The Role of hMLH3 in Familial Colorectal Cancer

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ABSTRACT

Hereditary nonpolyposis colorectal cancer (HNPPC) is commonly associated with at least three currently known DNA mismatch repair genes: (a) hMSH2; (b) hMLH1; and (c) hMSH6. A majority of HNPPC families has identifiable mutations in hMLH1 and hMSH2. When these mutations cause an inherited risk of colorectal cancer, they are also most often associated with microsatellite instability in the tumors. Recently, hMLH3 was suggested to be causative in HNPPC. We screened 70 index patients suggestive of a genetic predisposition for germ-line mutations in hMLH3 with denaturing high-performance liquid chromatography. One frameshift mutation and 11 missense mutations were identified in 16 index patients (23%). Most families presented evidence against hMLH1 as a high-risk factor in familial colorectal cancer, and most of the mutations were found in the low-risk patients, suggesting hMLH3 to be a low-risk gene for colorectal cancer. We demonstrate in one family that a hMLH3 mutation segregated with disease together with a missense mutation in hMSH2, which makes us hypothesize that these mutations work together in an additive manner and result in an elevated risk of colorectal tumors in the family. None of the tumors with hMLH3 mutations showed microsatellite instability, which demonstrates that hMLH3 does not make its contribution to carcinogenesis through an impaired DNA mismatch repair function.

INTRODUCTION

HNPPC, also known as Lynch syndrome, is an autosomal dominantly inherited cancer syndrome predisposing to a variety of cancers. It is believed to account for 1% of all CRC cases in the Western world (1, 2).

HNPPC is commonly associated with at least three currently known DNA MMR genes: (a) hMSH2; (b) hMLH1; and (c) hMSH6 (3–7). A majority of HNPPC families has identifiable mutations in hMLH1 and hMSH2. When these mutations cause an inherited risk of CRC, they are also most often associated with MSI in the tumors (8).

However, some mutations, in particular missense mutations, in these genes are believed to cause CRC without MSI; thus, the mechanism for tumorigenesis is not always associated with a deficient DNA MMR function (9). Germ-line mutations in hMSH6 seem to be responsible for disease in a limited proportion of atypical, as well as typical, HNPPC families (10, 11), and tumors in these families show MSI to a less extent (12). Many families do not segregate mutations in the MMR genes but still show a dominant trait of an inherited risk of CRC without MSI. These families are likely to segregate putative unknown predisposing genes (13, 14) and are referred to as HCRC families. Furthermore, there are other families in which individuals with two affected first-degree relatives have an empirically increased CRC risk (14). We call this group of families TCR.

A newly identified DNA MMR gene, MLH3, was shown to interact with MLH1 (15–17) and suggested to be another candidate gene for HNPPC (18). The first two studies of germ-line mutations in hMLH3 did not support this hypothesis (19, 20). Recently, however, Wu et al. (21) screened 39 unrelated HNPPC families fulfilling the Amsterdam Criteria and 288 index patients suggested of having HNPPC for germ-line mutations in hMLH3. They identified 10 different germ-line variants, one frameshift, and nine missense mutations in 12 index patients with suspected HNPPC. Although it was not shown whether any of these mutations did segregate with disease in the families or not, they still suggested some might be causative in the families based on the observation that these mutations were not prevalent among 188 Dutch normal controls (21). Besides, they also reported that the tumors from the index cases could be MSI positive or negative (21).

However, a recently published paper presenting that MLH3-deficient mice do not show MSI (22) suggests that any colorectal tumors thought to be associated with hMLH3 mutations are likely to be MSI negative.

To investigate a possible role of hMLH3 as predisposing to CRC, we screened hMLH3 for germ-line mutations in index patients from 70 families suggestive of a genetic predisposition for CRC. None of the families had classical or attenuated familial adenomatosis polyposis. MSI status of all tumors had been established previously, and germ-line mutations in hMLH1, hMSH2, and hMSH6 were excluded in all but two families (families 21 and 199; Refs. 10 and 23).

MATERIALS AND METHODS

Patients. This study involves 70 unrelated individuals recruited through oncogenetic counseling at the Cancer Family Clinic at the Karolinska Hospital. Family history was obtained, and all diagnoses were confirmed through medical records or death certificates. The families were classified as described in Table 1. In the same table were also shown 33 families (not included in this study) where we identified germ-line mutations previously in other MMR genes. All members of the families at increased risk for CRC were counseled and offered surveillance with regular colonoscopy.

For controls, we used 96 unrelated healthy relatives of patients at the Department of Clinical Genetics at the Karolinska Hospital. For comparison, we also used 96 unrelated sporadic cases with CRC.

DNA Extraction. Blood or paraffin tissues from living or deceased patients/relatives were collected from the members of each family after informed consent according to institutional guidelines. DNA was extracted using the protocols established previously in our lab.

PCR. Seventeen published primers were used for PCR (19). Exon 1 was divided into seven overlapping fragments, and exons 8 and 9 were amplified together because of their small sizes and the small intron in between. PCR was carried out in 50 μl of reaction volume containing 100 ng of genomic DNA, 1 × PCR buffer solution, 1.5 mM Mg2+, 100 μM each deoxynucleotide triphosphate, 0.2 μM each primer, and 1.25 units of Ampli-TaqGOLD polymerase. The length of the PCR fragments, primer sequences, and corresponding annealing temperature for each fragment are available on request. Touch-Down PCR Programs were used for all exons. The following cycles of PCR were used: for exon 1 (seven fragments in total), 95°C for 9.5 min, 7 cycles of
frameshift mutation and 11 missense mutations were identified in 16

95°C for 30 s, 58°C for 45 s, −1°C per cycle, and 72°C for 45 s, followed by
26 cycles of 95°C for 30 s, 51°C for 45 s, and 72°C for 45 s. The final
extension was 72°C for 7 min, and for exon 2 to 12, 95°C 10 min, 30 cycles
at 95°C for 45 s, corresponding annealing temperature for 45 s, and 72°C for
45 s. Final extension was 72°C for 10 min. All PCR products were examined
for specificity and quantity with 2% agarose gel electrophoresis.

**DHPLC.** Before DHPLC analysis, PCR products were denatured at 95°C
for 5 min and gradually cooled down to room temperature at 1.5°C per cycle
decrements on a PCR machine to enable the efficient formation of heteroduplexes.
DHPLC was carried out in a Transgenomic Wave DNA Fragment
Analysis System, an automated DHPLC instrumentation equipped with a
dNASep column (Transgenomic, Crewe, United Kingdom). The mobile phase
gradient and running column temperature selected for optimal hetereoduplex
separation were determined for each amplicon using WAVERMAKER 3.4
software provided with the instrument. Samples of 10 μl were injected onto the
dNASep Column, followed by elution with a mixture of buffer A (0.1 M
triethylammonium acetate) and buffer B (0.1 M triethylammonium acetate
and 25% Acetonitrile; Bie & Berntsen AS) at a flow rate of 0.9 ml/min. Abnormal
elution profiles were identified by visual inspection of the chromatogram on
the basis of the appearance of one or more additional earlier eluting peaks.
Temperatures used for DHPLC analysis are shown in Table 2.

**Direct Sequencing.** For all samples exhibiting abnormal DHPLC profiles,
genomic DNA was reamplified. After purification of DNA using QIA quick
PCR Purification Kit (Qiagen Ltd., Hilden, Germany), sequence changes were
examined in cases and controls, one silent variant and 3 intronic variants
were considered likely polymorphisms (Table 3). Besides one missense variant equally common in cases and controls, one silent variant and 3 intronic variants
were considered likely polymorphisms (Table 3b). Only 2 variants, Lys231Gln and Glu1451Lys, were in the conserved homologous regions of Mutl proteins at the NH2 and COOH terminals, respectively (17, 24), and none of the variants were evolutionarily conserved
in yeast or *Escherichia coli*.

Among 16 families, 1 was classified as HNPCC, 4 as HCRC, 1 as
autosomal dominantly inherited endometrial cancer, and 10 as TCR
families. To elucidate whether the 11 variants identified segregated
with the disease or not in the families, we tried to obtain DNA from as
many as possible of related affected and unaffected family members.
We evaluated the MSI status, and all tumors from the index cases were MSI negative. All variants were tested for prevalence in controls and sporadic CRC patients (Table 3a).

The frameshift mutation, 885delG (Fig. 1), was obviously the one
most expected to be causative because it meant a truncation very early in
the protein. This mutation was found in 2 cases with CRC, one
woman with endometrial cancer and one of three unaffected relatives,
all aged >75, in family 141 (Fig. 2). Besides, this mutation was not
detected in any controls or sporadic CRC patients. Thus, the result is
consistent with this mutation being associated with disease but with
reduced penetrance.

The missense mutation A691C was found in the index cases in four
families: (a) 12; (b) 26; (c) 70; and (d) 265 (Fig. 2). The mutation did not
segregate well with the disease in any of the families tested. In
families 12 and 70, family members affected with CRC did not have
the mutation. In family 26, 2 patients with tubular adenomas did not have
the mutation, whereas 2 elderly women with clean colonoscopies
did. Family 265 was not used for test of segregation. Furthermore, the
A691C mutation was found in 6% of controls and 10% of sporadic
cases.

The A1234G variant was found in family 143, but only the index
case and one healthy sib had the mutation (Fig. 2). The variant was
also found in 2 cases of sporadic CRC.

The alteration, G1870C, published previously in two families (21),
was found in 1 index case in family 91. None of six other affected
family members shared this variant, and it was also found in 3 controls
and 1 sporadic CRC case.

The G2221T mutation was identified in family 54, which was an
endometrial cancer family (Fig. 2). The index case and her daughter,

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**Table 1** Families included in the study

<table>
<thead>
<tr>
<th>Family type</th>
<th>Criteria</th>
<th>No. of families with unidentified MMR mutations</th>
<th>No. of families with identified MMR mutations</th>
<th>No. of families with identified MMR mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNPCC</td>
<td>1 Amsterdam criteria II, MSl positive</td>
<td>10</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>2 Amsterdam criteria II, MSl negative</td>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3 Amsterdam criteria II, MSl unknown</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HCRC</td>
<td>1 Non-Amsterdam criteria, MSl positive</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2 Non-Amsterdam criteria, MSl negative</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TCR</td>
<td>1 Two first-degree relatives, MSl positive</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2 Two first-degree relatives, MSl negative</td>
<td>21</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>OCR</td>
<td>1 One case of early age colorectal cancer, MSl positive</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2 One case of early age colorectal cancer, MSl negative</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ENDO</td>
<td>1 Familial endometrial cancer, MSl positive</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2 Familial endometrial cancer, MSl negative</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3 Familial endometrial cancer, MSl unknown</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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**Table 2** Oven temperatures used for DHPLC analysis of hMLH3

<table>
<thead>
<tr>
<th>Exon-fragment</th>
<th>Temperature (°C)</th>
<th>Exon-fragment</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1-1</td>
<td>59</td>
<td>E4</td>
<td>57, 59</td>
</tr>
<tr>
<td>E1-2</td>
<td>57, 58</td>
<td>E5</td>
<td>56, 59</td>
</tr>
<tr>
<td>E1-3</td>
<td>56, 57, 59</td>
<td>E6</td>
<td>58, 60</td>
</tr>
<tr>
<td>E1-4</td>
<td>55, 56, 58</td>
<td>E7</td>
<td>60, 64</td>
</tr>
<tr>
<td>E1-5</td>
<td>54, 55, 57</td>
<td>E8</td>
<td>57, 60</td>
</tr>
<tr>
<td>E1-6</td>
<td>58</td>
<td>E9/10</td>
<td>58, 59</td>
</tr>
<tr>
<td>E1-7</td>
<td>56, 57, 59</td>
<td>E11</td>
<td>58, 62</td>
</tr>
<tr>
<td>E2</td>
<td>55, 56, 62</td>
<td>E12</td>
<td>58, 63</td>
</tr>
<tr>
<td>E3</td>
<td>57, 60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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* HNPCC, hereditary nonpolyposis colorectal cancer.
* HCRC, hereditary colorectal cancer.
* TCR, two close relatives with colorectal cancer.
* OCR, one close relative with colorectal cancer.
* MSI, microsatellite instability.
* MMR mutations, germ-line mutations in one of the mismatch repair genes, hMLH2, hMLH1, and hMSH6.

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both affected with endometrial cancer, as well as one unaffected aunt >80 years of age, all had this mutation, which is consistent with the role of one causative mutation with reduced penetrance in this family. This variant was not found in normal controls or sporadic CRC cases. The C2825T variant was found in two families, 3 normal controls and 2 sporadic CRC cases. In family 210, it was not possible to find out if the affected sib had this mutation, but his daughter, who had a history of two tubular adenomas during surveillance, did not (Fig. 2). In family 239, only the index case had this variant. Although studying DNA from her daughter with CRC at age 28, it was discovered that the daughter had another variant, G2797T instead, likely to have been inherited from her unaffected father (Fig. 2). The G2797T variant was not found among controls or sporadic cancer cases.

The T2896C mutation was found in three families: (a) 26; (b) 21; and (c) 66 (Fig. 2). In family 26, it was not possible to test the other subjects with CRC. However, the fact that none of five unaffected children of one CRC patient (H:1) had this mutation suggested this affected person not to be a carrier. Moreover, in the same family, two subjects with adenomas did not have this mutation, whereas two old female carriers of the mutation never had any polyps during surveillance. In family 21, three affected members in three consecutive generations shared the variant. In family 86, the index patient and her mother shared this variant but not the affected uncle. The T2896C mutation was also found in 2 sporadic CRC cases and 2 controls.

The G2911A variant was reported previously in two families, at least one of which segregated an hMSH6 mutation (21). This mutation was found in the index case of family 60. The mutation was not identified in her sister with colon cancer but in her sister with endometrial cancer, as well as her four daughters. This variant was not found among sporadic CRC cases or controls.

Table 3b Polymorphisms found in hMLH3

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Reportage</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C2531T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Pro844Leu</td>
<td>Present study</td>
<td>Many</td>
</tr>
<tr>
<td>1</td>
<td>C2838A</td>
<td>Ser947Ser</td>
<td>Present study</td>
<td>Many</td>
</tr>
<tr>
<td>3</td>
<td>IVS3 + 37 del T&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Unknown</td>
<td>Present study</td>
<td>Many</td>
</tr>
<tr>
<td>9/10</td>
<td>IVS9 + 66 A → T</td>
<td>Unknown</td>
<td>Present study</td>
<td>Many</td>
</tr>
<tr>
<td>9/10</td>
<td>IVS11 + 13 C → G</td>
<td>Unknown</td>
<td>Present study</td>
<td>Many</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbering is according to the cDNA starting at the A in the start codon.

<sup>b</sup> Numbering is according to the GenBank accession no. AF19568a.
used single-strand conformation polymorphism analysis to
the role of this gene in inherited CRC susceptibility (19–21).

screen for germ-line mutations, and 5 seemingly polymorphic variants in the hMLH3 gene for a representative sample. A, DHPLC plot; B, sequence analysis of a wild-type control and an index patient with the heterozygous G885del G mutation.

DISCUSSION

The gene MLH3 was first published as a new member of the DNA MMR genes in yeast and later mapped to the region of the mouse complex trait locus colon cancer susceptibility I (18). Although no germ-line mutations were found in these mice, the hMLH3 gene was still suggested a candidate predisposing colon cancer gene in humans (18). In this study, we found 1 frameshift mutation, 11 missense mutations, and 5 seemingly polymorphic variants in hMLH3 among 70 cases with familial CRC. The discovery that all but 2 missense mutations identified were not in the conserved region and none of them changed a codon conserved in yeast and E. coli does not rule out their possible pathogenic consequences. In fact, a large proportion of reported missense mutations in hMLH1, another MutL homologue, is within the least conserved regions. Three studies have tried to verify the role of this gene in inherited CRC susceptibility (19–21). Loukola et al. used single-strand conformation polymorphism analysis to screen for germ-line hMLH3 mutations in 52 Finnish probands with family histories of CRC and without known germ-line mutations in hMLH1 or hMSH2. Two obviously common missense variants and two intronic polymorphisms were found (19). Lipkin et al. (20) used DHPLC to screen 60 CRC cases with a suggested genetic background for their disease. No germ-line mutation was found. Wu et al. used denaturing gradient gel electrophoresis to screen 39 unrelated HNPCC families fulfilling the Amsterdam Criteria and 288 index patients suspected of having HNPCC. They identified 26 different germ-line variants, one frameshift and 25 missense, in 8% of the patients. No members of the families could be used to find out if any of the variants did segregate with disease in families. However, 10 of the variants could not be identified among 188 controls, which suggested them to be pathogenic. Besides, the gene hMSH6 seemed to be involved in the initiation of the tumors in only two families, and, for the remaining cases, the involvement of hMLH1, hMSH2, and hMSH6 had been excluded, which further prompted the authors to conclude that it was possible that some of the hMLH3 variants found were associated with an increased risk for CRC (21).

The present study in 70 probands with a likely genetic predisposition to colon cancer revealed a somewhat higher frequency of hMLH3 germ-line variations (23%). The different prevalence of sequence variants detected in these four studies likely reflects the different methods used and various numbers of cases studied. The Finnish study used the least sensitive method, single-strand conformation polymorphism, whereas the rest used the more sensitive techniques denaturing gradient gel electrophoresis and DHPLC. Moreover, it is plausible to think that the mutation prevalence discrepancy could also reflect the fact that our study involved more low risk families than the previous studies. We considered the DHPLC analysis in our study very robust and reproducible. It was somewhat surprising that so few similar variants were identified among the studies, without taking into account the 16 unpublished variants in the Dutch study. This could be attributable to the possibility that genetic polymorphisms vary among the Finnish, American, Dutch, and Swedish populations.

We tried to establish whether the mutations segregated with disease in every family. Three of the variants, including the frameshift one, showed a kind of segregation pattern consistent with a monogenic predisposing risk factor in families 141, 54, and 199. Furthermore, none of these three were detected among controls or sporadic cases. However, these families were small, and the affected individuals were all first-degree relatives, which provides quite weak evidence for hMLH3 serving as a high penetrant predisposing CRC gene even in these three families. For all of the other variants, there was not a very good segregation with disease in families, and/or they were present with equal frequencies in sporadic cases and normal controls.

Although all other mutations presented some evidence against a monogenic explanation for the familial aggregation of CRC, we would still like to propose that some or all of these variants constitute low risk genes, perhaps with an additive predisposing risk for CRC. Our hypothesis is that, in similarity to what was recently suggested for breast cancer (25), a majority of CRCs is caused by a number of low risk alleles, which act on their own or interact in a recessive/additive manner. Thus, in high risk families, a monogenic susceptibility is still suggested as the first choice of genetic explanation for the disease, whereas in low risk groups, a polygenic inheritance of susceptibility alleles is suggested. In the latter families, we expect to find more than one low risk gene alteration. This model fits well with the risk figures for close relatives in low risk families. Subjects with two affected first-degree relatives have a 4-fold increased lifetime risk of CRC, whereas those with one first-degree and one second-degree relative affected have only a 2-fold increased risk (26). This difference in risk is consistent with the difference in the numbers of alleles shared between the subjects. As comparison, first-degree relatives to CRC patients in high risk families have a 6–8-fold increased lifetime risk of disease (13, 14). The theory with low risk genes is supported by the common Jewish APC variant, J1307K, known not to segregate perfectly with disease in families but still to be associated with a relative CRC risk of 1.6 (27). Moreover, an example was recently published where missense mutations in the DNA repair gene MYH were reported to act in a recessive manner and cause CRC in three sibs with compound heterozygous missense mutations in this gene (28). Both mutations were also found as heterozygous mutations in healthy members of the same family and normal control individuals (28). Although, in this family, the predisposition seems to be inherited in a recessive manner, it is possible that heterozygous mutations in this gene also act in an additive manner as a low risk gene. Interestingly, a recent study in yeast demonstrated that functional interactions between weak alleles in the majority of known MMR genes (MLH1,
PMS1, MSH2, MSH3, EXO1, and POL30) and other genes function in DNA replication (POL32 and RNR1). These alleles caused weak mutator phenotypes as single mutants but caused strong mutator phenotypes when combined with each other (29). We further support our hypothesis with one example from this study (Fig. 3). Two families were included in this study despite the already identified missense MMR gene mutations of unclear pathogenicity (Table 1). One of them, family 199, is an HNPCC family with five affected members. In this family, we identified an hMSH2 missense variant previously, E198G, in all four sibs and their father (23). The fact that all tumors were MSI negative and the father displayed a somewhat weaker phenotype (a tubular adenoma with cancer at age 80), as well as the fact that missense variations in hMSH2 are not often reported to be pathogenic, made us hesitate about the consequence of this hMSH2 variant alone. The finding of the missense hMLH3 variant T3826C, inherited from the mother and present in all four sibs in the present study (the chance for this to occur is 1 in 256), suggests that mutations in the two genes could have contributed together to the phenotype in the four siblings. The finding in this family supports our hypothesis of the existence and action of low penetrant additive CRC risk alleles.

On the basis of our results of hMLH3 screening in 70 patients at risk for HCRC, we propose that this gene might occasionally be involved...
as a high risk gene predisposing to CRC and endometrial cancer. However, most of the variants were found in the low risk population of TCR, and, in general, mutations did not segregate well with disease in families. Thus, we hypothesize a model where hMLH3 works as a low risk allele, which contributes to the risk of CRC in many families sometimes together with other low risk alleles. Furthermore, tumors in all index cases with hMLH3 mutations were clearly MSI negative, suggesting the mechanism in carcinogenesis is not likely to involve a deficient MMR. This is in agreement with the MLH3 function in yeast (15) and mice (22). The definite answer to the open question of which deficient MMR. This is in agreement with the MLH3 function in yeast: missense variants in this study could rely on future association studies could present with a low CRC predisposition among the identified missense variants in this study could rely on future association studies where large numbers of patients and controls are involved.

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REFERENCES


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