, other mechanisms must be involved in the deletion event.

Unclassified sequence variants were present in five of the 31 patients with a loss of expression in immunohistochemistry. The tumors of three patients with the sequence variants MSH6 T1102T, MLH1 L653L, and MSH2 V161D showed a loss of immunohistochemical staining of the corresponding MMR protein. The possible pathogenic effect of these sequence variants has to be studied further. In our patient, the variant MSH2 D133D occurred in combination with MSH2 V161D, the first is classified as non-pathogenic (Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff). The variants MLH1 E234E and MLH1 I565F were not associated with an immunohistochemical loss of MLH1. Finally, 28 patients remain highly suspect for a deficient MMR pathway and inactivation of MLH1 or MSH2. Unclassified sequence variants might be responsible, the disease-causing mutations in the other cases remain obscure.
Addressing technical issues, a reduction of the dosage quotient can indicate a nucleotide exchange located in the target hybridization sequence, which was shown here for the sequence variation D38E in exon 1 of MSH2 (Fig. 1). With this experience in mind, we decided to sequence all one-exon deletions to exclude artifacts due to sequence variations in the probe ligation site. In another case, we could rule out a deletion of exon 15 in MSH2 as an unclassified sequence variant (c.1693A>T; I565F) next to the ligation site causing a reduction of the MSH2 exon 15 ratio to 0.5 (Fig. 3). These two sequence variants, MSH2 D38E and MLH1 I565F, have not been described before. D38E hits an amino acid conserved in S. cerevisiae and coincides at least in our patient with a deletion of MSH2 exon 3–5. The unclassified variant I565F in MLH1 does not hit a conserved amino acid. As the corresponding tumor shows microsatellite instability and loss of MSH2 and MSH6 expression, this variation appears unlikely to be disease-causing. However, further studies are needed before a classification of this missense mutation is possible.

Furthermore, the methods applied here miss genomic re-arrangements that do not result in deletions. To further clarify this, we are currently setting up several BAC clones, covering the genomic region of MLH1 and MSH2 to confirm large deletions encompassing several exons (Fig. 2) and to check for genomic re-arrangements in fiber-FISH experiments.

Another disease-causing mutation mechanism might be an inherited mutation that results in epigenetic modifications, for example, of the MLH1 promoter region leading to gene silencing (Herman and Baylin, 2003). Although promoter methylation has been excluded in several studies as a disease-causing mechanism in HNPCC (Deng et al., 2004), it might be worthwhile to check this issue again at least for the 16 mutation-negative patients where the MLH1 gene seems to be involved but no point mutation or deletion/duplication was found.

Further possibilities are mutations in the promoter regions of MSH2 and MLH1 which cannot be detected by the methods used here (Shin et al., 2002). Polymorphic sequence alterations could be used to check for biallelic expression of MLH1 and MSH2 in blood lymphocytes. Another consideration is the existence of mutations in proteins interacting with MLH1 or MSH2 such as PMS1 (MIM no. 600258), PMS2 (MIM no. 600259), or MLH3 (MIM no. 604395) (Wang et al., 1999).

We consider the MLPA deletion screening as a robust and reliable method for deletion screening. Apparently, single exon deletions can be mimicked by mismatches close to the ligation site. Therefore resequencing of these exons is recommended. Marker analyses could help to confirm deletions and to estimate the range of terminal deletions.

There is an ongoing discussion whether immunohistochemistry for MLH1, MSH2, and MSH6 or microsatellite analysis is the most sensitive method to detect tumors caused by mutations in DNA mismatch repair genes. Immunohistochemistry is hampered by disease-causing missense mutations, where a non-functioning protein might be synthesized (Christensen et al., 2002). Another example is given here, as the disease-causing in-frame deletion in MSH2 exon 3–5 did not result in a loss of expression of the corresponding protein. Microsatellite analysis revealed a MSI-H phenotype for all tumors analyzed, which was due to a genomic deletion in MLH1 or MSH2. On the other hand, DNA repair mutations have been shown not to express the MSI-H phenotype. The last has been described for MSH6 mutations (Peterlongo et al., 2003; Schiemann et al., 2004).

Finally, both methods have their limitations and will not detect 100% of the tumors caused by mutations in MLH1 or MSH2. The highest pick-up rate is achieved by the combination of family history, immunohistochemistry and microsatellite analysis. 22% of the pre-selected 64 patients in the study cohort, mostly with MSI-H tumors or loss of expression of MLH1, MSH2, or both MSH2 and MSH6, and no truncating point mutations in MLH1, MSH2, or MSH6 turned out to have deletions in MLH1 or MSH2 for one or several exons.

The remaining patients do not harbor genomic deletions; three of them, however, had unclassified sequence variants matching with the corresponding IHC results (Table 3).

So, at least for 50% of the patients of the study cohort with pathological results in immunohistochemistry, other disease-causing mechanisms must be present. No deletions were found for the Amsterdam patients with MSS tumors. They very probably define another molecular entity of hereditary colorectal cancer.

The present identification of 14 genomic deletions in German colorectal cancer families has widened the mutational spectrum of MMR genes, increasing the number of informative patients who can benefit from molecular screening. Deletions constitute 17% of the pathogenic germline alterations in the genes MLH1 and MSH2 in HNPPC families. These data and others (Charbonnier et al., 2002; Wang et al., 2003) show that large genomic deletions in MSH2 and to a lesser extent in MLH1 are involved in MMR gene inactivation and cause HNPPC. Thus, screening for genomic rearrangements as deletions and duplications in the two major MMR genes MSH2 and MLH1 should be a further step in the molecular diagnosis of HNPPC.

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