Table 2: Genotype and allele frequencies of the *MUTYH* and *OGG1* nucleotide variations found in 34 Japanese patients with early-onset colorectal carcinoma.

Gene	Variant	Nucleotide		Genotype frequency			Allele frequency	
		wt	vt	wt/wt	wt/vt	vt/vt	wt	vt
MUTYH	c.36+11C>T	C	T	32 (94.1%)	2 (5.9%)	0 (0%)	66 (97.1%)	2 (2.9%)
MUTYH	c.55C>T (p.Arg19*)	C	T	33 (97.1%)	1 (2.9%)	0 (0%)	67 (98.5%)	1 (1.5%)
MUTYH	c.325C>T (p.Arg109Trp)	C	T	33 (97.1%)	1 (2.9%)	0 (0%)	67 (98.5%)	1 (1.5%)
MUTYH	c.504+35A>G	A	G	25 (73.5%)	9 (26.5%)	0 (0%)	59 (86.8%)	9 (13.2%)
MUTYH	c.934−2A>G	A	G	33 (97.1%)	1 (2.9%)	0 (0%)	67 (98.5%)	1 (1.5%)
MUTYH	c.1014G>C (p.Gln338His)	G	C	15 (44.1%)	15 (44.1%)	4 (11.8%)	45 (66.2%)	23 (33.8%)
MUTYH	c.1118C>T (p.Ala373Val)	C	T	33 (97.1%)	1 (2.9%)	0 (0%)	67 (98.5%)	1 (1.5%)
MUTYH	c.1431G>C (p.Thr477Thr)	G	C	32 (94.1%)	2 (5.9%)	0 (0%)	66 (97.1%)	2 (2.9%)
MUTYH	c.1477−40C>G	C	G	2 (5.9%)	7 (20.6%)	25 (73.5%)	11 (16.2%)	57 (83.8%)
OGG1	c.−23A>G	A	G	32 (94.1%)	2 (5.9%)	0 (0%)	66 (97.1%)	2 (2.9%)
OGG1	c.−18G>T	G	T	32 (94.1%)	2 (5.9%)	0 (0%)	66 (97.1%)	2 (2.9%)
OGG1	c.294G>A (p.Lys98Lys)	G	A	33 (97.1%)	1 (2.9%)	0 (0%)	67 (98.5%)	1 (1.5%)
OGG1	c.748−15C>G	C	G	13 (38.2%)	14 (41.2%)	7 (20.6%)	40 (58.8%)	28 (41.2%)
OGG1	c.949−89G>T	G	T	33 (97.1%)	1 (2.9%)	0 (0%)	67 (98.5%)	1 (1.5%)
OGG1	c.966C>T (p.Asp322Asp)	C	T	33 (97.1%)	1 (2.9%)	0 (0%)	67 (98.5%)	1 (1.5%)
OGG1	c.977C>G (p.Ser326Cys)	C	G	11 (32.4%)	17 (50.0%)	6 (17.6%)	39 (57.4%)	29 (42.6%)

wt: wild-type, vt: variant type.

and p.Asp208Asn recombinant proteins were expressed in *E. coli* and were purified to a high level of homogeneity (Figure 2(a)). Their molecular size of approximately 61 kDa was determined using SDS-PAGE/CBB staining and Western blot analysis, and this size corresponded to the size calculated from the cDNA sequence (Figures 2(a) and 2(b)). Then, the DNA glycosylase activity of the MUTYH proteins was examined by determining its capacity to cleave a double-stranded oligonucleotide containing an adenine mispaired with 8OHG (Figure 2(c)). No clear cleavage products were detected when an oligonucleotide containing an unmodified A:T base pair was exposed to any of the MUTYH proteins, but cleavage products with the same mobility as the marker oligonucleotide were detected when WT MUTYH protein, but not a p.Asp208Asn negative control protein, was allowed to react with an oligonucleotide containing an A:8OHG base pair (Figures 2(d) and 2(e)). Importantly, a significantly smaller amount of cleavage products was detected in the reaction with p.Arg81Trp than in the reaction with WT (% incision: 1.8% versus 29.3%) (Figures 2(d) and 2(e)). These results indicate that the DNA glycosylase activity of p.Arg81Trp was severely decreased.

3.4. Impaired Suppressive Activity of MUTYH Type 2 p.Arg81Trp Variant against Mutations Caused by 8OHG. To investigate the ability of MUTYH p.Arg81Trp variant to suppress mutations caused by 8OHG in human cells, we planned to use the piggyBac transposon vector system [31] to establish human cells capable of inducibly expressing MUTYH protein and to perform a *supF* forward mutation assay using the shuttle plasmid pMY189, which contains a single 8OHG in the *supF* gene. First, we established human H1299 cell lines capable of inducibly expressing WT, p.Arg81Trp, or p.Asp208Asn MUTYH using the piggyBac transposon vector system [31]. The expression of MUTYH protein after cumate induction was examined using a Western blot analysis with an anti-MUTYH monoclonal antibody (Figure 3(a)). MUTYH protein was abundantly expressed in cells in which a WT, p.Arg81Trp, or p.Asp208Asn MUTYH expression vector, but not an empty vector, was transposed. An immunofluorescence analysis also showed abundant MUTYH protein expression in cells in which a WT, p.Arg81Trp, or p.Asp208Asn MUTYH expression vector, but not an empty vector, was transposed (Figure 3(b)). The p.Arg81Trp variant, as well as the WT MUTYH protein, was localized in the nucleus, suggesting that the amino acid changes in p.Arg81Trp were unlikely to alter the subcellular localization of the protein in human cells.

Next, the mutation frequencies were compared among the empty vector-transposed human cells and the cumate-inducible stable cells expressing WT or a variant MUTYH

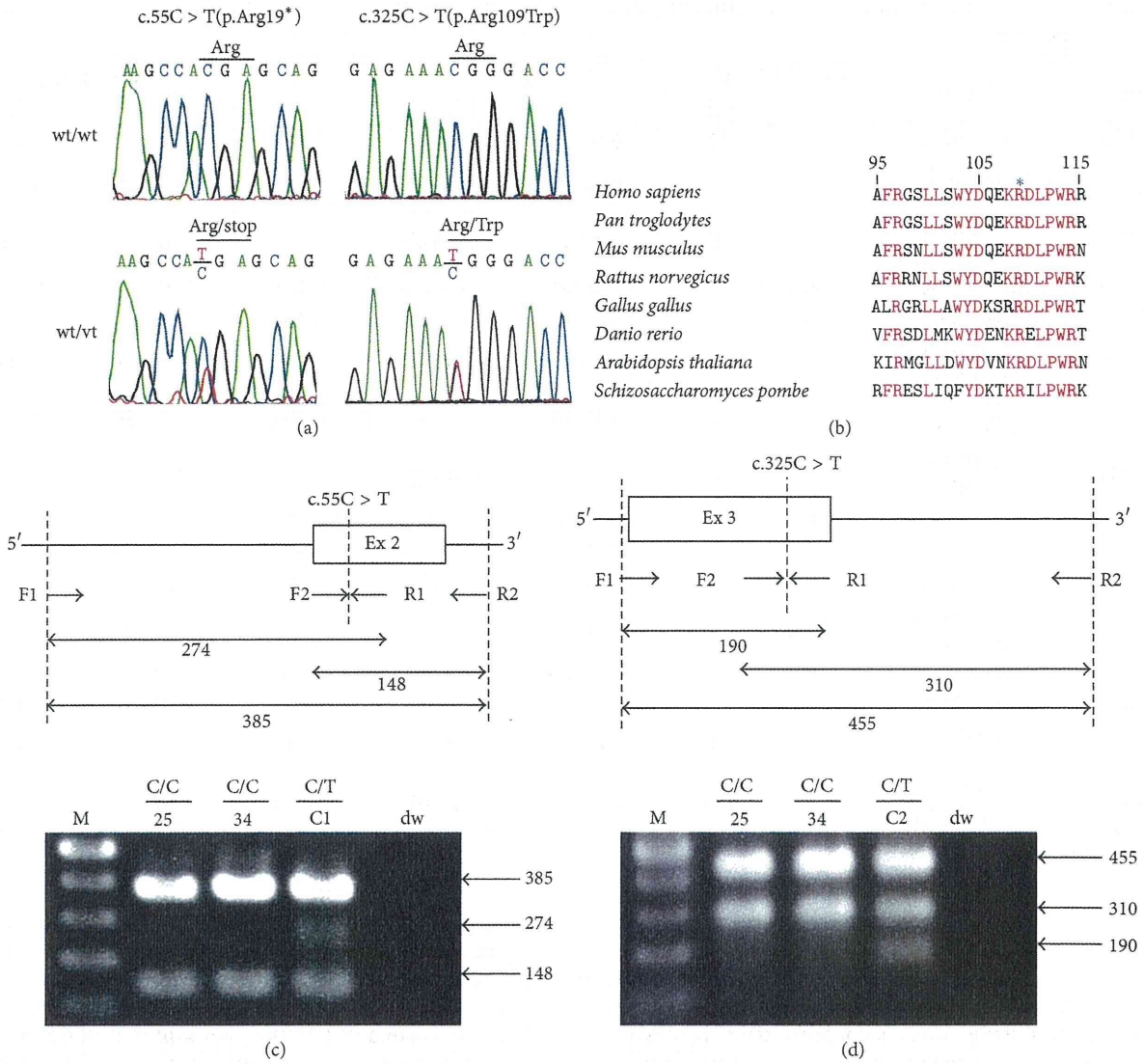

FIGURE 1: Identification and genotyping of c.55C>T (p.Arg19*) and c.325C>T (p.Arg109Trp) variants of the *MUTYH* gene in the Japanese population. (a) Identification of c.55C>T and c.325C>T variants of the *MUTYH* gene in Japanese patients with early-onset CRC. Sequencing electropherograms show a C to T variation at the c.55 and c.325 positions (lower panels). (b) Amino acid sequence alignment of a section of MUTYH among different species. The human MUTYH protein sequence from p.Ala95 to p.Arg115 was compared with the MUTYH sequences of other species. Amino acids exhibiting ≥75% identity among the species are shown in red. The position of p.Arg109 is marked by an asterisk. (c and d) Genotyping of the c.55C>T (c) and c.325C>T (d) variants of the *MUTYH* gene in Japanese individuals without CRC (control individuals). The schematic diagrams of PCR-CTPP used to genotype the c.55C>T and c.325C>T variants are shown in the upper panel. The PCR primers are indicated by the horizontal arrows; and F and R mean forward primer and reverse primer, respectively. The location of each variant is indicated by a vertical dashed line. The PCR product sizes for the primer pairs of F1 and R1, F2 and R2, and F1 and R2 are shown. Representative results of agarose gel electrophoresis of the PCR-CTPP products are shown in the lower panel. The number on the panel indicates the assigned number of control individuals, "C1" and "C2" indicate a case with a variant allele, and "dw" indicates the no template DNA in the PCR. M indicates a size marker.

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 3.2×10^{-2} in the 8OHG-containing pMY189 plasmid and 2.3×10^{-4} in the WT pMY189 in empty vector-transposed cells (Figure 3(c)), representing a 139-fold increase in the mutation frequency with the introduction of 8OHG. The mutation frequency of *supF* in the 8OHG-containing pMY189 plasmid in the WT MUTYH-transposed and p.Arg81Trp variant-transposed, but not p.Asp208Asn-transposed, cells was significantly lower than that in the empty vector-transposed cells (Figure 3(c)). Importantly, the

Table 3: Characteristics of the *MUTYH* and *OGG1* nucleotide variations found in 34 Japanese patients with early-onset colorectal carcinoma.

Gene	Variant	Position[a]	dbSNP ID[b]	PolyPhen-2 prediction (score)[d]	SIFT prediction (score)[d]	PROVEAN prediction (score)[d]	Allele frequency in a Japanese SNP database[e]
MUTYH	c.36+11C>T	45805880	rs2275602	—	—	—	0.048
MUTYH	c.55C>T (p.Arg19*)	45800165	NA[c]	—	—	—	0.002
MUTYH	c.325C>T (p.Arg109Trp)	45799108	NA	Probably damaging (1)	Damaging (0)	Deleterious (−7.22)	NS[f]
MUTYH	c.504+35A>G	45798555	rs3219487	—	—	—	0.12
MUTYH	c.934−2A>G	45797760	rs77542170	—	—	—	0.026
MUTYH	c.1014G>C (p.Gln338His)	45797505	rs3219489	Benign (0.343)	Tolerated (0.136)	Neutral (−1.03)	0.434
MUTYH	c.1118C>T (p.Ala373Val)	45797401	rs35352891	Possibly damaging (0.506)	Tolerated (0.128)	Neutral (−2.324)	0.01
MUTYH	c.1431G>C (p.Thr477Thr)	45796899	rs74318065	—	—	—	0.051
MUTYH	c.1477−40C>G	45796269	rs3219493	—	—	—	0.885
OGG1	c.−23A>G	9791948	rs1801129	—	—	—	0.039
OGG1	c.−18G>T	9791953	rs1801126	—	—	—	0.033
OGG1	c.294G>A (p.Lys98Lys)	9792785	rs1801127	—	—	—	0.015
OGG1	c.748−15C>G	9798140	rs2072668	—	—	—	0.452
OGG1	c.949−89G>T	9798656	NA	—	—	—	NS
OGG1	c.966C>T (p.Asp322Asp)	9798762	NA	—	—	—	NS
OGG1	c.977C>G (p.Ser326Cys)	9798773	rs1052133	Benign (0.121)	Tolerated (0.176)	Neutral (−0.647)	0.446

[a]Genome positions of *MUTYH* and *OGG1* variants are shown according to the reference sequences (GRCh37) of chromosome 1 and chromosome 3, respectively. [b]Identification number of variants according to the database of single nucleotide polymorphisms (dbSNP) located on the homepage of the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/SNP/). [c]NA, not assigned. [d]The accession numbers for the reference proteins of MUTYH and OGG1 are E5KP25 and O15527, respectively. [e]Variant allele frequency in a reference database of genetic variations in the Japanese population (http://www.genome.med.kyoto-u.ac.jp/SnpDB/). [f]NS, not shown.

supF mutation frequency in the p.Arg81Trp-transposed cells was significantly higher than that in the WT MUTYH-transposed cells (1.6×10^{-2} versus 3.3×10^{-3}), meaning that the suppressive activity of the p.Arg109Trp variant against mutations caused by 8OHG in human cells was severely decreased when compared with that of WT MUTYH.

We further investigated what kind of mutation is contained in the *supF* mutant colony in the *supF* forward mutation assay. PCR and gel electrophoresis for the *supF* region of the mutants revealed that the percentage of mutant clones with the same mobility as a WT clone was significantly lower in the WT MUTYH-transposed cells (52%) than the empty vector-transposed cells and the p.Arg81Trp-transposed or p.Asp208Asn-transposed cells (>92%), meaning that the activity to decrease *supF* alterations of the base substitutions or small insertions/deletions caused by 8OHG is lower in the p.Arg81Trp variant than in WT MUTYH (Table 4). Further sequencing analysis of the mutants revealed that a G:C to T:A transversion at position 159 of *supF* among the 8OHG-containing pMY189 was predominant (>91%) in empty vector-transposed cells and p.Arg81Trp-transposed or p.Asp208Asn-transposed cells, while the proportion of the G:C to T:A transversion was significantly reduced in the WT MUTYH-transposed cells (46%) (Table 4, Figure 3(d), Supplementary Figure S2). These results suggest that the suppressive activity of the p.Arg81Trp variant against G:C to T:A mutations caused by 8OHG in human cells was severely reduced, compared with that of WT MUTYH.

4. Discussion

In this study, no biallelic pathogenic mutations were found in 34 Japanese patients with early-onset CRC, although a total of 9 *MUTYH* variants and 7 *OGG1* variants were detected. Among them, the p.Arg19* and p.Arg109Trp *MUTYH* variants were identical to variants previously reported by Vogt et al. [28] in non-Japanese patients with multiple colorectal adenomas and a carcinoma. According to Vogt's report, both the p.Arg19* and p.Ala385Profsx25 mutations were detected in one male patient with multiple (60–70) colorectal adenomas and a carcinoma, and both the p.Arg109Trp and p.Gly396Asp mutations were detected in another male

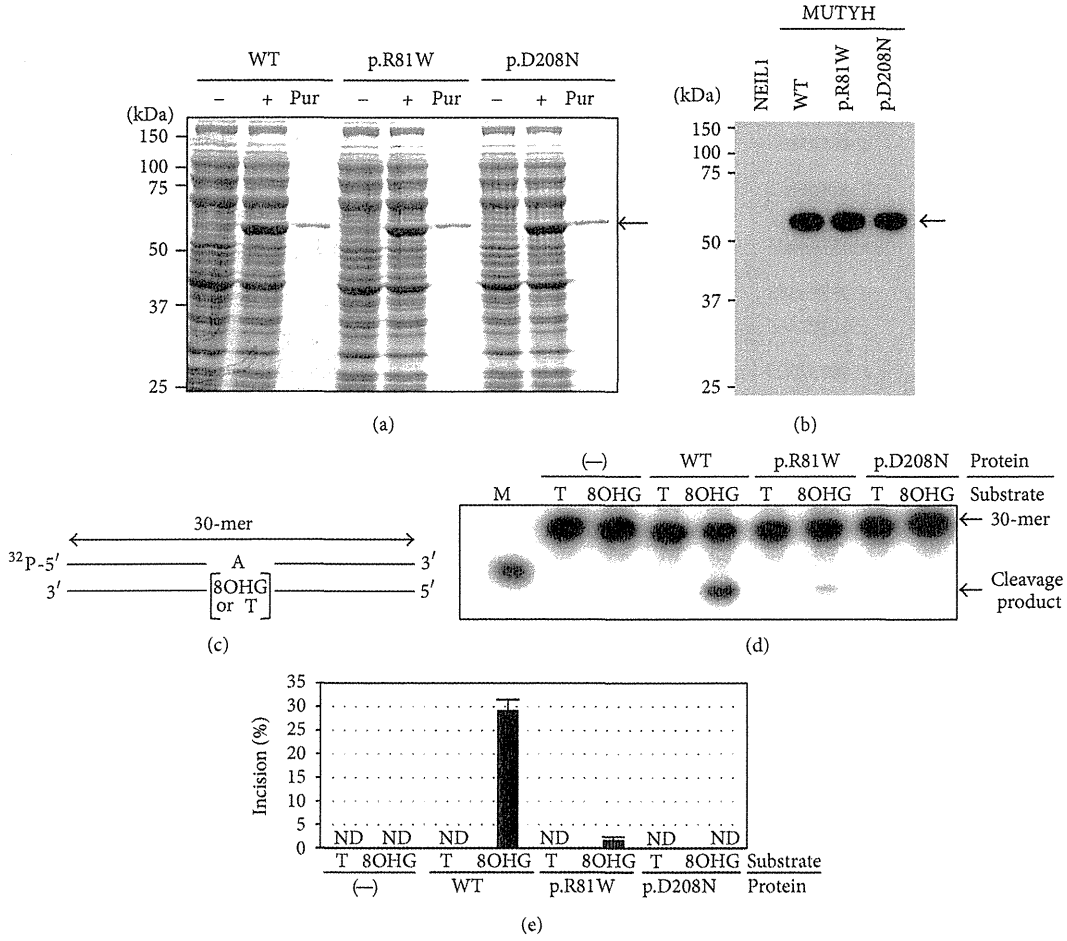

FIGURE 2: Comparison of the DNA glycosylase activity of WT MUTYH and p.Arg81Trp variant MUTYH protein using a DNA cleavage assay. (a) Purification of WT, p.Arg81Trp, and p.Asp208Asn MUTYH type 2 recombinant proteins. The MUTYH proteins were overexpressed and purified using the pET system and TALON metal affinity resins. Representative results for the expression and purification of MUTYH proteins resolved by SDS-PAGE and stained with CBB are shown. "–" and "+" mean the absence and presence, respectively, of IPTG induction, and "Pur" means purified MUTYH type 2 proteins. The arrow points to the MUTYH-His_6 protein band. (b) Western blot of purified MUTYH type 2 proteins. MUTYH-His_6 proteins are indicated by the arrow. Purified recombinant DNA glycosylase NEIL1-His_6 protein, which was previously prepared using the same system as that used for the MUTYH-His_6 protein [38], was included as a negative control. (c) Substrate used in the DNA cleavage assay. ^{32}P-labeled 30-mer double-stranded oligonucleotides containing or not containing a single 8OHG mispair were prepared. (d) Measurement of the DNA glycosylase activity of WT, p.Arg81Trp, and p.Asp208Asn MUTYH type 2 protein on double-stranded DNA containing an 8OHG using the DNA cleavage assay. The reaction mixture was subjected to 20% PAGE. The intact 30-mer oligonucleotides and cleavage products are indicated by the arrows. "M" means a marker oligonucleotide. The amount of cleavage products as a proportion of the total oligonucleotides was calculated as the % incision, and the values are shown in (e). The values are the means ± standard errors of data from three independent experiments. ND means not detected.

patient with multiple (50–100) colorectal adenomas and a carcinoma. Since both patients had the clinical symptoms of MAP and p.Ala385Profsx25 and p.Gly396Asp are pathogenic mutations frequently found in non-Asian MAP patients [12, 28], p.Arg19* and p.Arg109Trp were speculated to be pathogenic mutations. The p.Arg19* and p.Arg109Trp variants correspond to p.Arg5* and p.Arg81Trp, respectively, in the type 2 form. p.Arg5* is strongly suspected to have a defective DNA repair activity because of its extremely short structure, but the p.Arg81Trp missense variant has not been functionally characterized. Therefore, we investigated type 2 p.Arg81Trp MUTYH using a DNA cleavage assay and a *supF* forward mutation assay and found that the abilities of p.Arg81Trp to cleave A:8OHG-containing DNA and to suppress mutations caused by 8OHG were severely reduced. These results suggest that biallelic *MUTYH* or *OGG1* pathogenic mutations are very rare or nonexistent in Japanese patients with early-onset CRC; however, they also suggest that the *MUTYH* alleles of p.Arg19* and p.Arg109Trp detected in our patient series are associated with functional

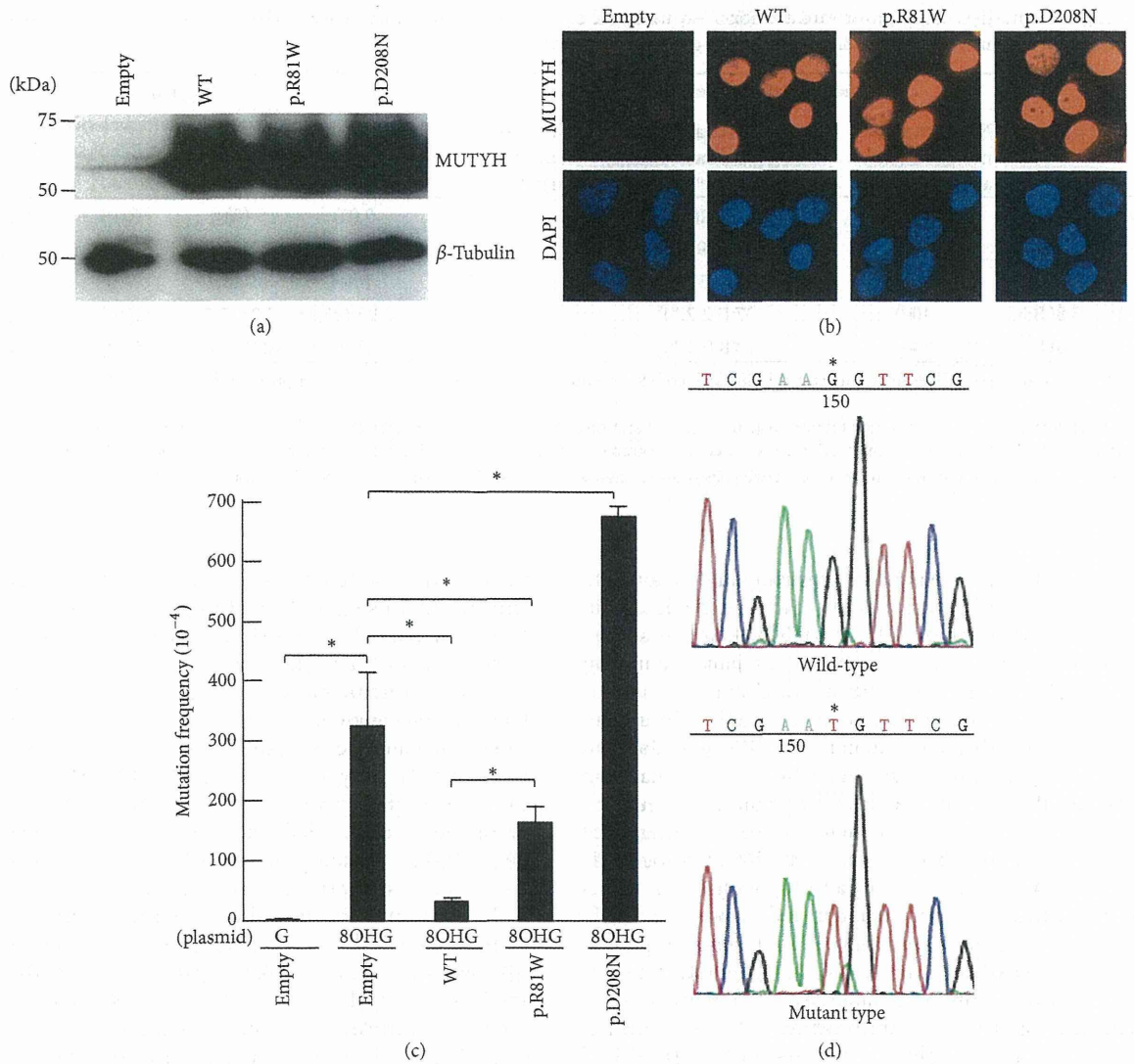

FIGURE 3: Comparison of the activity to suppress the mutation caused by 8OHG between H1299 human cell lines inducibly expressing WT MUTYH and p.Arg81Trp variant MUTYH protein using a *supF* forward mutation assay. (a) Detection of MUTYH proteins in cumate-inducible stable cell lines expressing MUTYH using a Western blot analysis with an anti-MUTYH antibody. Lysates from empty vector-transposed cells and cells inducibly expressing WT type 2 MUTYH, type 2 p.Arg81Trp MUTYH variant, or p.Asp208Asn negative control in the presence of cumate 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 DAPI (blue). (c) 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 proteins. The cell lines used in (a) 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. "8OHG" indicates a pMY189 plasmid containing an 8-hydroxyguanine residue at position 159 of *supF*, while "G" indicates a pMY189 plasmid containing the WT *supF* gene. The data are shown as the means ± standard error. (d) A representative result of a *supF* mutation in an 8OHG-containing pMY189 replicated in empty vector-transposed H1299 cells. Sequencing electropherograms show a G to T (G:C to T:A) mutation at position 159 (marked by asterisks) of the *supF* gene.

impairment. This information would be of great help in diagnosing MAP worldwide, judging from the existence of alleles in both Japanese and other ethnicities.

p.Tyr179Cys and p.Gly396Asp are major pathogenic *MUTYH* mutations for MAP in many ethnicities other than Asian, and some ethnic-specific *MUTYH* mutations, for example, p.Glu480del (Southern Europe), p.Tyr104* (Pakistan), and p.Glu480* (India), have been reported [12, 32]. Regarding pathogenic *MUTYH* mutations in the Japanese population, p.Gly286Glu is the only *MUTYH* mutation for

Table 4: *supF* mutations in a *supF* forward mutation assay using the pMY189 plasmid containing 8-hydroxyguanine (8OHG) at position 159 of *supF* in H1299 human cell lines inducibly expressing MUTYH protein.

Cell line[a]	Plasmid[b]	PCR and gel electrophoresis		Sequencing				
		Number of mutant clones analyzed	Number of mutant clones showing the same mobility as a WT clone (%)	Number of mutant clones analyzed	Number of mutant clones containing a mutation at position 159 of *supF* (%)			
					Total	G:C to T:A	G:C to A:T	G:C to C:G
Empty	G (WT)	22	3 (13.6)	3	0 (0)	0 (0)	0 (0)	0 (0)
Empty	8OHG	40	38 (95.0)[c]	24	24 (100)	22 (91.7)[d]	1 (4.2)	1 (4.2)
WT	8OHG	25	13 (52.0)[c]	13	8 (61.5)	6 (46.2)[d]	1 (7.7)	1 (7.7)
p.R81W	8OHG	40	37 (92.5)[c]	24	23 (95.8)	22 (91.7)[d]	0 (0)	1 (4.2)
p.D208N	8OHG	40	38 (95.0)[c]	24	23 (95.8)	23 (95.8)[d]	0 (0)	0 (0)

[a]Empty vector-transposed H1299 human cancer cell line and H1299 cells inducibly expressing type 2 MUTYH protein of WT, p.Arg81Trp, or p.Asp208Asn were used.
[b]The shuttle plasmid pMY189, containing 8-hydroxyguanine (8OHG) at nucleotide position 159 of *supF*, or a wild-type (WT) pMY189 plasmid was used.
[c]The *P* value for the difference in the proportion between cells transfected with an 8OHG-containing pMY189 plasmid was <0.0001 (Fisher exact test).
[d]The *P* value for the difference in the proportion between cells transfected with an 8OHG-containing pMY189 plasmid was <0.001 (Fisher exact test).

which the resulting protein was experimentally shown to be defective in DNA repair activity and to be found in the Japanese population [14]. The p.Gly286Glu mutation was found as a homozygous mutation in a Japanese patient with colorectal multiple polyps and a carcinoma by Yanaru-Fujisawa et al. [14], and in the paper, mouse MUTYH mutant protein corresponding to the human p.Gly286Glu was shown to have an impaired repair activity. However, this mutation has not been detected in other *MUTYH* mutation screenings performed in Japanese CRC patients [13–15], including the current study, and whether the p.Gly286Glu pathogenic mutation is common in the Japanese population remains unclear. The p.Arg19* detected in our analysis was previously found as a heterozygous mutation in one Japanese patient with CRC reported by Kuno et al. [15], suggesting that it could be relatively common impaired *MUTYH* mutation in the Japanese population. On the other hand, the p.Arg109Trp also detected in our analysis is the first demonstration of such a variant in the Japanese population. Since neither the p.Arg19* nor the p.Arg109Trp variation was observed in our screening of 100 Japanese control individuals, these variants are considered to be relatively rare among the general Japanese population. In addition to the fact that the two major pathogenic *MUTYH* mutations of p.Tyr179Cys and p.Gly396Asp have not been seen in Japanese individuals in previous studies [13–15] or the present study, the p.Arg19* and p.Arg109Trp variations as well as the p.Gly286Glu variation, rather than the p.Tyr179Cys and p.Gly396Asp variations, are thought to account for functionally impaired *MUTYH* alleles in the Japanese population. A combination of these *MUTYH* variations would cause an even higher susceptibility to MAP.

The type 2 MUTYH protein is a nuclear form of MUTYH [4–6], and somatic *APC* (MIM #611731) and *KRAS* (MIM #190070) mutations occur in the nuclear DNA of MAP tumors [9, 10, 33]; therefore, we believed that it would be more appropriate to use type 2, rather than type 1, in a comparative study of MUTYH variants, and we analyzed the DNA repair function of the variant type 2 form *in vitro* and *in vivo* in this study. As a result, an impaired cleavage activity of type 2 p.Arg81Trp towards A:8OHG-containing DNA was clearly demonstrated using a DNA cleavage assay, and a severely reduced activity of the protein to suppress mutations caused by 8OHG in human cells was also clearly revealed using a *supF* forward mutation assay. A combination of the results of two distinct analyses, that is, *in vitro* and *in vivo* analyses, would provide more definitive proof of the pathogenicity of the p.Arg109Trp (type 2 p.Arg81Trp) MUTYH variant. The existence of a patient with multiple colorectal adenomas and a carcinoma, who carried both the p.Arg109Trp variant and the p.Gly396Asp pathogenic mutation, in the report by Vogt et al. [28] also supports the pathogenicity of the p.Arg109Trp MUTYH variant. 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 variant proteins encoded by the two *MUTYH* alleles of the patient are severely reduced, when *MUTYH* gene variations are found in the patient by mutation screening, information on the levels of the repair activities of the MUTYH variant proteins is indispensable for the proper diagnosis of MAP. Thus, our evaluation of the repair activity of the p.Arg109Trp (type 2 p.Arg81Trp) MUTYH variant is clinically useful.

So far, no analyses of the crystal structure of the full-length human MUTYH polypeptide have been reported; therefore, it is difficult to explain fully why an amino acid substitution in p.Arg109Trp leads to a functional impairment. However, p.Arg109 in human MUTYH protein is conserved among *Homo sapiens*, *Pan troglodytes*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Danio rerio*, *Arabidopsis thaliana*, and *Schizosaccharomyces pombe* (Figure 1(b)). Furthermore, mutations resulting in an amino acid exchange from Arg to Trp in codon 185 or 241 have been previously revealed to be pathogenic mutations by functional analyses [34, 35]. In addition, the PolyPhen-2, SIFT, and PROVEAN programs predicted that an amino acid substitution in p.Arg109Trp would alter its protein function (Table 3). Moreover, the screening for nonacceptable polymorphisms (SNAP) program, which predicts the effect of single amino acid substitutions

on protein function (http://www.rostlab.org/services/SNAP) [36], also predicted that the MUTYH type 2 p.Arg81Trp variation was nonneutral. In conjunction with the fact that other various single missense *MUTYH* mutations also exist as pathogenic mutations [12, 33, 37], the notion that p.Arg109Trp is a functionally impaired allele is thought to be acceptable. In the future, a crystal structure analysis of the full-length MUTYH protein and its covalent complex with DNA, in conjunction with the present findings regarding the p.Arg109Trp variant, should contribute to establishing further correlations between the structure and repair function of the MUTYH protein.

The p.Arg19* *MUTYH* variant was detected heterozygously in a patient diagnosed with CRC at 43 years of age and a pathological stage of IIIa, while the p.Arg109Trp *MUTYH* variant was detected heterozygously in a patient diagnosed with CRC at 43 years of age and a pathological stage of I, as summarized in Table 1. The histological classification of CRCs of both patients was well-differentiated adenocarcinoma. Regarding their colorectal polyp status, both patients were recorded as non-FAP, and no other information was available. Therefore, we concluded that the two patients were unlikely to have exhibited any specific clinicopathological characteristics other than early-onset CRC.

In this paper, no biallelic pathogenic mutations in the *MUTYH* and *OGG1* genes were found in 34 Japanese patients with early-onset CRC. Since the sample size was relatively small, we could not make a robust conclusion; however, this result suggests that biallelic *MUTYH* or *OGG1* pathogenic mutations are very rare or possibly nonexistent in Japanese patients with early-onset CRC. A future study with a large number of Japanese patients with early-onset CRC is needed to obtain a robust conclusion regarding this issue.

In conclusion, our results suggested that biallelic *MUTYH* or *OGG1* pathogenic mutations are rare among Japanese patients with early-onset CRC; however, they also suggested that the p.Arg19* and p.Arg109Trp *MUTYH* variants that were detected in our Japanese patient group are functionally impaired alleles. This information is likely to be very useful in the diagnosis of MAP worldwide. Additionally, since recent technological progress in genome sequencing analysis has contributed to efficient and rapid genome screening, an increase in the number of novel *MUTYH* variants can be expected in the future. Our analysis system for determining the repair abilities of MUTYH variants, as successfully performed in this study, might be useful for characterizing such newly detected variants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Genes to Cells

YB-1 promotes transcription of *cyclin D1* in human non-small-cell lung cancers

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Cyclin D1, an oncogenic G1 cyclin, and YB-1, a transcription factor involved in cell growth, are both over-expressed in several human cancers. In human lung cancer, the functional association between YB-1 and cyclin D1 has never been elucidated. In this study, we show YB-1 is involved in the transcription of *cyclin D1* in human lung cancer. Depletion of endogenous YB-1 by siRNA inhibited progression of G1 phase and down-regulated both the protein and mRNA levels of cyclin D1 in human lung cancer cells. Forced over-expression of YB-1 with a cyclin D1 reporter plasmid increased luciferase activity, and ChIP assay results showed YB-1 bound to the *cyclin D1* promoter. Moreover, the amount of *YB-1* mRNA positively correlated with *cyclin D1* mRNA levels in clinical non-small-cell lung cancer (NSCLC) specimens. Immunohistochemical analysis also indicated YB-1 expression correlated with cyclin D1 expression in NSCLC specimens. In addition, most of the cases expressing both cyclin D1 and CDC6, another molecule controlled by YB-1, had co-existing YB-1 over-expression. Together, our results suggest that aberrant expression of both cyclin D1 and CDC6 by YB-1 over-expression may collaboratively participate in lung carcinogenesis.

Introduction

Lung cancer is the leading cause of cancer death worldwide, including in Japan (Jemal *et al.* 2011). In particular, non-small-cell lung cancer (NSCLC) accounts for 85% of human lung cancers. Despite significant progress in NSCLC treatment, such as chemotherapy, radiotherapy, and surgery, the prognosis for patients with NSCLC has been improved only minimally and the 5-year survival rate remains at 15% (Molina *et al.* 2008). Recent advances in the molecular characterization of NSCLC have enabled the identification of numerous cell growth and proliferation pathways that are disrupted in these tumors. These findings have provided insights into the mechanisms of tumor development in various histologic subtypes of NSCLC and have pointed toward targeted treatment strategies (Sanders & Albitar 2010). Li Ding *et al.* identified several mutated genes in NSCLC, including tumor suppressor and tyrosine kinase genes that may function as proto-oncogenes. Furthermore, the authors found a significant excess of mutations and copy number alterations in genes from

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the MAPK, p53, Wnt, cell cycle, and PI3K mTOR signaling pathways, suggesting that these pathways are linked to the disease development and progression (Okudela *et al.* 2007; Ding *et al.* 2008). Many of the genes and proteins involved in lung cancer pathogenesis can be categorized into three major pathways: cell cycle regulation, apoptosis, and angiogenesis. The molecules in these three pathways have been also investigated from the standpoint of their influence on the clinical outcome of NSCLC (Singhal *et al.* 2005).

Cyclins and their associated cyclin-dependent kinases (CDK) are the central machinery that control cell cycle progression. In G1 phase, once the Rb protein is phosphorylated by the cyclin D1/CDK complex, E2F is released, allowing transcription of other CDKs, cyclins, and S phase proteins, thereby promoting the transition from G1 to S phase of the cell cycle. During S phase, cyclin D1 is phosphorylated by glycogen synthase kinase 3β, which promotes nuclear export and ubiquitination of cyclin D1 by the SCF^{Fbox4}-αB crystalline complex, leading to proteasome-dependent degradation of cyclin D1. Other F-box proteins, such as Fbxw8, Fbox31, and Skp2, have also been reported as E3 ligases for cyclin D1, but knockout mouse analyses of these genes indicated that the contribution of these reported E3s toward degradation of cyclin D1 were not significant (Kanie *et al.* 2012). Therefore, rather than the ubiquitin proteasome-mediated degradation system, transcriptional regulation may be more important in controlling cyclin D1 expression.

Induction of cyclin D1 in the G1 phase depends on several growth factors, such as EGF and IGF, and several hormones, including estrogen (17β-estradiol:E2) and angiotensin II, until the restriction point (Klein & Assoian 2008; Witzel *et al.* 2010). Until the point of irreversible transition from G1 to S phase, cyclin D1 expression is tightly regulated at the level of transcriptional activation. Several transcriptional factors, including TCF/LEF, CREB, NF-κB, AP-1, and SP1, have been found to transactivate the *cyclin D1* promoter, and some transcriptional suppressors, such as Tob1 and Jumonji, have been reported to down-regulate *cyclin D1* gene promoter activity (Guttridge *et al.* 1999; Lee *et al.* 1999; Shtutman *et al.* 1999; Bakiri *et al.* 2000; Nagata *et al.* 2001; Boulon *et al.* 2002; Klein & Assoian 2008; Witzel *et al.* 2010). Over-expression of cyclin D1 is thought to enhance cell cycle progression from G1 to S phase and increase cell proliferation. The cyclin/CDK kinase complexes also target substrates that play important roles in centrosome duplication, mitochondrial function, cell growth, cell adhesion and motility, and cytoskeletal modeling. Therefore, elucidation of the transcriptional regulation of *cyclin D1* is essential to understand its role in the tumorigenesis of NSCLC (Musgrove *et al.* 2011).

The Y-box-binding protein 1 (YB-1) is a member of the cold-shock domain protein superfamily that binds to an inverted CCAAT box, named the Y-box sequence, in the promoter regions of target genes. YB-1 is a multifunctional protein and regulates translation and transcription in the nucleus and cytoplasm. YB-1 has been reported to be a negative prognostic factor for several cancers, including breast, ovarian, and lung cancers and synovial sarcoma (Kohno *et al.* 2003). YB-1 has also been shown to up-regulate the transcription of cell-cycle-related molecules, including *cyclin A*, *cyclin B* (Jurchott *et al.* 2003), and *CDC6* (Basaki *et al.* 2010). However, we recently showed that YB-1 binds and represses the CDK inhibitor $p16^{ink4}$ gene (Kotake *et al.* 2013). This implies that YB-1 participates in cell cycle progression both via positive and negative regulatory pathways, that is, functioning as both an accelerator and a brake.

In lung cancer, increased expressions of not only YB-1 but also cyclin D1 were found independently, but the correlated expression and functional relationship between YB-1 and cyclin D1 have never been addressed in lung cancer (Eliseeva *et al.* 2011; Lasham *et al.* 2013). In the present study, we investigated whether YB-1 controls cyclin D1 expression in human lung cancers, with particular focus on our identification of several Y-boxes in the *cyclin D1* promoter. Moreover, we also investigated the correlation of CDC6, another target of YB-1, with expression of cyclin D1 and YB-1 in clinical samples of NSCLC.

Results

Effects of YB-1 knockdown in lung cancer cell lines

Based on the reports suggesting that YB-1 promotes cell cycle progression, we first evaluated whether the cell cycle of human lung cancer cell lines was positively regulated by YB-1. We investigated the effects of YB-1 knockdown on cellular proliferation of two lung cancer cell lines (A549 and H1299) by transfecting cells with YB-1 siRNA (YB-1-i #1) for 48 h. The proliferation of A549 and H1299 cells was suppressed to 41.9% and 45.7%, respectively, by YB-1 depletion (Fig. 1A), suggesting that YB-1 participates in the cell proliferation of these lung cancer cells. We next examined effects of YB-1 depletion on the cell cycle. A549 and H1299 cells were transfected with YB-1 siRNA (YB-1-i #1 or