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Functional characterization of human MutY homolog (hMYH) missense mutation (R231L) that is linked with hMYH-associated polyposis

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Abstract

The MutY homolog (MYH) can excise adenines misincorporated opposite to guanines or 7, 8-dihydro-8-oxo-guanines (8-oxoG) during DNA replication; thereby preventing G:C to T:A transversions. Germline mutations in the human *MYH* gene are associated with recessive inheritance of colorectal adenomatous polyposis (MAP). Here, we characterize one newly identified MAP-associated *MYH* missense mutation (R231L) that lies adjacent to the putative hMSH6 binding domain. The R231L mutant protein has severe defects in A/GO binding and in adenine glycosylase activities. The mutant fails to complement *mutY*-deficiency in *Escherichia coli*, but does not affect binding to hMSH6. These data support the role of the hMYH pathway in carcinogenesis.

Keywords

DNA repair; MYH mutation; colorectal cancer; genome stability

1. Introduction

There are three common forms of hereditary colorectal cancer: non-polyposis colorectal cancer (HNPCC), familial adenomatous polyposis (FAP), and MYH-associated polyposis (MAP). HNPCC is associated with mutations in the long-patch mismatch repair genes (reviewed in [1–3]). FAP is associated with germline mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene (reviewed in [4]). Biallelic germline mutations in the human *MYH* gene are associated with recessive inheritance of multiple colorectal adenomas and carcinoma [5–9]. MAP is similar to, but slightly differs from, familial adenomatous polyposis coli (FAP) in its mode of transmission, later age of onset, less florid form of polyposis, and fewer extra

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colonic manifestations [10]. MYH mutations can cause G:C to T:A mutations of the adenomatous polyposis coli (*APC*), *K-ras*, and other genes that control cellular proliferation in the colon [5,11,12]. The mutations in *APC* gene found in MAP occur at hot spots containing GAA sequences [5].

G:C to T:A transversions are a characteristic result of failure to repair 7,8-dihydro-8-oxo-guanine (8-oxoG or GO), one of the most stable products of oxidative DNA damage, and has the significant deleterious effects. The eukaryotic GO defending system contains four major players [13]. The *Escherichia coli* MutT homolog (MTH) hydrolyses 8-oxoG-dGTP into 8-oxo-dGMP and therefore prevents 8-oxo-dGTP from incorporating into DNA [14–16]. OGG1, a functional homolog of *E. coli* MutM, can remove both ring-opened purine lesions and 8-oxoG when they are paired with cytosines [17–19]. If C/GO is not repaired by OGG1, adenines are frequently incorporated opposite GO during DNA replication [20,21]. The MSH2/MSH6 (MutS α)-dependent mismatch repair removes the mismatched A on the daughter DNA strand [22,23]. The *Escherichia coli* MutY homolog (MYH) is an adenine glycosylase that removes adenines paired with GO [24–28]. MYH can also excise adenines misincorporated opposite G or C, remove 2-hydroxyadenines misincorporated with template A, G, or GO, and weakly excise G from a G/GO mismatch [24,26,27,29–31]. We have shown that hMYH physically interacts with MutS α and that hMYH glycosylase activity can be stimulated by hMutS α [32]. If the A/GO mismatches are not repaired by MYH or MutS α , G:C to T:A transversions will form after the next round of DNA replication [21,33–35].

Screening for the *hMYH* mutation in FAP-like patients without inherited *APC* mutation has shown that biallelic mutations in the *hMYH* gene account for approximately 25% of such cases [7–9,36–39]. More than 20 mutations in the *hMYH* gene have been identified in MAP patients to date [10,13]. The two most common *hMYH* mutations, which account for approximately 80% of MAP in Caucasians, are Y165C and G382D. The effects of Y165C and G382D mutations and two additional variants of hMYH (R227W and V232F) associated with MAP have been characterized biochemically [5,40–42]. Y165C, G382D and R227W of hMYH expressed in *E. coli* are defective in adenine glycosylase activity on A/GO mismatches. Moreover, expression of these three hMYH mutants in *E. coli* is unable to complement the mutator phenotype of a *mutY* mutation. hMYH(V232F) has reduced enzyme activity both *in vitro* and *in vivo*. These studies indicate that the high frequency of somatic G:C to T:A transversions in tumor tissues of MAP patients is caused by a reduced hMYH base excision repair of A/GO mismatches.

In this study, we characterized the functional effect of another pathogenic missense mutation of hMYH(R231L). This mutation is located adjacent to the putative hMSH6 binding domain [32], but it did not affect the hMYH binding to hMSH2/hMSH6 complex. hMYH(R231L) had no detectable DNA binding and glycosylase activities towards A/GO mismatches, and is unable to complement the *mutY*-deficiency in *E. coli*. These results provide additional evidence for the linkage between the deficiency of hMYH base excision repair pathway and MAP.

2. Materials and methods

2.1. hMYH missense mutation

A homozygous 692G→T mutation (i.e. R231L) in the *hMYH* gene (GenBank Accession number BC003178) was identified in a 57 year-old man of Italian ancestry with hundreds of adenomatous polyps.

2.2. *E. coli* strains

E. coli mutY-mutant strain PR70 (Su- *lacZ* X74 *galU galK* Sm^r *micA68::Tn10*Kan) was obtained from M. S. Fox. The strain CC104 [43] and its derivative CC104 *mutM::mini-kan mutY::mini-Tn10* were from J. H. Miller. DE3 lysogens were constructed according to the procedures described by Invitrogen.

2.3. Expression of hMYH(R231L) mutant

Plasmids pGEV-hMYH, pGEX-4T-hMYH, and pKK-hMYH have been previously described [40]. pGEV-hMYH(R231L), pGEX-4T-hMYH(R231L), and pKK-R231L were derived from pGEV-hMYH, pGEX-4T-hMYH and pKK-hMYH, respectively, by the QuickChange mutagenesis method that is described in the manufacturer's instructions (Stratagene). The mutagenesis employed two complementary oligonucleotides, Chang519 (sense strand) (5'-GCACGGGTGCTGTGCCTCGTGCGAGCCATTGGTGCT) and Chang520 (antisense strand) (5'-CGTGCCCCACGACACGGAGCACGCTCGGTAACCACGA). The underlined sequences represent the mutated 692G→T for R231L and a silent 693T→C to generate a BssSI site. The mutated hMYH open reading frame was first screened by BssSI cleavage and then by sequencing.

2.4. Protein expression, purification and Western blot analysis

Mutant and wild-type GB1-hMYH were expressed and partially purified as described previously [40]. The concentrations of partially purified GB1-hMYH were determined by comparing with known amounts of GB1-fused *E. coli* MutY in a Western blot analysis. The antibody used to detect the GB1 tag was anti-annexin-2 (BD Bioscience).

2.5. GST-hMYH pull-down assay

GST-hMYH fusion proteins were immobilized on glutathione-sepharose 4B (GE Health). A coomassie-stained gel was used to estimate the concentrations of wild-type and mutant GST-hMYH associated with the beads. Equal amounts of the wild-type and the mutant GST-hMYH on the beads were incubated with purified hMSH2/hMSH6 (hMutSα) complex as described [32]. The hMutSα in the pellet was fractionated by a 10% SDS-polyacrylamide gel followed by Western blot analysis with antibodies against hMSH2 (BD biosciences).

2.6. Assay of glycosylase and DNA binding activities of hMYH

The DNA substrate used for the glycosylase assay was an A/GO-containing 44-base-pair duplex oligonucleotide:

5' AATTGGGCTCCTCGAGGAATTAGCCTTCTGCAGGCATGCCCCGG 3'

3' TTAACCCGAGGAGCTCCTTAAOCGGAAGACGTCCTGACGGGGCC 5'

(where O = GO). The DNA substrate used in the gel shift assay was an A/GO-containing 20-base-pair, instead of a 44-base-pair duplex, to reduce non-specific binding to DNA:

5' CCGAGGAATTAGCCTTCTG 3'

3' GGCTCCTTAAOCGGAAGAC 5'.

The 5' end of the top strand was labeled with [γ -³²P]ATP as described [44]. The Glycosylase reaction was carried out in a 10 μ l volume containing 1.8 fmol DNA substrate, 25 mM HEPES (pH 7.0), 5 mM EDTA, 1.5% glycerol, 50 μ M ZnCl₂, and 0.02–0.1 μ M of partially purified hMYH. The reaction mixture was incubated at 37°C for 1.5 hr and then 2 μ l of 1 M NaOH was added for additional incubation at 90°C for another 30 min. The reaction was supplemented

with 5 μ l formamide dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) and 8 μ l of the mixture was loaded onto a 14% polyacrylamide gel containing 7 M urea. The DNA binding of hMYH was carried out in a 20 μ l reaction containing 1.8 fmol DNA substrate, 10 mM Tris-HCl (pH 7.6), 0.5 mM DTT, 0.5 mM EDTA, 1.5% glycerol, 20 ng poly(dI-dC) and 5–50 nM of partially purified hMYH. The reaction was carried out at 37°C for 30 min before adding 2 μ l of 50% glycerol and loading onto a 6% polyacrylamide gel in 50 mM Tris borate (pH 8.3), 1 mM EDTA and 2.5% glycerol. The gel image was detected by PhosphorImager (GE Health).

2.7. Measurement of mutation frequency

The mutation frequencies of *E. coli* strains CC104 [43], CC104*mutMmutY*, and CC104*mutMmutY* containing plasmids pKK223-3, pKK-hMYH, and pKK-R231L were compared. Four independent colonies of each strain were grown in LB medium to OD₆₀₀ of 0.6, induced by adding IPTG to final concentration of 0.4 mM, and then incubated for an additional 16 hrs at 20°C. The total viable cell numbers and rifampicin-resistant (Rif^R) cell numbers were determined by plating the cell cultures on LB agar (10⁻⁶ dilution) and LB agar containing 0.1 mg/ml rifampicin, respectively. The mutation frequency was calculated as a ratio of Rif^R cells to the total viable cells. The averages and standard deviations were obtained from a minimum of three replicates.

3. Results

3.1. Clinical summary

A 57 year-old male patient of Italian ancestry presented symptoms of abdominal pain and rectal bleeding. At colonoscopy, the patient had adenomatous polyposis. He underwent a colectomy and had hundreds of adenomatous and hyperplastic polyps documented throughout the resected colon. The polyps were more numerous and larger in the proximal versus the distal colon. There was no family history of colorectal pathology, but limited information was available. DNA sequencing and deletion studies of the *APC* gene in lymphocyte DNA did not reveal a germline pathogenic mutation. A selected polyp did not exhibit micro-satellite instability, and had normal expression of three DNA mismatch repair genes (*MLH1*, *MSH2*, and *MSH6*) in both polyp and adjacent normal tissue. Thus, a diagnosis of hereditary non-polyposis colorectal cancer (HNPCC) or familial adenomatous polyposis (FAP) was unlikely. A partial sequencing screen of the *APC* gene in the DNA isolated from the selected polyp revealed the presence of multiple transversion mutations predicting missense amino acid substitutions, and one G:C to T:A transversion resulting in a premature stop at codon 1344 (*APC* p.Ser1344X). These mutations are suggestive of a mutator phenotype causing somatic mutation in the *APC* gene, and are consistent with the type of mutations often found in MYH-associated polyposis (MAP) patients [5,10]. Consequently, we then considered that the patient's disease may be due to MAP. Sequencing of the *hMYH* gene in lymphocyte DNA demonstrated that the patient was homozygous for a 692G→T mutation, which causes a R231L mutation. There were no other variants noted in the *hMYH* gene. Because R231L has not been reported in MAP patients to date, we then assessed the effects of such a mutation. We expressed recombinant hMYH (R231L) protein in pGEX-4T-2 for interaction with hMutS α , in pGEV1 for enzyme assays, and in pKK223-3 for *in vivo* complementation with the *E. coli mutYmutM* mutator phenotype.

3.2. hMYH (R231L) interacts with hMutS α

Because the R231L mutation of hMYH is located next to the hMSH6 binding region (residues 232–254) [32], we examined whether the mutant protein could still interact with hMSH2/hMSH6 (hMutS α) by GST pull-down assay. As shown in Fig. 1, equal amounts of GST-hMYH (wild-type) and GST-hMYH(R231L) immobilized on glutathione sepharose beads could pull

down similar amounts of hMutS α . Therefore, hMYH(R231L) is not defective in the interaction with hMSH2/hMSH6 (hMutS α).

3.3. Impaired glycosylase and binding activities of the hMYH(R231L) protein

To improve hMYH protein solubility, we cloned the mutated gene in pGEV1 to express a fusion protein with streptococcal protein G (GB1 domain) at its N-terminus and 6-His at its C-terminus. Plasmid pGEV-hMYH(R231L) was expressed in *E. coli* PR70 (DE3) containing pRARE, which provides rare tRNA gene for efficient translation. The hMYH (wild-type) and hMYH(R231L) proteins were partially purified by passing through a nickel-agarose column. Both of the partially purified hMYH proteins were applied to 10% SDS-PAGE for Coomassie blue staining and for Western blot against the GB1 domain to determine the proteins' purity and concentrations (Fig. 2B). Both proteins were partially purified to ~15% homogeneity. There was some degradation of the proteins (Fig. 2B). The protein concentrations of wild-type and R231L were estimated to be 22 and 77 μ g/ml, respectively.

The adenine removal from a 44-base-pair duplex containing an A/GO mispair was compared using various amounts of partially purified wild-type and R231L mutant hMYH proteins. hMYH(R231L) protein showed no detectable glycosylase activity with A/GO mismatches even at 0.1 μ M concentration (Fig. 3A, lane 6). Compared with the wild-type protein, hMYH(R231L) protein also showed no DNA binding activity to a 20-base-pair duplex containing an A/GO mispair (Fig. 3B, lanes 7–10). All three DNA-protein complexes, which were observed with wild-type hMYH (Fig. 3B, lanes 3–6), were absent in the hMYH(R231L) reaction (Fig. 3B, lanes 7–10). Therefore, the missense mutation R231L of hMYH exhibited neither adenine glycosylase nor DNA binding activities towards A/GO mismatches.

3.4. In vivo complementation activity of hMYH(R231L)

Next we checked the ability of hMYH(R231L) to reduce the mutator phenotype of *E. coli* *mutYmutM*. The high mutation frequency of the *mutYmutM* strain can be reduced substantially by the expression of MutY from plasmid [45,46]. The resistance to rifampicin was used to measure the mutation frequency. In the case of low DNA repair capacity, the mutation frequency in the gene of RNA polymerase increases. *E. coli* cells, containing mutated RNA polymerase defective in binding rifampicin, can then grow on rifampicin-containing plates. The *mutMmutY* double mutant is a strong mutator as reported previously (Table 1, compare rows 1 and 2). CC104*mutMmutY* cells containing pKK223-2 vector have a similar mutation frequency as the cells without the vector (Table 1, compare rows 2 and 3) ($P = 0.257$). Expression of wild-type hMYH significantly lowered the mutation frequency in CC104*mutMmutY* (Table 1, compare rows 3 and 4) ($P = 0.008$). However, CC104*mutMmutY* expressing R231L had a similar mutation frequency as the cells with only the vector (Table 1, row 3 and row 5) ($P = 0.310$). Thus, R231L is unable to complement the defect of *E. coli* *mutY* mutation.

4. DISCUSSION

Reactive oxygen species are believed to play a causative role in degenerative diseases such as aging and cancer. Constant scanning and repair of damaged DNA are essential to reduce mutagenic and cytotoxic accidents. Oxidative DNA damage is removed by several repair pathways that are usually redundant. The OGG1-mediated base excision repair pathway removes GO lesions paired with cytosines in DNA. If C/GO mismatches are not repaired, adenines are frequently incorporated opposite GO lesions during DNA replication [20,21]. MYH and MSH2/MSH6 (MutS α)-dependent mismatch repair removes the mismatched A on the daughter DNA strand to increase replication fidelity [22–28]. Failure to remove the mutagenic 8-oxoG on DNA can cause G:C to T:A transversions. Knockouts of the *Myh* or

Ogg1 gene in mice have unexpectedly mild consequences [47–49]. This has been explained by the redundant repair pathways to cope with oxidative stress in the mouse cell. It has been reported that the *Myh* knockout mice were cancer free [50]; however, a higher frequency of intestine tumor was observed in *Myh* knockout mice that were 18 months old [48]. Remarkably, the combined deficiency in *Myh* and *Ogg1* predisposes mice to tumors, predominantly lung and ovarian tumors, and lymphomas [49]. In humans, biallelic *hMYH* mutations are the underlying genetic basis of a substantial fraction of patients with adenomatous polyposis [5, 6, 45, 51]. It is interesting to note that the types of tumors developed in the double knockout mice are different from those found in human MAP with colon cancer. The reason for this is unclear. Nevertheless, oxidative DNA damage appears to play a causal role in carcinogenesis.

Human *MYH* mutations can cause G:C to T:A mutations of the adenomatous polyposis coli (*APC*), *K-ras*, and other genes that control cellular proliferation in the colon [5, 11, 12]. Four MAP-related missense mutations of hMYH (Y165C, G382D, R227W and V232F) have been defined biochemically [5, 40–42]. In the present study, we characterized one newly identified variant of hMYH(R231L) which is mutated homozygously in a patient with hundreds of adenomatous and hyperplastic polyps throughout the resected colon. The R231L mutant of hMYH is inactive in both binding and glycosylase activity to A/GO mismatches, although it still retains the ability to physically interact with hMutS α . The biochemical properties of hMYH (R231L) are similar to those of hMYH(R227W). The R227 and R231 of hMYH are conserved in the MutY family and are involved in a consensus sequence R(V/L)XXR of helix-hairpin-helix (HhH) superfamily of glycosylases containing a [4Fe-4S]²⁺ cluster [52]. According to the recent X-ray structure of BsMutY-DNA complex [53], R227 of hMYH is predicted to be in the proximity to the phosphate group 5' to the mismatched adenine. R231 is predicted to be located on the same alpha helix containing the conserved Asp at the active center and the side chain of R231 is pointed into the [4Fe-4S]²⁺ pocket. The iron-sulfur cluster region has been shown to be involved in the substrate binding, which in turn interferes with the catalysis of base removal (reviewed in [54]). The mutation of a positive charged Arg231 to a hydrophobic Leu may disrupt the local structure of iron-sulfur cluster and thus affect the substrate binding and catalysis.

MYH has been shown to physically interact with MSH2/MSH6 resulting in stimulation of MYH DNA binding activity [13]. Although the mutations R227W, R231L, and V232F lie adjacent to the hMSH6 binding domain, all three mutant proteins retain the ability to interact with hMSH6. Therefore, it remains to be determined whether a defect in the hMYH-MSH6 interaction contributes to the cause of MAP.

Because the repair capacity of the mutant hMYH(R231L) is severely defective, the biallelic germ-line mutation R231L in *hMYH* gene found in the identified patient is likely to be responsible for the MAP phenotype. Since the discovery of the linkage of germ-line mutations in the *hMYH* gene with MAP in 2002, studies on MutY and MYH repair have been greatly stimulated [10]. The identification of individuals affected by MAP polyposis brings new and important implications for the diagnostic, screening, genetic counseling, follow up, and therapeutic options in these patients [51]. However, only five out of more than 20 mutations in the *hMYH* gene associated with MAP have been biochemically characterized thus far. Further analyses of the effects of hMYH mutations will elucidate the pathogenesis of this emerging FAP-like colorectal adenomas and carcinoma.

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Fig 1.

Physical interaction between hMutS α and hMYH(R231L). Purified hMutS α (hMSH2/hMSH6 complex, 460 ng) pulled down by GST-hMYH beads was fractionated by a 10% SDS-polyacrylamide gel followed by Western blot analysis using antibodies against hMSH2. Equivalent concentrations of wild type and mutant GST-hMYH proteins were used in the assay. Purified hMutS α (46 ng) was applied to lane 1. For control, hMutS α was applied to beads containing GST alone (lane 2). hMutS α bound to both wild-type (lane 3) and hMYH(R231L) (lane 4).

Fig 2. Analyses of hMYH(R231L) mutant protein. (A) Partially purified GB1-hMYH-His fusion protein detected by Coomassie blue staining. Wild type (WT) and mutant proteins were expressed in *E. coli* PR70/DE3 containing pRARE and partially purified by Ni-agarose affinity chromatography. Protein samples (2 μ l) were applied to 10% SDS-PAGE and stained with Coomassie blue. (B) Partially purified GB1-hMYH-His fusion proteins detected by Western-blotting. Samples are similar to (A) but detected by Western-blotting with an antibody against annesin-2 to detect GB1 tag.

Fig 3.

Functional analyses of hMYH(R231L) mutant enzyme. (A) Glycosylase activity of partially purified hMYH(R231L) mutant. 5'-end labeled 20-base-pair DNA duplex (1.8 fmol) containing an A/GO mismatch was incubated with partially purified GB1-hMYH-His proteins for 1.5 hr at 37°C before cleavage of the phosphodiester backbone by NaOH. The reaction products were fractionated on a 14% polyacrylamide sequencing gel containing 7 M urea. Lanes 1–3 are with wild-type (WT) hMYH and lanes 4–6 are with hMYH(R231L). The hMYH protein concentrations are indicated on the top of each lane. Intact (I) and nicked (N) DNA are marked. (B) DNA binding activity of hMYH(R231L) mutant. Labeled DNA (20-mer) substrate containing an A/GO mismatch was incubated with *E. coli* MutY (lane 2) and partially purified wild-type (lanes 3–6) and hMYH(R231L) (lanes 7–10) at 37°C for 30 min. Protein-DNA complexes were run on a non-denaturing 6% polyacrylamide gel. The hMYH protein concentrations are indicated on the top of each lane. The arrows mark the hMYH-DNA complex (Y-DNA) and free DNA substrate (Free).

Table 1
Mutation frequencies of *E.coli mutYmutM* expressing mutant hMYH proteins

Strains ^a	Mutation Frequency ^b (Rif ^R 10 ⁸ cells)	Increase (fold) ^c
1. CC104+ (wild type)	0.6 ± 0.2	1
2. CC104- (<i>mutMmutY</i>)	329 ± 25	532
3. CC104-/pKK223-3 (vector)	278 ± 64	450
4. CC104-/pKK223-hMYH (wild type)	106 ± 26	172
5. CC104-/pKK223-R231L (mutant)	231 ± 63	375

^a *E.coli* strains CC104+ and CC104- contain wild-type and mutated *mutY* and *mutM* genes, respectively.

^b The *in vivo* activities of wild-type and mutant hMYH were measured by the frequency of rifampicin resistant colonies (average at least three separate experiments, with errors reported as the standard deviations).

^c Fold increase compared with the wild-type strain.