Biochemical Characterization of MLH3 Missense Mutations Does Not Reveal an Apparent Role of MLH3 in Lynch Syndrome

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So far 18 MLH3 germline mutations/variants have been identified in familial colorectal cancer cases. Sixteen of these variants are amino acid substitutions of which the pathogenic nature is still unclear. These substitutions are known as unclassified variants or UVs. To clarify a possible role for eight of these MLH3 UVs identified in suspected Lynch syndrome patients, we performed several biochemical tests. We determined the protein expression and stability, protein localization and interaction of the mutant MLH3 proteins with wildtype MLH1. All eight MLH3 UVs gave protein expression levels comparable with wildtype MLH3. Furthermore, the UV-containing proteins, in contrast to previous studies, were all localized normally in the nucleus and they interacted normally with wildtype MLH1. Our different biochemical assays yielded no evidence that the eight MLH3 UVs tested are the cause of hereditary colorectal cancer, including Lynch syndrome. © 2009 Wiley-Liss, Inc.

INTRODUCTION

Mismatch repair (MMR) proteins form a highly conserved group of proteins that play a crucial role in correcting DNA mismatches that have escaped the proofreading activity of DNA polymerases. In the human MMR system the mismatch recognition component is fulfilled by a heterodimeric protein complex composed of two MutS homologous (MSH) proteins. The major MSH-heterodimer consists of MSH2 and MSH6 (MUTSα). This heterodimer is able to recognize and bind to base-base mismatches as well as to small insertion/deletion loops. A minor and partially redundant mismatch recognizing/binding heterodimer called MUTSβ consists of the MSH2 and MSH3 proteins. This protein complex recognizes and binds mainly to larger insertion/deletion loops (Chung and Rustgi, 2003). During the mismatch repair process, when MUTSα/ MUTSβ recognize DNA mismatches that arise during DNA replication, the protein complex binds to the mismatch, thereby inducing a conformational change of the heterodimer and allowing ATP to bind and activate the protein complex. After activation of the MUTS complex, a heterodimer of two MutL homologous (MLH) proteins will bind to the DNA/protein complex. Two heterodimers composed of either MLH1 or PMS2 (MUTLα) or MLH1 and MLH3 (MUTLγ) are proven interactors with the MSH2-containing DNA-protein complex. These MLH complexes are thought to coordinate the downstream repair events, involving different proteins such as exonucleases (e.g., EXO1) and DNA polymerases (Tishkoff et al., 1997; Tran et al., 2001; Liberti and Rasmussen, 2004). The heterodimer MLH1-PMS2 (MUTLα) interacts with both MUTSα and MUTSβ, whereas the heterodimer MLH1-MLH3 (MUTLγ) is believed to participate in insertion/deletion loop (IDL) repair.

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mutations and an MLH3 to tumor development (Perucho, 1999). These coding sequence mutations that contribute function results in unrepaired mutations in non-encoded mutated protein. This loss of MMR translation and thus in loss-of-function of the identified result in premature termination of maki and Vasen, 2004). Many of the mutations are associated with tumor development. Germline mutations in four MMR genes, namely MLH1, MSH2, PMS2, and MSH6, have been identified in the majority of families with hereditary nonpolyposis colorectal cancer or Lynch syndrome (Peltonak and Vasen, 2004). Many of the mutations identified result in premature termination of translation and thus in loss-of-function of the encoded mutated protein. This loss of MMR function results in unrepaired mutations in non-coding but also in coding sequences. It is mainly these coding sequence mutations that contribute to tumor development (Peruro, 1999).

Recently, we identified nine MLH3 missense mutations and an MLH3 frameshift mutation in patients suspected of having Lynch syndrome (Wu et al., 2001). The missense mutations will be called UVs throughout this article because it is not yet known whether these DNA variants contribute to disease development. Identifying these DNA variants, in combination with the identification of somatic MLH3 mutations in three tumors of patients with these DNA variants (Wu et al., 2001), led us to hypothesize that, besides the four known MMR genes, MLH3 might also play a role in Lynch syndrome development. This thought is further strengthened by other articles describing the association of germ-line MLH3 mutations with esophageal, colorectal and endometrial cancer susceptibility (Liu et al., 2006; Taylor et al., 2006; Kim et al., 2007). However, as all but two of the variations identified so far were UVs, the role of MLH3 in the development of Lynch syndrome is still under debate. We biochemically tested eight identified MLH3 UVs to see whether there is a possible role for MLH3 in Lynch syndrome.

MATERIALS AND METHODS

Cell Lines

HEK293T cells, human embryonic kidney cells that lack MLH1 and MLH3 expression due to hypermethylation of the promoter regions of both genes (Cannavo et al., 2005), were grown in DMEM, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and l-Glutamine (all from Invitrogen, Breda, the Netherlands). HeLa cells (ATCC CCL-2; a cervical carcinoma cell line) were grown in DMEM containing 10% FBS and 1% penicillin-streptomycin (all from Gibco, Taastrup, Denmark).

In Silico Analysis of the Unclassified Variants in MLH3

A description of the different MLH3 UVs that were tested and the clinical characteristics of the mutation carriers are given in Table 1 and in Figure 1. Alignments with MLH3 homologous proteins from six other vertebrates were obtained by blasting the complete MLH3 protein to assess whether the variant amino acids had been evolutionarily conserved. The program used was M-Coffee (http://www.tcoffee.org) (Moretti et al., 2007). The following sequences were used in the Blast search: Homo sapiens, Rattus norvegicus, Mus musculus, Canis familiaris, Bos taurus, Gallus gallus, Xenopus tropicalis, Pan troglodytes, and Saccharomyces cerevisiae.

All eight MLH3 UVs were further analyzed in silico for putative functional effects by determining the Grantham’s chemical distance that considers composition, polarity and volume (Grantham, 1974) and by using the Align GVGD algorithm (Grantham variation and Grantham deviation), a web-based program that combines the biophysical characteristics of amino acid and protein multiple sequence alignments to predict where missense substitutions in genes of interest fall in a spectrum from enriched deleterious to enriched neutral (http://agvgd.iarc.fr/agvgd_input.php; Tavtigian et al., 2005; Mathe et al., 2006). We also evaluated the biochemical effects
TABLE 1. Genetic and Clinical Data of the Tested MLH3 UVs

<table>
<thead>
<tr>
<th>Additional mutations in MSH2, MLH1, and/or MSH6</th>
<th>Somatic (second)</th>
<th>MLH3 mutation</th>
<th>Ternanucleotide instability</th>
<th>Mononucleotide instability</th>
<th>Dinucleotide instability</th>
<th>Exon</th>
<th>change</th>
<th>protein change</th>
<th>Cancer and age of diagnosis</th>
<th>MSI status a</th>
<th>Instability</th>
<th>IHC MSH2/MLH1/MSH6</th>
<th>MSI-H</th>
<th>MSI-L</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 c.70C&gt;G</td>
<td>Yes</td>
<td>H2</td>
<td>04 (0 %)</td>
<td>4/11 (36 %)</td>
<td>5/9 (56 %)</td>
<td>+/++/++</td>
<td>+/++/++</td>
<td>11 c.4180G&gt;A</td>
<td>p.Ala1394Thr</td>
<td>Yes</td>
<td>CRC</td>
<td>44</td>
<td>b0/4 (0 %)</td>
<td>4/12 (33 %)</td>
<td>5/9 (56 %)</td>
</tr>
<tr>
<td>1 c.1496A&gt;G</td>
<td>No</td>
<td>CRC</td>
<td>62</td>
<td>H2</td>
<td>20/4 (0 %)</td>
<td>9/11 (82 %)</td>
<td>7/9 (78 %)</td>
<td>+/++/++</td>
<td>1 c.2449A&gt;G</td>
<td>MSH6</td>
<td>G</td>
<td>p.Ser817Gly</td>
<td>IVS9+43 ins10 bp</td>
<td>No</td>
<td>EC</td>
</tr>
<tr>
<td>1 c.1870G&gt;C</td>
<td>No</td>
<td>L1</td>
<td>56</td>
<td>1/4 (25 %)</td>
<td>4/11 (45 %)</td>
<td>6/9 (67 %)</td>
<td>+/++/++</td>
<td>1 c.2578delA</td>
<td>p.N860IfsX13</td>
<td>No</td>
<td>CRC</td>
<td>43</td>
<td>L</td>
<td>1/4 (25 %)</td>
<td>3/10 (30 %)</td>
</tr>
<tr>
<td>1 c.2941G&gt;A</td>
<td>No</td>
<td>L</td>
<td>24</td>
<td>0/4 (0 %)</td>
<td>4/10 (40 %)</td>
<td>5/8 (63 %)</td>
<td>+/++/++</td>
<td>1 c.3020A&gt;G</td>
<td>p.Asn1007Ser</td>
<td>Yes</td>
<td>CRC</td>
<td>49</td>
<td>L</td>
<td>b0/4 (0 %)</td>
<td>1/11 (9 %)</td>
</tr>
<tr>
<td>11 c.4180G&gt;A</td>
<td>Yes</td>
<td>CRC</td>
<td>44</td>
<td>b0/4 (0 %)</td>
<td>4/12 (33 %)</td>
<td>5/9 (56 %)</td>
<td>+/++/++</td>
<td>12 c.4351G&gt;A</td>
<td>MSH6</td>
<td>c.651dupT</td>
<td>No</td>
<td>CRC</td>
<td>41</td>
<td>L</td>
<td>0/4 (0 %)</td>
</tr>
</tbody>
</table>

None of the families fulfilled the Amsterdam II criteria; all families fulfilled the Bethesda guidelines.

CRC, colon cancer; EC, endometrial cancer; OV, ovarian cancer; H, MSI-H; L, MSI-L; ND, not determined.

aMSI status determined by the use of the five consensus markers.

bThese patients have no PMS2 germline mutation.

cA recent study on a Korean population identified c.4351G>A as a SNP (Kim et al., 2007).
dLoss of MSH6 is due to the truncating mutation identified.

Melanoma UVs: Netgene2 (http://www.cbs.dtu.dk/services/NetGene2), Splicesitefinder (http://violin.genet.sickkids.on.ca/~ali/splicesitefinder.html), and SpliceSiteFinder (http://www.cbs.dtu.dk/services/splice.html). As input we gave a fragment containing the exon sequence plus 200 nucleotides upstream and 200 bp downstream of the exon. This was done for exons 1, 11, and 12 since the eight UVs tested lie within these exons.

We also ran ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), a program that searches for sequences that act as binding sites for four members of the serine/arginine rich family of splicing enhancer proteins. Input sequences are screened for consensus binding sequences for the SR proteins CF2/ASF, SC35, SRp40, and SRp55. Regions with scores above a certain threshold value are predicted to act as SR protein binding sites, and thus function as ESEs (exonic splice enhancers). The wildtype (or UV-containing) MLH3 cDNA sequence (AB039667) was used as input. The program was run in an exon-by-exon manner.

**MLH3 Vectors and MLH3 Mutants**

To clone the MLH3 cDNA (wildtype) in pAS2 (a yeast two-hybrid vector containing a GAL4 DNA binding domain) and pACT2 (a yeast two-hybrid vector containing a GAL4 activation
domain), MLH3 was first cloned into pBluescript. MLH3 was PCR-amplified using primers containing the 5' end and the 3' end of the coding sequence coupled to a BamHI (forward) and an Eco47III (reverse) restriction site. An extra AG was inserted between the BamHI site and the first codon of MLH3 (to get MLH3 in the correct reading frame after subsequent subcloning into pACT2 and pAS2). After cloning this PCR-amplified MLH3 in pBluescript, the insert was sequenced and subcloned in pACT2 and pAS2 using the BamHI and Eco47III restriction sites. Mutations [i.e., the eight UVs mentioned in Table 1, one known polymorphic missense variant (p.Ser845Gly) and the identified frameshift mutation (see Table 1)] were introduced in these plasmids using the Stratagene QuickChange XL Mutagenesis Kit (La Jolla, CA). This was done according to the manufacturer’s instructions. After mutagenesis the MLH3 inserts were sequenced to confirm the mutation status of the plasmids.

Wildtype MLH3 (from pBluescript-MLH3) was cloned directly, in-frame with YFP, into pEYFPC1 (Clontech Laboratories, Woerden, the Netherlands) and the constructed YFP-MLH3 vector was used for subcellular localization studies. Mutations [i.e., the eight UVs mentioned in Table 1, one known polymorphic missense variant (p.Ser845Gly) and the truncating mutation we identified (see Table 1)] were introduced in WT-YFP-MLH3 using the Stratagene QuickChange XL Mutagenesis Kit (La Jolla, CA). This was done according to the manufacturer’s instructions. After mutagenesis all inserts were verified by sequencing.

Protein Expression of MLH3 in the HEK293T Cell Line

HEK293-T cells were seeded in a 6-well plate 24 hr before transfection to ensure 60% confluence on the day of transfection. Four micrograms of YFP-MLH3 vector (WT or mutant) was transfected using 10 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA). WT-CFP-MLH1 and WT-YFP-MLH3 were used as positive controls. Transfected HEK293T cells were lysed 48 hr after transfection using a nondenaturing lysis buffer (20 mM Tris-HCl, pH 8, 135 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% Triton X-100, 10% glycerol, protease inhibitors). Protein concentration determinations were performed according to Bradford (1976) and 75 µg of the cell lysate was loaded and size separated on a 6% SDS-PAGE gel. After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Bio-Rad, Veenendaal, the Netherlands). MLH3 was detected with specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or with anti-GFP (GeneTex, San Antonio, TX) and anti-mouse IgG HRP-conjugated secondary antibody (Pierce Biotechnology, Rockford). Beta-tubulin (a housekeeping protein) was used as an internal loading control and detected with an anti-beta-tubulin antibody (Sigma-Aldrich, St. Louis, MO) and anti-mouse IgG HRP-conjugated secondary antibody (Pierce Biotechnology, Rockford). Signal visualization was performed with the Supersignal West Dura Extended Duration Substrate kit (Pierce Biotechnology, Rockford).
Subcellular Localization of MLH3

On the day prior to transfection, 100,000 cells were seeded in small glass Petri dishes (20 mm in diameter). On the day of transfection, the media were refreshed. For transfection 1 µg of plasmid DNA (also 1 µg in total for the double transfection experiments) and 3 µl Fugene6 per Petri dish was used. Fugene6 (3 µl) was mixed with 50 µl optimem and incubated for 5 min at room temperature. DNA was added and mixed, and this mixture was incubated for 20 min after which it was added to the cells. The cells were then incubated for ~24 hr. After incubation, the transiently expressed, fluorescently labeled proteins were visualized by confocal laser scanning microscopy (Zeiss LSM510, Carl Zeiss MicroImaging GmbH, Jena, Germany).

Yeast Two Hybrid Analysis

To investigate the ability of UV-containing MLH3 proteins to form MLH1-MLH3 heterodimers, yeast two-hybrid assays were performed essentially as described by Rasmussen et al., (2000). pAS2-MLH1 and pACT2-MLH3 were cotransformed into the Saccharomyces cerevisiae strain Y190. Transformants were selected on synthetic dextrose minimal medium (SD) lacking tryptophan, leucine and histine (SD/-Trp/-Leu/-His). At least five colonies were streaked onto an SD/-Trp/-Leu/-His plate that was supplemented with 25 mM 3-amino-1,2,4-triazole (3-AT). Colonies were further tested on SD/-Trp/-Leu/-His/3-ATþ plates supplemented with 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) plates. Blue colonies that can grow on these selection plates express GAL4-tagged proteins that are able to interact. Several attempts to introduce pACT2-MLH3 p.E1451K into Y190 failed. The reason for this is unknown. The two-hybrid vectors containing MLH3 or MLH1, in combination with the empty vectors, were used as negative controls.

RESULTS

In Silico Analysis of the Unclassified Variants in MLH3

The alignment depicted in Figure 2 shows that one of the eight UVs changed a reasonably well-conserved amino acid (p.Gly981Ser), whereas the other seven of the eight UVs changed highly conserved amino acids. The results of the other in silico analyses of the MLH3 UVs are presented in Table 2. The Grantham scores and the “Russel’s prediction” show that all amino acid substitutions are not likely to interfere with the function of MLH3. Seven of the eight UVs were predicted to be benign based on PolyPhen. Only UV, p.Asn499Ser, was predicted to be possibly damaging based on the PolyPhen algorithm. Although SIFT predicts effects on protein function for p.Gln24Glu and p.Ala1394Thr, these predictions have a low confidence.

An in silico study for the possible splicing effects of the UVs, as determined by NetGene2, SpliceSiteFinder, Splice Site Predict and ESEfinder, revealed that none of the UVs were likely to cause splice defects. This analysis included donor and acceptor site changes as well as inactivation of exonic splicing enhancer sequences.

These in silico experiments suggest that the MLH3 UVs are likely to be benign, with the possible exception of p.Asn499Ser.

Transient Expression of MLH3 in HEK293T Cells

To evaluate the stability of the WT- or Mutant-YFP-MLH3 proteins, we transiently expressed Mutant-YFP-MLH3 in HEK293T cells. Notably, HEK293T cells are deficient for both MLH1 and MLH3 due to hypermethylation of the promoter regions of both genes (Cannavo et al., 2005). The wildtype MLH3 protein was stably expressed, even in the absence of MLH1 and the MLH3 expression levels of all UV-containing proteins were comparable with the expression level of the WT-YFP-MLH3 and that of one known missense polymorphism (p.Ser845Gly) (see Fig. 3A). The MLH3 protein variant with a frameshift mutation, p.Asn860IfsX13, was not detectable (see Fig. 3A). This was caused by shortening of the protein leading to a loss of the epitope recognized by the MLH3-antibody. The MLH3 (H-2) antibody is a mouse monoclonal antibody raised against amino acids 1228-1453 of human MLH3. To verify the presence of the truncated protein, we reprobed the blot with an anti-GFP antibody. A protein with the size expected for the truncated protein was detected (around 126 kDa), see Figure 3B. These results suggest normal expression of all UV-containing MLH3 proteins.

Subcellular Localization of MLH3

To evaluate whether the UV-containing MLH3 proteins were transported correctly into
the nucleus, WT-YFP-MLH3 and Mutant-YFP-MLH3 were transfected into HEK293T cells or into HeLa cells. Figure 4 shows representative results of these experiments in HeLa cells (similar results were obtained in HEK293T cells, data not shown). In the HeLa and HEK293T cell lines, being MLH3-proficient and -deficient, respectively, all mutated and WT proteins were mainly localized in the nucleus. Some cytoplasmatic staining was seen, although this was independent of the presence of MLH1. These results suggest that the subcellular localization of the mutated proteins is normal. The subcellular localization for the MLH3 protein containing a truncating mutation clearly differs from the UV-containing mutations since, besides the nuclear staining, a strong cytoplasmatic staining was also observed.

**Yeast Two-Hybrid Analysis**

To determine whether MLH1 and UV-containing or wildtype MLH3 are able to form protein dimers in vivo, we performed a yeast two-hybrid screen. The screen, as shown in Figure 5, showed the MLH3 UVs analyzed, one known MLH3 missense polymorphism (p.Ser845Gly), and the MLH3 frameshift mutation identified (see Table 1). All mutated MLH3 proteins were able to interact with WT-MLH1 and this interaction was comparable with WT-MLH3-WT-MLH1 interaction and with that of the known missense polymorphism. The data suggest that the UV-containing MLH3 proteins can bind to WT-MLH1 in vivo.

The control experiments also showed colonies for the combination of the two empty vectors and the combination of pAS2 (empty vector) with
pACT2-MLH3. The observed colonies, however, were white whereas the colonies observed for the WT-MLH1 with WT- or MUTANT-MLH3 were all blue, as expected. We also observed interaction between the truncating MLH3 mutation (p.Asn860IfsX13) and WT-MLH1.

**DISCUSSION**

Previously, nine MLH3 missense mutations and an MLH3 frameshift mutation in patients suspected of having Lynch syndrome were detected (Wu et al., 2001). To clarify a possible role for eight of these MLH3 UVs identified in suspected Lynch syndrome patients, we performed several biochemical tests.

Transfection of mutant and WT-MLH3 in an MLH3-deficient cell line, HEK293T, showed no difference in protein levels for the UVs tested. Our data also showed that the MLH3 protein is stable without a heterodimeric or other partner, corroborating previous findings (Cannavo et al., 2005). To repair mismatches in vivo, the MLH3 protein needs to be present in the nucleus. Recent localization experiments (Korhonen et al., 2007) suggested that endogenous MLH3 is mainly localized in the cytoplasm whereas MLH1 and PMS2 are localized in the nucleus in a human MMR-proficient cell line. It was also shown by Korhonen et al. (2007) that when MLH3 was transiently expressed in HCT116 (a human cell line deficient for MLH1 and PMS2), the MLH3 protein also localized in the cytoplasm. Only after cotransfection with MLH1, Korhonen et al. (2007) saw that MLH3 was partially transported into the nucleus. They conclude that nuclear localization of MLH3 is dependent on MLH1 and competitive with PMS2. Our data do not corroborate these findings as we observed with experiments performed in two independent labs that both WT and UV-containing MLH3 proteins were largely located in the nucleus and only partially in the cytoplasm, regardless of whether MLH3 was transfected alone or cotransfected with MLH1 and regardless of the cell line we used for transfection. When transfecting the vector expressing the truncated MLH3 protein (p.Asn860IfsX13) however, more cytoplasmatic staining can clearly be seen (see Fig. 4). The protein, however, is normally not expressed as the naturally occurring unprocessed mRNA containing such a frameshift mutation will be removed by the nonsense mediated RNA decay pathway.

**TABLE 2. In Silico Analyses of the Eight Tested MLH3 UVs**

<table>
<thead>
<tr>
<th>MLH3 UVs</th>
<th>Polarity change</th>
<th>Grantham scores</th>
<th>Splice defect prediction</th>
<th>EMBL prediction</th>
<th>Distance (0-215)</th>
<th>Deviation</th>
<th>Predictiona</th>
<th>Predictionb</th>
<th>Polarity by PolyPhen (score followed by prediction)</th>
<th>Polarity by SIFT (score followed by prediction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Gln24Glu (p.Q24E)</td>
<td>P!, AP</td>
<td>1.184; benign</td>
<td>0.00; tolerated</td>
<td>Class C0</td>
<td>Neutral</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Asn499Ser (p.N499S)</td>
<td>P!, P</td>
<td>1.056; possibly damaging</td>
<td>0.70; tolerated</td>
<td>Class C0</td>
<td>Neutral</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Glu624Gln (p.E624Q)</td>
<td>P!, AP</td>
<td>1.471; benign</td>
<td>0.05; affects protein function</td>
<td>Class C0</td>
<td>Favored</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Ser817Gly (p.S817G)</td>
<td>P!, NP</td>
<td>0.008; benign</td>
<td>0.48; tolerated</td>
<td>Class C0</td>
<td>Neutral</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Gly981Ser (p.G981S)</td>
<td>NP!, P</td>
<td>0.052; benign</td>
<td>1.00; tolerated</td>
<td>Class C0</td>
<td>Neutral</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Asn1007Ser (p.N1007S)</td>
<td>P!, P</td>
<td>0.185; benign</td>
<td>1.00; tolerated</td>
<td>Class C0</td>
<td>Neutral</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Ala1394Thr (p.A1394T)</td>
<td>NP!, P</td>
<td>1.413; benign</td>
<td>0.29; tolerated</td>
<td>Class C0</td>
<td>Neutral</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Glu1451Lys (p.E1451K)</td>
<td>AP!, P</td>
<td>1.267; benign</td>
<td>0.29; tolerated</td>
<td>Class C0</td>
<td>Neutral</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P, polar amino acid (AA); NP, non-polar AA; AP, acidic charged polar AA; BP, basic charged polar AA.
aThe classification is ordered from most likely to interfere with function (Class C65) to least likely to interfere with function (Class C0). bThere is low confidence in this prediction.
Figure 3. A: Expression of UV-containing and wildtype MLH3 in HEK293T cells. This Western blot, using an MLH3 specific antibody, shows no differences in MLH3 expression on comparing cells transfected with WT-MLH3 and those transfected with UV-containing MLH3. B: Expression of MLH3 in HEK293T cells. This Western blot, using a GFP antibody shows in all but one lane the expected band of 190 kDa. In lane 7 (extracts from cells transfected with p.Asn860fsX13-MLH3), a lower band is visible (126 kDa). Data shown are representative of three independent experiments. In this figure, an aspecific band of ~150 kDa is visible in all lanes.

Figure 4. Subcellular localization of CFP-MLH1 and YFP-MLH3, WT-MLH3, the UV-containing MLH3s, and one truncating mutation containing MLH3 in HeLa cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
To study whether the identified MLH3 UVs influence the interaction with MLH1 in vivo, we tested the MLH3 mutants in a yeast two-hybrid system. Yeast two-hybrid studies showed that all of the analyzed MLH3 mutants were able to interact with WT-MLH1 and that these interactions were comparable with a WT-MLH3-WT-MLH1 interaction. Surprisingly, we also observed interaction between the truncating MLH3 mutation (p.Asn860IfsX13) and WT-MLH1. A plausible explanation for this would be that a truncated MLH3 protein is produced, as shown in Figure 3B (see Results section, protein expression/stability studies). This truncated protein might still be able to interact with MLH1 to form a heterodimer with MLH1. Notably, the N-terminal MLH1 interaction domain was still present in the truncated form of MLH3, whereas the entire COOH-terminal MLH1 interaction domain was lacking (Fig. 1).

In conclusion, these biochemical assays do not show a change in the function of the mutated proteins we tested. Not finding a functional defect in these assays does, however, not preclude the MLH3 UVs being involved in Lynch syndrome. The assays described above...
investigate only specific biochemical aspects of MMR protein. It is conceivable that the UVs tested can cause a functional defect of the protein that was not detectable with the assays used in this study. We cannot therefore fully exclude these UVs from being pathogenic.

Besides data from biochemical assays, we also collected theoretical arguments that might help in determining the pathogenic nature of the MLH3 amino acid substitutions identified. We determined conservation in nine organisms and showed that seven of the amino acids that were mutated are highly conserved (Fig. 2). We also looked for polarity changes and saw again that several of the UVs give rise to substantial changes in polarity. However, when all of the data of the in silico analyses were analyzed we had to conclude that only based on Polyphen one of the eight UVs could be considered as possibly causative. The PolyPhen software combines structural, evolutionary and physicochemical properties. Notably, validation of the program showed a proper prediction in only 80% of known deleterious mutations, and thus false-positive or - negative findings can be expected (Sunyaev et al., 2000; Ng and Henikoff, 2002). However, the prediction results are in line with the biochemical assays described above, with the exception of pASN499Ser, which was predicted to be possibly damaging. Our in silico analysis on splicing showed that splicing abnormalities were not predicted for any of the UVs.

On the basis of the results from the biochemical and the theoretical prediction algorithms, we conclude that we have no convincing evidence that the MLH3 UVs tested are involved in the development of Lynch syndrome.

The above findings leave us with the question whether mutations in MLH3 can contribute to the development of Lynch syndrome? Our data do not support any involvement of the MLH3 UVs identified in Lynch syndrome. Our data are supporting a recent study on the MLH3 missense mutations p.Q24E, p.S817G, p.A1494T, and p.E1451K that were also biochemically evaluated in our study. Korhonen et al., (2008) show that these substitutions and three others (p.R647C, G933C, and W1276R) do not affect in vitro mismatch repair. Whether or not MLH3 UVs involvement in Lynch syndrome can be concluded for the two reported MLH3 frameshift mutations (Wu et al., 2001; Liu et al., 2003) remains unanswered. The fact that the tumors of these patients were MSI-low might be an argument against involvement in tumorigenesis. On the other hand, the finding of somatic mutations, knocking out the second allele in the tumor in three of the nine patients, argues in favor of their involvement (Wu et al., 2001). Importantly, these patients do not carry a germline mutation in one of the other important MMR genes (based on DNA sequence analysis and immunohistochemistry stainings) that could account for the second hit of MLH3. Furthermore, PMS2 screening was performed for the carriers of MLH3 missense mutations p.Gln24Glu or p.Ala1494Thr and PMS2 is not mutated in these patients. Finding MSI-L tumors in the MLH3 frameshift mutation carriers should also be no surprise since it was shown that only a small but significant (20%) repair of both G/T mismatches and +1 insertion/deletion loop substrates was observed when MMR-deficient HEK293T nuclear protein extracts were supplemented with high amounts of MUTLγ. This suggests that MUTLγ might play a backup role in human MMR (Cannavo et al., 2005). In addition, it might be that MLH3 is more involved in tetranucleotide instability as shown in Table 1. In the article of Wu et al., (2001) it has already been shown that the use of five most informative markers (3 dinucleotide and 2 tetranucleotide markers) show that 77% of the tumors of MLH3 missense mutation carriers show an MSI-H phenotype.

A low activity is also reflected in the presence of low amounts of endogenous MLH3 protein in human cell lines. Semiquantitative Western analysis of HeLa cells revealed endogenous MLH3 levels 60 times less abundant than PMS2 and 6 times less abundant than PMS1 (Cannavo et al., 2005). Finally, as previously shown by others (Cannavo et al., 2005), and now confirmed in this study, MLH3 is stable in the absence of MLH1. As the other proteins that are facultative partners of MMR components (PMS2, MSH6), are unstable in the absence of their partner, MLH3 seems to behave differently compared with the other MMR proteins which could be seen as an argument against involvement in MMR.

In conclusion, we analyzed the functional significance of eight MLH3 UVs by in silico analyses and biochemical assays. Our assays show that the MLH3 UVs are likely to be as functional as the wildtype MLH3 protein, suggesting that MLH3 is not a major player in Lynch syndrome. However, we cannot fully exclude a role for MLH3 as a modifier in tumorigenesis.
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