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

Germline mutations in the mismatch repair (MMR) genes are associated with Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPPC) 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*-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.

Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPPC), is an autosomal dominant cancer predisposition disorder. Apart from colorectal cancers, affected individuals are also predisposed to an array of 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 intestinal cancers (reviewed in Lynch and de la Chapelle, 2003).

In vitro reconstitution of MMR shows that MutLα-directed excision of the mismatch, in addition to factors such as PCNA, RPA and EXO1 (Dzantiev et al, 2004). Components of the MMR pathway are highly conserved from bacteria to human (Modrich, 2006). In eukaryotes, heterodimers of MSH2-MSH6 (MutSζ) and MSH2-MSH3 (MutSβ) detect mismatches and insertion–deletion loops and form complexes with heterodimers of MLH1-PM2 (MutLζ), 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).
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 plasmids 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-Lahit 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-Lahit 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.Gly67Glut perturbs the protein’s ability to prevent the accumulation of mutations. This loss-of-function is similar to a previously described variant (Shcherbakova and Kunkel, 1999). 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 yMLH1G67E for 5'-AGGGAGGCGCTGAA and hMLH1G67Erev for 5'-CAAATAACAGATAACGGATCTGAAATTAATAAAGCAGA-3' mutation in hMLH1

GTGTATTCAGATCAGAAGACATGGCA CGGAGATCCGG-3' and hMLH1G67Erev for 5'-GGGGTTTGCTCAGAGGCTGCAGAAATGCA TCAAAGC-3'. Plasmids were introduced into the haploid yeast strain Y39 (Y55 background, MATa, his4-R1, leu2-R1, ade1-1, lys2::InhE-A3, can1, ura3-N). Plasmid yMLH1-G64E was generated as above using plasmid yMLH1-G64R as a template and primers yMLH1G64E for 5'-CAAAATACAGATAACGGATCTGAAATTAATAAAGCAGA CTCGCCA-3' and yMLH1G64Erev for 5'-TGGCAGGTCTGCTTTTA TTTACCATGCGTTCGTTATTTGGG-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 × 10⁷ 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 goat anti-mouse antibody conjugated to horseradish peroxidase (Amersham Biosciences, Amersham, UK), which was probed with a goat anti-mouse antibody conjugated to horseradish peroxidase (Amersham Biosciences, Amersham, UK), which was probed with a mouse anti-β-actin antibody (1:1000, clone A5441, Sigma, St Louis, USA). The membranes were washed and the blots were developed with an enhanced 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
expression of four independent transformants shows that the 'vector only' has no discernible effect on mutation rates of lys2::InsE-A14 (Figure 1A). In contrast, introduction of 'vector + hMLH1' causes an increase in the mutation rate of lys2::InsE-A14, 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-A14. Introduction of hMLH1 causes a 18 700-fold and 5.1-fold increase in the mutation rate estimates of lys2::InsE-A14 and CAN1, respectively, whereas hMLH1-G67R did not enhance the mutation rates (1.2-fold for lys2::InsE-A14 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.

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 yMLH1 causes a 30-fold and 19 000-fold increase in the mutation rates for CAN1 and lys2::InsE-A14, 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,
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). 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 pMS2. Moreover, cytos of ATP-binding and hydrolysis have been suggested to regulate MutLz 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-p.Gly67Arg (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-G64R.

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

|        | Rate | lys2::InsE-Au ± 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             |
| mhi1   | 1.9×10⁻⁴ | 5.8×10⁻⁴              | 19,000          | 1.3×10⁻⁵ | 1.2×10⁻⁶      | 30              |
| mhi1-G64R | 8.7×10⁻⁸ | 1.9×10⁻⁴              | 8,300           | 1.7×10⁻⁵ | 7.1×10⁻⁷      | 39              |
| mhi1-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 mhi1 or expressing mutant versions of yeast mhi1. Deletion of mhi1 greatly elevated mutation rates as expected. The single-point mutation of yMLH1 of G64R or G64E also increased the mutation rates significantly. Mutation rate estimated as mutation/cell/generation.
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