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The G67E mutation in *hMLH1* is associated with an unusual presentation of Lynch syndrome

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Germline mutations in the mismatch repair (MMR) genes are associated with Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC) syndrome. Here, we characterise a variant of *hMLH1* that confers a loss-of-function MMR phenotype. The mutation changes the highly conserved Gly67 residue to a glutamate (*G67E*) and is reminiscent of the *hMLH1-p.Gly67Arg* mutation, which is present in several Lynch syndrome cohorts. *hMLH1-Gly67Arg* has previously been shown to confer loss-of-function (Shimodaira *et al*, 1998), and two functional assays suggest that the hMLH1-Gly67Glu protein fails to sustain normal MMR functions. In the first assay, hMLH1-Gly67Glu abolishes the protein's ability to interfere with MMR in yeast. In the second assay, mutation of the analogous residue in yMLH1 (*yMLH1-Gly64Glu*) causes a loss-of-function mutator phenotype similar to *yMLH1-Gly64Arg*. Despite these molecular similarities, an unusual spectrum of tumours is associated with *hMLH1-Gly67Glu*, which is not typical of those associated with Lynch syndrome and differs from those found in families carrying the *hMLH1-Gly67Arg* allele. This suggests that hMLH1 may have different functions in certain tissues and/or that additional factors may modify the influence of *hMLH1* mutations in causing Lynch syndrome.

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Genetics and Genomics

Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC), is an autosomal dominant cancer predisposition disorder. Apart from colorectal cancers, affected individuals are also predisposed to an array or other cancers, such as endometrial cancer, transitional cell carcinomas of the upper urothelial tract, carcinoma of the stomach, hepatobiliary and small intestinal cancers (reviewed in Lynch and de la Chapelle, 2003). Sets of criteria that trigger investigation of the possibility of a Lynch syndrome diagnosis include the revised Bethesda and the Amsterdam I and II criteria, which consider the combinations of tumours in individuals and families, the age of tumour onset and pathological features of tumours (Vasen *et al*, 1999; Umar *et al*, 2004).

In the 1990s, a number of studies linked Lynch syndrome to mutations in the mismatch repair (MMR) genes (Fishel *et al*, 1993; Bronner *et al*, 1994; Nicolaides *et al*, 1994; Papadopoulos *et al*, 1994; Li, 2003). The MMR proteins prevent the accumulation of DNA replication errors by detecting and effecting the removal of base – base mismatches as well as small insertion-deletion loops from the newly synthesised strand. Tissue samples from tumours and human cell lines in which MMR has been inactivated show variation in the length of repetitive DNA sequences (microsatellites), a phenomenon known as microsatellite instability (MSI). These include mononucleotide repeats of adenine, which are often mutated in MMR-defective cells, and are present in DNA damage repair genes *MRE11*, *RAD50* and *BRCA1*, checkpoint component *CHK1* (Yoshikawa *et al*, 2000; Menoyo *et al*, 2001; Giannini *et al*, 2002; Kim *et al*, 2007) as well as *TGFβ-R2*, *PTEN*, *APC* and *MBD4* (Markowitz *et al*, 1995; Huang *et al*, 1996; Kong *et al*, 1997; Tashiro *et al*, 1997; Riccio *et al*, 1999).

Components of the MMR pathway are highly conserved from bacteria to human (Modrich, 2006). In eukaryotes, heterodimers of MSH2-MSH6 (MutSα) and MSH2-MSH3 (MutSβ) detect mismatches and insertion – deletion loops and form complexes with heterodimers of MLH1-PMS2 (MutLα), and to a lesser extent with MLH1-MLH3 (MutLβ). hPMS2 contains endonuclease activity that can initiate the removal of the newly synthesised strand, which contains the incorrectly inserted base (Kadyrov *et al*, 2006). *In vitro* reconstitution of MMR shows that MutLα is required for 3′-directed excision of the mismatch, in addition to factors such as PCNA, RPA and EXOI (Dzantiev *et al*, 2004).

More than 450 germline abnormalities of MMR genes have been described (InSiGHT, http://www.insight-group.org). Whether a specific missense variant in *hMSH2* or *hMLH1* confers pathogenicity can be difficult to determine, as it requires the evaluation

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of multiple parameters, including statistical analyses of the segregation of the variant with the phenotype and functional assessment of the variant protein. Segregation of the variant within a family may provide some evidence, but this could in theory be caused by another closely linked mutation. Small family size, incomplete penetrance of mutations and lack of confirmatory records on tumours from older generations can often limit the power of such analyses. Collation of data on variants from different families is helpful, but is not available if variants are rare or novel.

There are several functional tests available to assess variant protein activity, although they each have their limitations. Mismatch repair-deficient tumour-derived cell lines can be transfected with plasmid containing variant *MLH1* genes and assessed for mutator phenotype and/or drug-resistance phenotypes (Brieger *et al*, 2002; Trojan *et al*, 2002; Blasi *et al*, 2006). For C-terminal mutations in hMLH1 and hPMS2, testing the variant proteins for interactions with their partner can be informative (Nystrom-Lahti *et al*, 2002). There are also *in vitro* tests for MMR activity, which rely upon the complementation of MMR-deficient extracts by the variant protein. However, these assays require expression and purification of recombinant MMR proteins (Nystrom-Lahti *et al*, 2002; Heinen *et al*, 2002), and although MMR repair *in vitro* may be deemed normal when sufficient quantities of the variant protein is used, some variants are poorly expressed, suggesting that the cellular levels of the variant may be lower (Kariola *et al*, 2002). All of these assays require tissue culture facilities and/or recombinant protein expression, significant skill, and can be costly. To overcome these limitations, several yeast and bacteria-based systems for the functional analysis of variant MMR proteins have been developed. In both yeast and bacteria, hMLH1 interferes with normal, endogenous yMMR/bMMR, resulting in enhanced mutation rates (Shimodaira *et al*, 1998; Quaresima *et al*, 2003). Several mutations in *hMLH1* abolish the capacity of the *hMLH1*-variant to interfere with yMMR/bMMR, thus restoring normal mutation rates (Shimodaira *et al*, 1998; Quaresima *et al*, 2003; Takahashi *et al*, 2007). Generally, *in vitro* assays concur with findings in such studies (Takahashi *et al*, 2007). Furthermore, there are alternative/complementary strategies for assessing *hMLH1* function in yeast. The N-terminus of MLH1 contains the highly conserved ATPase domain, allowing analogous variant *hMLH1* mutations to be introduced into *yMLH1* (Shcherbakova and Kunkel, 1999; Hoffmann *et al*, 2003). As this method is restricted to evolutionarily conserved N-terminus residues, hybrid mammal–yeast *MLH1* have been generated to assess MMR function of *hMLH1* variants in budding yeast (Polaczek *et al*, 1998).

Here, we describe the functional analysis of a variant hMLH1 protein following our description of a Lynch syndrome kindred with a unique collection of tumours rarely seen in this condition. Functional analysis of hMLH1 suggests that the mutation of p.Gly67Glu perturbs the protein's ability to prevent the accumulation of mutations. This loss-of-function is similar to a previously analysed allele, *hMLH1-Gly67Arg*, which has been described in Lynch syndrome families. Our observations suggest that the loss-of-function of hMLH1 is not sufficient to predict the type of tumours. We suggest that other genetic modifiers may be present to explain the phenotype and/or that the function of hMLH1 may differ from one cell type to another.

MATERIALS AND METHODS

Plasmids pRS315

Plasmids pRS315 containing *hMLH1* and *hMLH1-G67R* were kindly provided by Dr Shimodaira. The hMLH1-G67E and yMLH1-G64R mutations were generated using the Quick Change Site-directed Mutagenesis kit from Stratagene (La Jolla, CA, USA) using primers hMLHG67E for 5′-AGGGAGGCCTGAA GTTGATTCAGATCCAAGACAATGGCA CCGAGATCAGG-3′ and hMLHG67Erev for 5′-GGGGTTTGCTCAGAGGCTGCAGAAATGCA TCAAGC-3′. Plasmids were introduced into the haploid yeast strain Y39 (Y55 background, *MAT***a**, *his4-R1, leu2-R1, ade1-1, lys2*::*InsE-A*$_{14}$ *CAN1, ura3-N*). Plasmid yMLH1-G64E was generated as above using plasmid yMLH1-G64R as a template and primers yMLHG64E for 5′-CAAATAACAGATAACGGATCTGAAATTAATAAAGCAGA CCTGCCA-3′ and yMLHG64Erev for 5′-TGGCAGGTCTGCTTTA TTAATTTCAGATCCGTTATCTGTTATTTG-3′. The yMLH1-G64E mutation was integrated into the yeast genome by two-step gene replacement, as described previously (Hoffmann *et al*, 2003).

Yeast media

Yeast media were generated and used according to (Hoffmann *et al*, 2003).

Fluctuation tests

Fluctuation tests were carried out using the method of the median (Lea and Coulson, 1949; Reenan and Kolodner, 1992). We assessed 11 colonies from three independent transformants for each mutation.

Immunoblotting

Total protein was extracted from the transformants expressing the hMLH1variants. A total of 3.5×10^7 cells were disrupted by glass beads in 200 μl 20% trichloroacetic acid (TCA) in a FastPrep cell disrupter (Thermo Savant, Breda, Netherlands). The glass beads were removed and the precipitated protein was resuspended in 100 μl sample loading buffer (250 mM Tris-HCl, pH 8.0, 5% glycerol, 0.4% SDS, 1% β-mercaptoethanol, 0.02% Bromophenol Blue). Equal amounts of protein, as determined by the Bradford assay, were separated by SDS polyacrylamide gel electrophoresis (6%). Proteins were electrophoretically transferred onto a nitrocellulose membrane (Amersham Biosciences, Amersham, UK), which was probed first with a mouse hMLH1 antibody (1 : 500, G168-15, BD Biosciences, Erembodegem, Belgium), and secondly with a horseradish peroxidase-conjugated antibody raised against mouse IgG (1 : 3000, Dako, Glostrup, Denmark). Expressed protein was detected by chemiluminescence (Western Lightning Chemiluminescence Reagent, PerkinElmer, Waltham, MA, USA).

RESULTS

Genetic analysis and tumour spectrum of the *hMLH1-p. Gly76Glu* variant: a case study

A Caucasian male was seen in clinic with a history of male breast cancer and leiomyosarcoma of the thigh (presenting concurrently in his third decade), colon cancer (presented in his fourth decade) and prostate cancer (presented in his fifth decade). There were colorectal cancers with the age of onset in the fourth decade in his paternal grandfather and two paternal uncles. This family history is consistent with the revised Amsterdam criteria for the clinical diagnosis of Lynch syndrome (Vasen *et al*, 1999). His father had oesophageal cancer at the age of 47 years. Other unusual tumours in carriers of the variant included cervical adenosquamous carcinoma, oligodendroglioma and prostate cancer.

Initial genetic screening for mutations (by full sequencing) in the *TP53* gene responsible for the Li-Fraumeni syndrome was negative. The breast cancer susceptibility genes *BRCA1* and *BRCA2* also did not harbour any mutations as screened by dHPLC (denaturing high-performance liquid chromatography) and MLPA (multiplex ligation-dependent probe amplification).

Several of the tumours in this family including the proband's sarcoma showed microsatellite instability. Molecular analysis of

the proband's germline DNA identified a missense variant in the *hMLH1* gene in exon 2 at nucleotide position 200 (c.200 G>A). This results in an amino-acid substitution from glycine to glutamic acid (Gly67Glu/G67E). The Gly67Glu mutation segregates with cancer predisposition across the pedigree.

Expression of hMLH1-G67E does not interfere with yeast MMR

To determine whether hMLH1-p.Gly67Glu (denoted hMLH1-G67E henceforth) is functional in MMR, we used two different approaches.

We took the advantage of the findings that the expression of functional human MLH1 (hMLH1) interferes with normal MMR in the budding yeast, whereas several point mutants associated with Lynch syndrome fail to do so. For example, hMLH1-p.Gly67Arg (G67R), known to occur in HNPCC families, shows reduced ability to interfere with yeast MMR in this assay (Figure 1; Shimodaira *et al*, 1998). We transformed budding yeast (Y39) with vector only (pRS315), vector containing *hMLH1*, *hMLH1-G67R* or *hMLH1-G67E* and assessed the forward mutation rate of *CAN1* as well as the reversion rate of *lys2*::*InsE-A*$_{14}$. *CAN1* encodes an arginine permease that renders cells sensitive to the arginine analogue, canavanine. Mutations in *CAN1* include base substitutions as well as insertions and deletions, which cause cells to become resistant to canavanine. The *lys2*::*InsE-A*$_{14}$ contains a homopolymeric run of 14 adenines, which causes a +1 frameshift mutation in *LYS2* (Tran *et al*, 1997). Reversion reconstitutes *LYS2*, an α-amino-adipate reductase that allows cells to synthesise lysine. Qualitative assessment of four independent transformants shows that the 'vector only' has no discernible effect on mutation rates of *lys2*::*InsE-A*$_{14}$ (Figure 1A). In contrast, introduction of 'vector + *hMLH1*' causes an increase in the mutation rate of *lys2*::*InsE-A*$_{14}$, consistent with aberrant yMMR. Cells transformed with the previously characterized mutation, *hMLH1-G67R*, do not display an increased mutator phenotype, although some of the transformants do show a stochastic increase (data not shown). *hMLH1-G67E* behaved very similarly to *hMLH1-G67R* in that the majority of transformants did not show a mutator phenotype (Figure 1A and data not shown). These observations show that *hMLH1-G67E* displays a strongly reduced capacity for interfering with normal MMR function in budding yeast. We also quantified the mutation rates using the method of the median (see Materials and Methods) for both *CAN1* and *lys2*::*InsE-A*$_{14}$. Introduction of *hMLH1* causes a 18 700-fold and 5.1-fold increase in the mutation rate estimates of *lys2*::*InsE-A*$_{14}$ and *CAN1*, respectively, whereas *hMLH1-G67R* did not enhance the mutation rates (1.2-fold for *lys2*::*InsE-A*$_{14}$ and 0.6-fold for *CAN1* change in mutation rates as compared with 'vector only'; Table 1). *hMLH1-G67E* shows a similar phenotype to *hMLH1-G67R*, with 1.9-fold and 1.1-fold difference compared with 'vector only' (Table 1), suggesting that *hMLH1-G67E* has a similar loss-of-function phenotype as *hMLH1-G67R* in this assay.

The hMLH1 and hMLH1-G67R proteins are expressed stably in yeast cells (Shimodaira *et al*, 1998). To determine whether this is also the case for hMLH1-G67E, we carried out western blot analyses on the four transformants (Figure 1B). Protein extracts from cells containing hMLH1, hMLH1-G67R and the four independent transformants of hMLH1-G67E, but not vector only, showed the diagnostic 80 kD band. Thus, hMLH1-G67E is stably expressed in budding yeast cells, but shows reduced ability to interfere with normal MMR functions.

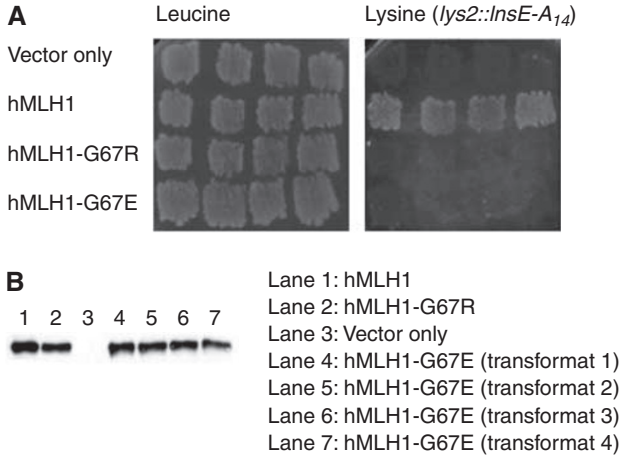

Figure 1 (**A**) Yeast cells were transformed with vector only (pRS315), vector containing *hMLH1*, *hMLH1-G67R* or *hMLH1-G67E* and assessed the forward mutation rate of *CAN1* as well as the reversion rate of *lys2*::*InsE-A*$_{14}$. The hMLH1-G67E mutant shows reduced ability to interfere with yeast MMR similar to the hMLH1-G67R mutation. (**B**) Western blot analyses of the four separate transformants of hMLH1-G67E. Yeast cells stably express wild-type hMLH1 protein as well as the hMLH1-G67R and the hMLH1-G67E mutants.

yMLH1-G64R confers increased mutation rates

The second yeast assay we employed is based on the observation that eukaryotic *MLH1* homologues are highly conserved in the N-terminal ATPase domain. Several Lynch syndrome mutations, including *hMLH1-G67R*, confer a loss-of-function phenotype for mutator activity when introduced into the analogous residue in yeast MLH1 (*yMLH1-G64R*; Shcherbakova and Kunkel, 1999). We introduced the G67E change directly into the budding yeast locus (*yMLH1-G64E*) and assessed the mutation rates (Table 2). Deletion of y*MLH1* causes a 30-fold and 19 000-fold increase in the mutation rates for *CAN1* and *lys2*::*InsE-A*$_{14}$, respectively, compared with the wild-type strain. The *yMLH1-G64R* mutant protein confers an intermediate phenotype, but nevertheless shows highly elevated mutation rates. The *yMLH1-G64E* mutation is similar to *yMLh1-G64R*, suggesting that changing the G64 residue in yMLH1 to either a positively charged arginine or a negatively charged glutamate confers similar inhibition of protein activity during MMR.

DISCUSSION

Characterising MMR gene alterations in Lynch syndrome families is important in clarifying risk and advising on management issues,

Table 1 Human *hMLH1-G67E* mutant does not interfere with yeast MMR

	Rate[a]	lys2::InsE-A$_{14}$ ± s.e.m.	Fold difference	Rate	CAN1 ± s.e.m.	Fold difference
Vector only	1.2×10^{-7}	1.1×10^{-8}	1.0	1.5×10^{-6}	1.3×10^{-7}	1.0
hMLH1	2.3×10^{-3}	1.4×10^{-3}	18,700	7.7×10^{-6}	1.2×10^{-6}	5.1
G67R	1.5×10^{-7}	4.0×10^{-8}	1.2	8.6×10^{-7}	3.4×10^{-7}	0.6
G67E	2.4×10^{-7}	1.3×10^{-7}	1.9	1.7×10^{-6}	4.9×10^{-7}	1.1

MMR = mismatch repair. Mutation rates are assessed in yeast strains expressing the various forms of human MLH1 proteins. Only wild-type hMLH1 was capable of interfering with the endogenous yeast MMR to increase mutation rates. [a]Mutation rate estimated as mutation/cell/generation.

Table 2 Mutation rates for the yeast *yMLH1-G64E* mutant are elevated

	Rate[a]	lys2::InsE-A_{14} ± s.e.m.	Fold difference	Rate	CAN1 ± s.e.m.	Fold difference
WT	1.0×10^{-7}	1.3×10^{-8}	1.0	4.2×10^{-7}	8.0×10^{-8}	1.0
mlh1Δ	1.9×10^{-3}	5.8×10^{-4}	19,000	1.3×10^{-5}	1.2×10^{-6}	30
mlh1-G64R	8.7×10^{-4}	1.9×10^{-4}	8,300	1.7×10^{-6}	7.1×10^{-7}	3.9
mlh1-G64E	4.4×10^{-4}	2.7×10^{-4}	4,200	3.7×10^{-6}	2.9×10^{-6}	8.8

WT = wild type. WT yeast cells are compared with yeast cells deleted for *mlh1* or expressing mutant versions of yeast *mlh1*. Deletion of *mlh1* greatly elevated mutation rates as expected. The single-point mutation of ymlh1 of *G64R* or *G64E* also increased the mutation rates significantly. [a]Mutation rate estimated as mutation/cell/generation.

such as cancer surveillance, treatment and prevention. Detailed study of a family with a tumour spectrum satisfying the Amsterdam criteria for Lynch syndrome has allowed us to characterise a variant in *hMLH1*. Interestingly, in addition to classic tumours, this family also exhibits a collection of atypical tumours. Functional analysis of this variant shows that it confers a loss-of-function mutator phenotype.

The *p.Gly67Glu (p.Gly64Glu* in yeast) mutation alters an amino-acid residue in the highly conserved N-terminus of hMLH1 (Ban and Yang, 1998). By analogy to the crystal structure of bacterial MutL, G67 is positioned on a loop, which is part of the ATP-binding pocket (Ban and Yang, 1998; Ban *et al*, 1999). ATP-binding induces conformational changes in the N-terminus that allows MutL monomers to dimerise (Ban *et al*, 1999), thus facilitating the interaction between hMLH1 and hPMS2. Moreover, cycles of ATP-binding and hydrolysis have been suggested to regulate MutLα activity (Tran and Liskay, 2000). Structure predictions would suggest that the *hMLH1-p.Gly67Glu* substitution alters the local conformation of the hMLH1 protein, analogous to that predicted for *hMLH1-pGly67Arg* (Ban and Yang, 1998). We tested this directly by comparing the G67E/G64E mutation to a previously characterised mutant in the same residue (G67R/G64R) in two functional assays. It is clear that expressing hMLH1-G67E does not appear to interfere with yeast MMR, which is not the case when normal hMLH1 is present. Furthermore, when the analogous substitution is made in the yeast protein, yMLH1-G64E, this protein does not sustain MMR fully and is as compromised as yMLH1-G64R. Thus, we conclude that *hMLH1-G67E* is a loss-of-function allele, similar to *hMLH1-G67R*.

We have identified that *hMLH1-G67E* segregates with disease in the family reported and is associated with atypical cancers. This is in sharp contrast to reported kindreds harbouring the *hMLH1-G67R* mutation who only showed classical elements of Lynch syndrome (Herfarth *et al*, 1997; Heinimann *et al*, 1999; Plevová *et al*, 2004). Furthermore, the *hMLH1-G67E* mutation has not been reported in 1688 individuals studied in the general population (Barnetson *et al*, 2008), consistent with the mutation being pathogenic. Our observations suggest that alleles that modify the effect of the G67E mutation (Scott, 2008) may be present in this family to explain the unusual spectrum. Alternatively, there may be functions of hMLH1 that are not assessed simply by determining whether the resulting change causes a mutator phenotype.

Because of the rarity of tumours in our Lynch syndrome family, this poses significant clinical management issues regarding recommendations on screening and treatment. Identification of more families with this pathogenic mutation could shed light on the mechanism for carcinogenesis in our kindred.

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Conflict of interest

We declare the authors do not have any competing interests.
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