

Impaired suppressive activities of human MUTYH variant proteins against oxidative mutagenesis

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Abstract

AIM: To investigate the suppressive activity of MUTYH variant proteins against mutations caused by oxidative lesion, 8-hydroxyguanine (8OHG), in human cells.

METHODS: p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants, which were previously found in patients with colorectal polyposis and cancer, were selected for use in this study. Human H1299 cancer cell lines inducibly expressing wild-type (WT) MUTYH (type 2) or one of the 4 above-mentioned MUTYH variants were established using the piggyBac transposon vector system, enabling the genomic integration of the trans-

poson sequence for MUTYH expression. MUTYH expression was examined after cumate induction using Western blotting analysis and immunofluorescence analysis. The intracellular localization of MUTYH variants tagged with FLAG was also immunofluorescently examined. Next, the mutation frequency in the *supF* of the shuttle plasmid pMY189 containing a single 8OHG residue at position 159 of the *supF* was compared between empty vector cells and cells expressing WT MUTYH or one of the 4 MUTYH variants using a *supF* forward mutation assay.

RESULTS: The successful establishment of human cell lines inducibly expressing WT MUTYH or one of the 4 MUTYH variants was concluded based on the detection of MUTYH expression in these cell lines after treatment with cumate. All of the MUTYH variants and WT MUTYH were localized in the nucleus, and nuclear localization was also observed for FLAG-tagged MUTYH. The mutation frequency of *supF* was 2.2×10^{-2} in the 8OHG-containing pMY189 plasmid and 2.5×10^{-4} in WT pMY189 in empty vector cells, which was an 86-fold increase with the introduction of 8OHG. The mutation frequency (4.7×10^{-3}) of *supF* in the 8OHG-containing pMY189 plasmid in cells overexpressing WT MUTYH was significantly lower than in the empty vector cells ($P < 0.01$). However, the mutation frequencies of the *supF* in the 8OHG-containing pMY189 plasmid in cells overexpressing the p.R154H, p.M255V, p.L360P, or p.P377L MUTYH variant were 1.84×10^{-2} , 1.55×10^{-2} , 1.91×10^{-2} , and 1.96×10^{-2} , respectively, meaning that no significant difference was observed in the mutation frequency between the empty vector cells and cells overexpressing MUTYH mutants.

CONCLUSION: The suppressive activities of p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants against mutations caused by 8OHG are thought to be severely impaired in human cells.

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Key words: 8-hydroxyguanine; Mutation; MUTYH; MUTYH-associated polyposis; Oxidative mutagenesis; *supF* forward mutation assay; piggyBac transposon; Colorectal polyposis

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INTRODUCTION

8-hydroxyguanine (8OHG) is an oxidatively damaged form of guanine^[1], and because 8OHG can pair with adenine as well as cytosine, the formation of 8OHG in DNA causes a G:C to T:A transversion mutation^[2]. To prevent such mutations, excision repair proteins, such as MUTYH (OMIM 604933), that act on 8OHG are present in human cells. The MUTYH protein is a DNA glycosylase that catalyzes the removal of adenine that is mispaired with 8OHG in double-stranded DNA^[3-7]. Two major MUTYH proteins, type 1 and type 2, are expressed in human cells as a result of multiple transcription initiation sites and the alternative splicing of mRNA transcripts^[4,7]. Because the type 1 protein contains a mitochondrial targeting signal (MTS) in its N-terminal, it is localized in the mitochondria. In contrast, the type 2 protein lacks the N-terminal 14 amino acids of type 1, and this absence leads to the destruction of the MTS; consequently, the type 2 protein is localized in the nucleus^[4,7].

Biallelic germline mutations in the *MUTYH* gene are responsible for MUTYH-associated polyposis (MAP) (OMIM 608456), which is a hereditary disease characterized by multiple colorectal adenomas and carcinomas^[8-12]. Most biallelic *MUTYH* carriers have between 10 and a few hundred colorectal polyps, meaning that MAP shows a phenotypic overlap with two other hereditary colorectal polyposis syndromes: familial adenomatous polyposis (FAP: OMIM 175100) and attenuated FAP (AFAP: OMIM 175100), both of which are caused by inactivation of the *APC* gene (OMIM 611731)^[13,14]. Therefore, screening for germline mutations in *MUTYH* and *APC* is important in candidate patients with multiple colorectal polyps. However, even when *MUTYH* gene variations are detected in the mutation screening, if information regarding the level of the repair activities of the MUTYH variants is lacking, a correct diagnosis of MAP is impossible to make. Thus far, 300 unique DNA variants of the

MUTYH gene have been reported in the Leiden Open Variation Database (http://www.lovd.nl/2.0/index_list.php)^[15], and the proportion of missense *MUTYH* variations in the database is larger than nonsense mutations or truncating mutations. For most of the genes, a functional analysis is needed to determine whether the activity of a protein encoded by a missense variant is severely reduced. Thus, the effect of *MUTYH* variations on repair activity should be examined; however, so far, only a small number of *MUTYH* variations has been investigated^[16-27]. In most of these studies, the DNA glycosylase activities of the variant recombinant proteins were analyzed using a DNA cleavage assay to test the abilities of the variants to cleave double-stranded oligonucleotides containing an A:8OHG mispair *in vitro*^[18,19,21,23-27]. However, because examining the repair activity of MUTYH variant proteins from multiple aspects would lead to a more definitive judgment of the pathogenicity of MUTYH variants and MUTYH has the ability to regulate the mutation frequency in human cells *in vivo*^[28-30], evaluating the mutation frequency in human cells is also valuable. However, at present, the activities of MUTYH variants in the regulation of mutation suppression in human cells *in vivo* have not been previously reported. Therefore, in this paper, we evaluated the suppressive activities of MUTYH variant proteins against oxidative mutagenesis in human cells. We recently determined the DNA glycosylase activities of 14 type 2 (nuclear form) MUTYH variants using a DNA cleavage assay^[27], and based on those results, p.R154H, p.M255V, p.L360P, and p.P377L type 2 proteins were chosen from the tested variants, and their abilities to suppress mutations caused by 8OHG in human cells were analyzed in this study. As far as we know, this is the first report to analyze the suppressive activities of MUTYH variants against oxidative mutagenesis in human cells.

The Human Genome Variation Society (<http://www.hgvs.org/>) recommends using the transcript variant $\alpha 5$ (NM_001128425.1), which encodes the longest isoform (549 amino acids), as a reference sequence. Therefore, the type 2 proteins p.R154H, p.M255V, p.L360P, and p.P377L used in this study correspond to the reference proteins p.R182H, p.M283V, p.L388P, and p.P405L, respectively.

MATERIALS AND METHODS

Cell line

The human cancer cell line H1299 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37 °C in RPMI1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin under a 5% CO₂ atmosphere. The study design was approved by an institutional review board.

Construction of expression plasmid

Human wild-type (WT) and variant (p.R154H, p.M255V, p.L360P, and p.P377L) MUTYH type 2 cDNAs were polymerase chain reaction-amplified with *PfuUltra* Hot-

start DNA polymerase (Stratagene, La Jolla, CA) and the MUTYH-type 2/pET25b(+) expression vector^[27] as a template; the amplified sequence was then inserted into a piggyBac cumate switch inducible vector (System Biosciences, Mountain View, CA) at the *NheI* and *NotI* restriction enzyme sites. A WT MUTYH type 2 expression vector with a C-terminal FLAG tag was previously constructed using the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA)^[31]; in this study, the variants were generated using site-directed mutagenesis with a QuikChange Site-directed Mutagenesis kit (Stratagene). All of the vectors were confirmed using DNA sequencing with a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and an ABI 3100 Genetic Analyzer (Applied Biosystems).

Transfection

A plasmid vector was transfected into H1299 cells using Lipofectamine 2000 reagent (Invitrogen) according to the supplier's recommendations.

Establishment of stable inducible cell lines

H1299 cells were transfected with the cumate switch inducible vector for MUTYH expression together with the piggyBac transposase vector (System Biosciences). To establish stable inducible cell lines, positively transposed cells were selected using puromycin (1 µg/mL). Because the inducible piggyBac vector features a tight cumate switch combined with the EF1-CymR repressor-T2A-Puro cassette to establish stable cell lines, the addition of cumate solution (System Biosciences) to the puromycin-selected cells led to the induction of MUTYH expression.

Western blotting analysis

Cells were harvested in lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100, 0.5% sodium deoxycholate, 100 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and the whole-cell extracts were mixed with an equal volume of 2 × SDS sample buffer and boiled. The extract was then subjected to SDS-polyacrylamide gel electrophoresis, and the proteins obtained were electrophoretically transferred to a polyvinylidene fluoride membrane (GE Healthcare Bio-Science, Piscataway, NJ). The membrane was blocked with nonfat milk at room temperature (RT) for 1 h and incubated with an anti-MUTYH monoclonal antibody (clone 4D10; Abnova, Taipei, Taiwan) or an anti-β-tubulin monoclonal antibody (clone 2-28-33; Sigma-Aldrich) at RT for 1 h. After washing with PBS containing 0.05% Tween-20 (PBS-T), the membrane was incubated with an anti-mouse HRP-conjugated secondary antibody (GE Healthcare Bio-Science) at RT for 1 h. The membrane was then washed with PBS-T, and immunoreactivity was visualized using an ECL chemiluminescence system (GE Healthcare Bio-Science).

Indirect immunofluorescence analysis

Cells were fixed with 10% formalin at RT or 4% paraformaldehyde at 4 °C. The cells were permeabilized with 1% Nonidet P-40 in PBS for 5 min and incubated with 10% normal goat serum blocking solution (DAKO, Kyoto, Japan) for 30 min. The cells were then probed with mouse anti-MUTYH monoclonal antibody (4D10) or mouse anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) at RT for 1 h. Indirect immunofluorescence labeling was performed by incubation with an Alexa Fluor 594-conjugated secondary antibody (Molecular Probes, Eugene, OR) at RT for 1 h, and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The immunostained cells were examined under a fluorescence microscope (Olympus BX-51-FL; Olympus, Tokyo, Japan) equipped with epifluorescence filters and a photometric CCD camera (Sensicam; PCO Company, Kelheim, Germany). The captured images were digitized and stored using an image analysis program (MetaMorph; Molecular Devices, Palo Alto, CA).

Shuttle vector plasmid and an indicator bacterial strain

The plasmid pMY189 and the indicator *Escherichia coli* (*E. coli*) strain KS40/pKY241 were used for the *supF* forward mutation assay, as reported previously^[30,32]. pMY189 is a shuttle vector containing the bacterial suppressor tRNA (*supF*) gene. KS40 is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 with genotype *lacZ* (*am*), *CA7070*, *lacY1*, *hsdR*, *hsdM*, Δ (*araABC-leu*)7679, *galU*, *galK*, *rpsL*, *thi*. The pKY241 plasmid contains a chloramphenicol resistance marker and the *gyrA* (amber) gene. *E. coli* KS40/pKY241 cells carrying the active *supF* gene are sensitive to nalidixic acid and form blue colonies on LB plates containing ampicillin, chloramphenicol, isopropyl-β-D-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), whereas cells carrying the mutated *supF* gene form white colonies on plates containing nalidixic acid, ampicillin, chloramphenicol, IPTG, and X-gal.

Construction of a shuttle vector plasmid containing an 8OHG residue

pMY189-8OHG, which is the shuttle plasmid pMY189 containing a single 8OHG:cytosine pair at nucleotide position 159 of the *supF* gene, was prepared according to a previously described method^[30]. Briefly, *E. coli* XL1-Blue MRF⁺ (Stratagene) and R408 Helper Phage (Stratagene) were used to prepare single-stranded pMY189 DNA, and 30 µg of the single-stranded pMY189 plasmid and a 5-fold molar excess of a 5'-phosphorylated 24-mer oligonucleotide with a single 8OHG at nucleotide position 159 of the *supF* gene [5'-CGA CTT CGA A (8OHG) G TTC GAA TCC TTC-3'] were annealed in a reaction mixture. Forty units of T4 DNA polymerase (New England Biolabs, Beverly, MA), 600 µmol/L of deoxynucleotide triphosphate (GE Healthcare Bio-Science), 36 Weiss units of T4 DNA ligase (New England Biolabs) and 1 mmol/L of ATP (Nacalai Tesque, Kyoto, Japan) were added to the

reaction mixture, and the mixture was incubated at 37 °C for 4 h. Closed circular pMY189-8OHG was isolated using cesium chloride-ethidium bromide density gradient centrifugation.

SupF forward mutation assay

Cells were cultured in the presence of cumate for 3 d for the induction of MUTYH expression, and they were then transfected with the shuttle plasmid pMY189 or pMY189-8OHG. After 48 h, the propagated plasmids were extracted from the cells using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and digested with *DpnI* restriction enzyme (New England Biolabs) to eliminate unreplicated plasmids with the bacterial methylation pattern. After purification with Amicon Ultra Centrifugal Filter Units (Millipore, Bedford, MA), the plasmids were introduced into the KS40/pKY241 indicator *E. coli* strain using electroporation. The transformants were plated onto LB agar plates containing 50 µg/mL of nalidixic acid, 150 µg/mL of ampicillin, and 30 µg/mL of chloramphenicol together with IPTG and X-gal. White colonies on were counted as *supF* mutants. The mutation frequencies were calculated as the number of *supF* mutants per the total number of transformants, which were counted on LB plates containing ampicillin, chloramphenicol, IPTG and X-gal.

Statistical analysis

The statistical analysis was performed using an unpaired *t*-test and JMP software, version 9 (SAS Institute, Cary, NC). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Establishment of human cells inducibly expressing MUTYH variants

To investigate the ability of MUTYH variants to suppress mutations caused by 8OHG in human cells, we used the piggyBac transposon vector system^[33] to establish human cells capable of inducibly expressing MUTYH variants and performed a *supF* forward mutation assay using the shuttle plasmid pMY189, which contains a single 8OHG in the *supF* gene. First, H1299 human cancer cells were transfected with a piggyBac cumate switch inducible vector for the expression of WT, p.R154H, p.M255V, p.L360P, or p.P377L MUTYH together with the piggyBac transposase vector; positively transposed cells were then selected with puromycin. We also transfected cells with an empty (parental) piggyBac cumate switch inducible vector and transposase vector. The expression of MUTYH protein after cumate induction was examined using Western blotting analysis using an anti-MUTYH monoclonal antibody (Figure 1A). MUTYH protein was abundantly expressed in cells in which a WT, p.R154H, p.M255V, p.L360P, or p.P377L MUTYH expression vector but not an empty vector was transposed. Immunofluorescence analysis also showed abundant MUTYH pro-

tein expression in cells in which a WT, p.R154H, p.M255V, p.L360P, or p.P377L MUTYH expression vector but not an empty vector was transposed (Figure 1B). In accordance with the previous finding that the MUTYH type 2 protein is the nuclear form^[4,7], WT MUTYH protein was localized in the nucleus. All of the MUTYH variants were also localized in the nucleus, suggesting that the amino acid changes in p.R154H, p.M255V, p.L360P, and p.P377L did not alter the subcellular localization of these proteins in human cells. With regard to endogenous MUTYH expression, low levels were detected in the immunofluorescence analysis, as shown in the panel of empty vector-transposed cells (Figure 1B). When the intensity of the MUTYH protein signal was enhanced with image-editing software, the signal was observed in both the nucleus and cytoplasm (Figure 1C), which is compatible with the existence of both the type 1 mitochondrial form and the type 2 nuclear form^[4,7]. Next, to confirm the nuclear localization of MUTYH type 2 variant forms, we constructed a vector to express MUTYH tagged with a FLAG peptide at the C-terminus and examined the localization of the MUTYH variants using immunofluorescence analysis with an anti-FLAG antibody (Figure 2). All of the variants showed nuclear localization, further suggesting that the amino acid changes in p.R154H, p.M255V, p.L360P, and p.P377L did not alter their subcellular localization in human cells. Together, the above findings indicate that human cells inducibly expressing the MUTYH variants (p.R154H, p.M255V, p.L360P, or p.P377L) and their control cells were properly prepared and were appropriate for evaluating the suppressive activities of MUTYH variants against oxidative mutagenesis in human cells.

Impaired activities of MUTYH variants in the suppression of oxidative mutagenesis in human cells in vivo

Next, mutation frequencies were compared for the empty vector-transposed human cells and the cumate-inducible stable cells expressing WT or variant MUTYH using a *supF* forward mutation assay with the shuttle plasmid pMY189. In this assay, we introduced a single 8OHG residue at position 159 of the *supF* gene in pMY189. The mutation frequency of *supF* was 2.2×10^{-2} in the 8OHG-containing pMY189 plasmid and 2.5×10^{-4} in WT pMY189 in empty vector-transposed cells (Figure 3), which was an 86-fold increase in mutation frequency with the introduction of 8OHG. The mutation frequency (4.7×10^{-3}) of *supF* in the 8OHG-containing pMY189 plasmid in cells overexpressing WT MUTYH was significantly lower than in the empty vector-transposed cells. However, the mutation frequencies of *supF* in the 8OHG-containing pMY189 plasmid in cells overexpressing the p.R154H, p.M255V, p.L360P, or p.P377L MUTYH variant were 1.84×10^{-2} , 1.55×10^{-2} , 1.91×10^{-2} , and 1.96×10^{-2} , respectively, meaning that no significant difference was observed in the mutation frequency between the empty vector-transposed cells and the cells overexpressing MUTYH variants. These results suggested that the suppressive activities of p.R154H, p.M255V, p.L360P,

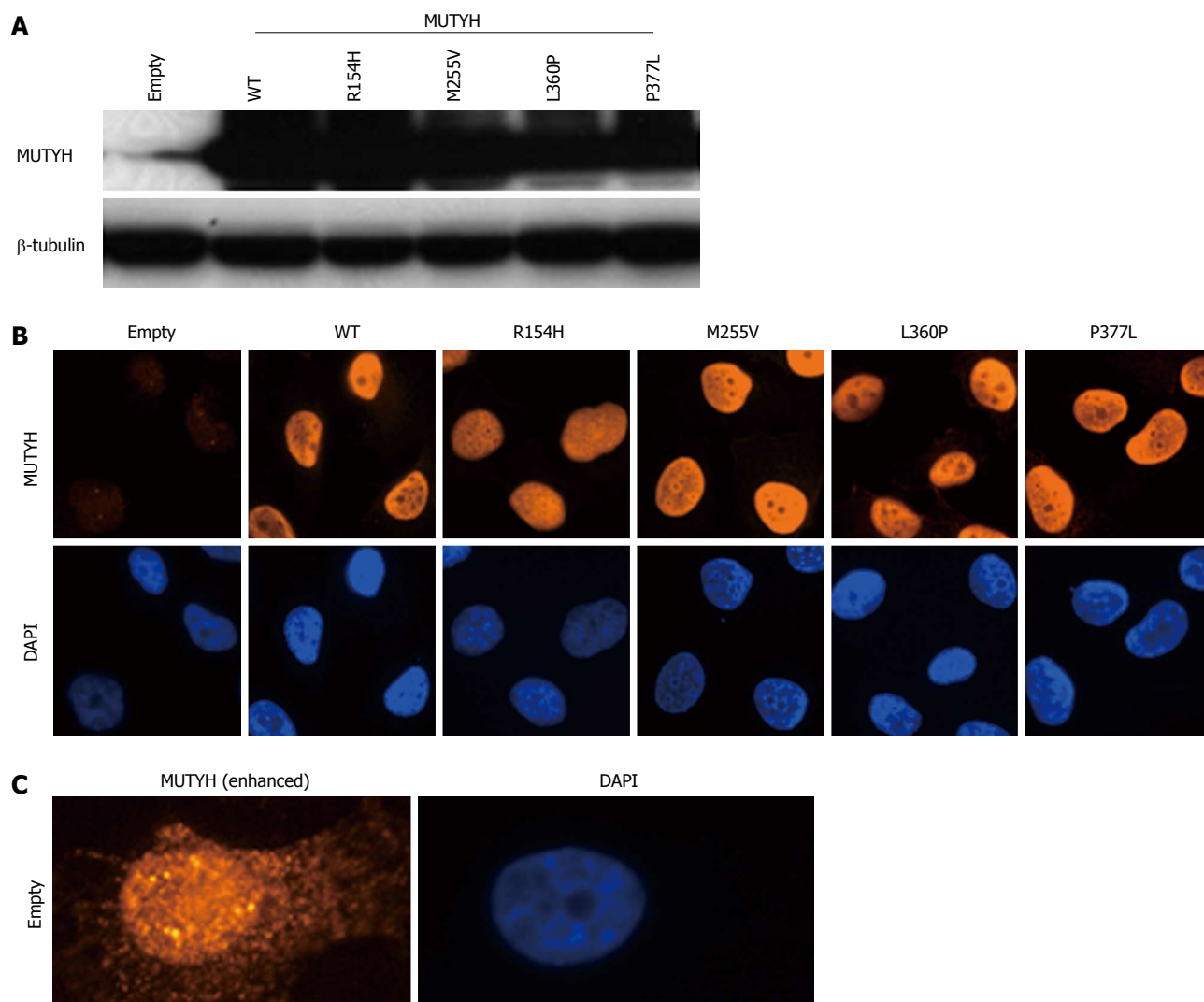


Figure 1 Establishment of H1299 human cell lines inducibly expressing MUTYH variant proteins. A: Detection of MUTYH proteins in cumate-inducible stable cell lines expressing MUTYH in the presence of cumate using Western blotting analysis with an anti-MUTYH antibody. Lysates from empty vector-transposed cells and cells inducibly expressing wild-type (WT) MUTYH or p.R154H, p.M255V, p.L360P, or p.P377L MUTYH variants were analyzed. β -tubulin protein was also analyzed as an internal control; B: Immunofluorescence detection of MUTYH proteins expressed in the cell lines used in (A) in the presence of cumate. The MUTYH protein (red) was stained with anti-MUTYH as the primary antibody and Alexa Fluor 594-conjugated goat anti-mouse IgG as the secondary antibody. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue); C: Immunofluorescence detection of endogenous MUTYH proteins in the empty vector-transposed cells as described in (B). The intensity of the signals of MUTYH protein (red) was enhanced with image-editing software to determine the subcellular localization of endogenous MUTYH protein. The nuclei were counterstained with DAPI (blue).

and p.P377L MUTYH variants against mutations caused by 8OHG were severely impaired in human cells.

DISCUSSION

In this study, human cell lines inducibly expressing MUTYH variants (p.R154H, p.M255V, p.L360P, or p.P377L) were established, and the abilities of these cells to suppress mutations caused by 8OHG were compared using a *supF* forward mutation assay with a shuttle vector containing an 8OHG residue in the *supF* gene. The assay showed that the suppressive activities of p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants against mutations caused by 8OHG were severely impaired in human cells. To the best of our knowledge, this is the first analysis of the suppressive activities of MUTYH variants against oxi-

dative mutagenesis in human cells *in vivo*.

The type 2 protein is the nuclear form of MUTYH^[4,7], and somatic *APC* and *KRAS* (OMIM 190070) mutations occur in the nuclear DNA of MAP tumors^[8,9,12]; therefore, we believed that it would be more appropriate to investigate type 2 rather than type 1 and we established cell lines expressing the type 2 form in this study. In the *supF* forward mutation assay using a shuttle vector containing 8OHG, no significant difference was observed in the mutation frequencies between empty vector-transposed cells and cells expressing 1 of the 4 MUTYH variants, indicating the severe impairment of the suppressive activities of the MUTYH variants against mutations caused by 8OHG in human cells *in vivo*. We previously showed that the adenine DNA glycosylase activity of the p.M255V protein was 10.7% of the level of the WT protein and that the DNA

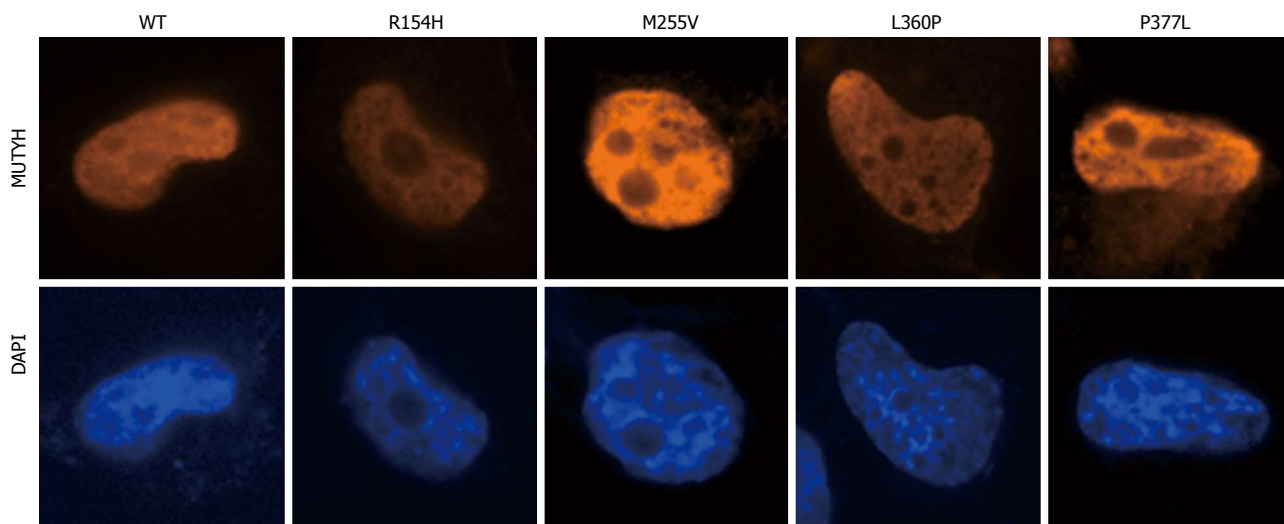


Figure 2 Nuclear localization of MUTYH variant proteins (p.R154H, p.M255V, p.L360P, and p.P377L). H1299 cells were transiently transfected with a vector expressing various types of MUTYH proteins tagged with FLAG, and MUTYH-FLAG protein (red) was stained with anti-FLAG M2 as the primary antibody and Alexa Fluor 594-conjugated goat anti-mouse IgG as the secondary antibody. The nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (blue). WT: Wild-type.

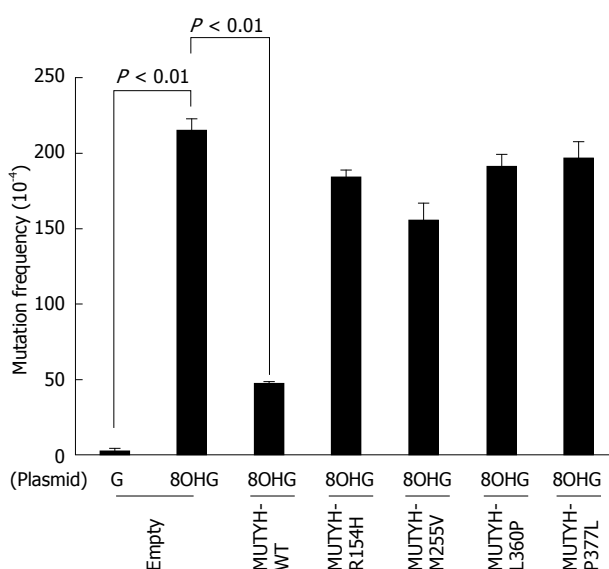


Figure 3 Measurement of the mutation frequency of the *supF* gene in the pMY189 plasmid using a *supF* forward mutation assay in H1299 human cell lines inducibly expressing MUTYH variant proteins. Empty vector-transfected cells and cells inducibly expressing wild-type (WT) MUTYH or p.R154H, p.M255V, p.L360P, or p.P377L MUTYH variants in the presence of cumate were transfected with a pMY189 shuttle plasmid, and the mutation frequency of *supF* in these human cell lines was measured. "8-hydroxyguanine (8OHG)" indicates a pMY189 plasmid containing an 8OHG residue at position 159 of *supF*, while "G" indicates a pMY189 plasmid containing the WT *supF* gene. The data are shown as the means \pm SE.

glycosylase activities of the p.R154H, p.L360P, and p.P377L proteins were severely impaired^[27]. Thus, the results regarding the regulation of the mutation frequency in the present study are in agreement with DNA glycosylase activity in the previous study. A combination of the results of two distinct analyses, i.e., *in vitro* and *in vivo* analyses, would provide more definitive proof of the pathogenicity of the p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants. Because the diagnosis of MAP depends on whether

(1) the clinical phenotypic characteristics of MAP are present in a candidate patient; and (2) the repair activities of the MUTYH variants encoded in the two *MUTYH* alleles of the patient are decreased, even when gene variations are found in the patient by *MUTYH* mutation screening, information on the levels of the repair activities of MUTYH variants is indispensable for properly diagnosing MAP. Regarding this point, our results are clinically useful.

Previous studies have provided contradictory results regarding the subcellular localization of MUTYH protein in MAP patients; one paper insisted that MUTYH protein was predominantly localized in the cytoplasm of colorectal tumor cells in MAP patients but not in non-MAP patients, while the other papers denied this localization^[34,35]. In the present study, the nuclear localization of the p.R154H, p.M255V, p.L360P, and p.P377L MUTYH type 2 variants was shown using two distinct experiments. Therefore, it seems that the amino acid changes of p.R154H, p.M255V, p.L360P, and p.P377L did not alter the subcellular localization of the MUTYH protein. Similarly, Molatore *et al.*^[26] recently reported that 3 missense MUTYH variants other than our variants were all localized in the nucleus.

In this paper, we successfully established cumate-inducible stable human cell lines by utilizing the piggyBac transposon vector system. Transposon technology is an attractive non-viral gene delivery model that allows for efficient genomic integration in a variety of cell types^[36]. Among the several transposon systems available, the piggyBac transposon, which was isolated from the genome of the cabbage looper moth (*Trichoplusia ni*), has been optimized for gene transfer into mammalian cells^[36,37]. In practice, the MUTYH expression status in our cell lines after puromycin selection in the presence of cumate clearly demonstrated abundant MUTYH expression in almost all of the cells. Because we performed transient transfection with a shuttle plasmid in the *supF* forward

mutation assay in this study, the genomic integration of the sequence for MUTYH expression using the piggyBac transposon system was well suited to our experiment.

In our experiments, the level of expression of exogenously introduced MUTYH was much higher than the level of expression of endogenous MUTYH. This scenario allowed us to effectively evaluate the activities of MUTYH variants to regulate the mutation frequency, and we believe that such an evaluation was successfully performed. However, we cannot completely exclude the possibility that the functional difference observed under experimental conditions of high MUTYH expression levels does not reflect a true functional difference.

The impaired activity of MUTYH variants was shown using H1299 human lung cancer cells in this study. We used this cell line because we believed that the ability of MUTYH variants to suppress mutations in H1299 cells is not different from their ability in human cells derived from the colorectum. If there are no organ type-specific systems to modulate MUTYH activity, then MUTYH activity is dependent on the MUTYH expression level and MUTYH variation. Moreover, we studied overexpressed and exogenous MUTYH variant proteins in this paper. Therefore, we believe our results can most likely be applied for colorectal cells. However, because it might be possible that the difference in organ type has an effect on the results of functional evaluation, we would like to evaluate this activity in human colorectal cells in the future.

Genetic screening for *MUTYH* mutations in the diagnosis of colorectal polyposis continues to be performed worldwide, and technological progress in genome sequencing analysis has contributed to efficient and rapid screening protocols. Therefore, increasing *MUTYH* nucleotide variants are likely to be detected in the future. For appropriate patient management, the levels of the repair activities of MUTYH variant proteins should be evaluated, and our system for determining the abilities of these variants to suppress oxidative mutagenesis in human cells *in vivo* may be of great use for such evaluations.

COMMENTS

Background

The *MUTYH* gene is responsible for MUTYH-associated polyposis (MAP), a relatively recently identified hereditary disease. Although 300 *MUTYH* variants have been found, only a small number of variants has been functionally characterized. Therefore, evaluations of the activities of MUTYH variant proteins are needed for the correct diagnosis of MAP.

Research frontiers

An *in vitro* DNA cleavage assay was performed to evaluate the repair activities of MUTYH variants. Despite the clinical importance of the multiplicity of functional analytical methods for evaluating the activities of MUTYH variant proteins, until now, the ability of MUTYH variants to suppress oxidative mutagenesis in human cells *in vivo* has not been previously analyzed.

Innovations and breakthroughs

Human cumate-inducible stable cell lines expressing various MUTYH variants were established using the piggyBac transposon vector system. This is the first report to utilize human cells expressing MUTYH variants encoded by an ectopically transposed gene. Moreover, this is the first report to analyze the suppressive activities of MUTYH variants against oxidative mutagenesis in human cells.

Applications

The results of the present study suggest that the suppressive activities of p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variant proteins against mutations caused by 8-hydroxyguanine (8OHG) are severely impaired in human cells. These *in vivo* results combined with results from our previous *in vitro* analysis provide definitive proof of the pathogenicity of p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants. This conclusion is valuable for the appropriate diagnosis of MAP.

Terminology

The base excision repair protein MUTYH is involved in the repair of the oxidative base lesion 8OHG in DNA. Patients with biallelic inactivating germline mutations in the *MUTYH* gene are predisposed to MAP, which is characterized by the development of multiple colorectal adenomas and carcinomas.

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This is a good study in which authors analyze the suppressive activity of MUTYH variant proteins against mutations caused by 8OHG in human cells. Towards understanding the impact of having so many missense mutations among MAP patients, the authors have steadily developed an infrastructure for serving the patients in the future. Through expression of MUTYH WT and 4 variants, subcellular localization, and mutation frequency counting, they suggested that anti-mutation activity of the four MUTYH variants were severely impaired in human cells.

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