with the cDNA and a QuantiTect SYBR Green PCR kit (Qiagen). The following PCR primers were used: 5′-AAA GTC TTT GCT AAA GCA AGA ATC ACA G-3′ and 5′-CTT CGA AGA GAA GGG ATG CG-3′ for the RSPO2 transcript, 5′-TTG AAG CCA CAT GTT TTT CCC-3′ and 5′-CAC CTT GGA AAG TGC CTT GAC-3′ for the RSPO3 transcript, and 5′-GCT CAG ACA CCA TGG GGA AG-3′ and 5′-TGT AGT TGA GGT CAA TGA AGG GG-3′ for the GAPDH transcript. The T/N ratios were calculated by dividing the normalized transcript amounts in the cancerous tissue by the amounts in the non-cancerous tissue.

Western blot

A western blot analysis using an anti-RSPO2 polyclonal antibody (Proteintech Group, Chicago, IL, USA), an anti-FLAG polyclonal antibody (Sigma-Aldrich, St. Louis, MO, USA), or an anti-β-tubulin monoclonal antibody (clone 2-28-33; Sigma-Aldrich) were performed using a previously described method [ ].

Immunohistochemical staining

Sections of formalin-fixed, paraffin-embedded tissue samples were used for immunohistochemical staining, which was performed using a Histofine Simple Stain MAX PO kit (Nichirei, Tokyo, Japan) as described previously [ ]. The primary antibodies were as follows: anti-CDX2 (BioGenex, San Ramon, CA, USA), anti-cytokeratin 7 (CK7) (DAKO, Tokyo, Japan), anti-cytokeratin 20 (CK20) (DAKO), anti-MLH1 (BD Pharmingen, San Diego, CA, USA), anti-MSH2 (clone FE11; Calbiochem, San Diego, CA, USA), and anti-p53 (clone DO7, DAKO). Hematoxylin-eosin (H&E) staining was also performed.

Mutation search

Genomic DNA were extracted from the tissue samples containing RSPO fusion transcripts using a DNeasy kit (Qiagen) and were examined for somatic mutations in the DNA sequences in the entire coding sequence of the *APC* and *p53* genes. PCR amplification was performed in 20-μL reaction mixtures containing HotStarTaq DNA polymerase (Qiagen) and a direct sequencing was performed. Information regarding the PCR primers is available upon request.

Establishment of stable inducible cell lines

The human *RSPO2* and *PTPRK-RSPO3* cDNAs with the FLAG sequence at the C-terminus were amplified using a PCR with *PfuUltra* Hotstart DNA polymerase (Stratagene, La Jolla, CA, USA) and the amplified sequences were then inserted into a piggyBac cumate switch inducible vector (System Biosciences, Mountain View, CA, USA) at the *Nhe*I and *Not*I restriction enzyme sites. A DLD-1 colon cancer cell line (American Type Culture Collection; Manassas, VA, USA) was transfected with the piggyBac cumate switch inducible vector for RSPO2 or PTPRK-RSPO3 expression, together with a piggyBac transposase vector (System Biosciences). To establish stable inducible cell lines, positively transposed cells were selected using puromycin (1.3 μg/mL). In the piggyBac cumate switch inducible vector system, the addition of cumate solution (System Biosciences) to puromycin-selected cells induces the expression of the inserted gene.

Cell proliferation assay

The cells were seeded, and the number of viable cells was counted after 72 h using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions.

Statistical analysis

The statistical analysis was performed using the Wilcoxon matched pairs test or Dunnett's test and JMP version 9 software (SAS Institute, Cary, NC, USA).

Results

In this study, 75 CRCs and 121 NSCLCs in the Japanese population were examined for EIF3E-RSPO2 and PTPRK-RSPO3 fusion transcripts using RT-PCR and subsequent sequencing analyses. Although the expression of EIF3E-RSPO2 or PTPRK-RSPO3 was not detected in any of the NSCLCs, EIF3E-RSPO2 and PTPRK-RSPO3 fusion transcripts were detected in two CRCs and one CRC, respectively (Fig. a). The RSPO fusion transcripts were expressed in the cancerous tissues but not in the non-cancerous tissues in the CRC cases (Fig. a). Sequencing of one of the RT-PCR products revealed that the fusion detected in case no. 15 was between exon 1 of EIF3E and exon 2 of RSPO2 (Fig. b). This fusion form was the same as that in a previous report [ ] and was expected to produce a full RSPO2 protein driven by the *EIF3E* promoter, since the first methionine of RSPO2 is located within exon 2. The sequencing of another EIF3E-RSPO2 fusion product detected in case no. 44 revealed the fusion of exon 1 of EIF3E to exon 2 of RSPO2 by a 351-bp insertion that was unrelated to EIF3E or RSPO2 (Fig. c). This was a novel fusion form of EIF3E-RSPO2 and was also expected to produce a full RSPO2 protein driven by the *EIF3E* promoter, similar to the situation for the form detected in case

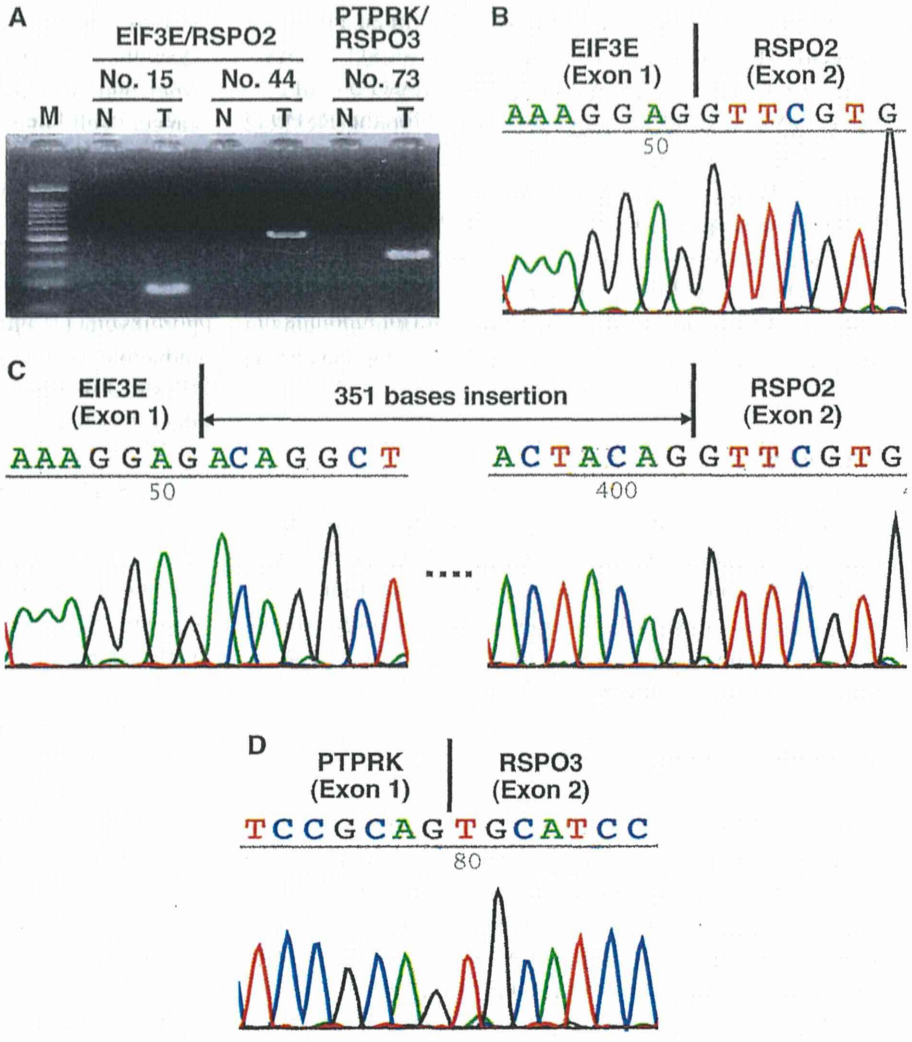

Fig. 1 Detection of RSPO fusion transcripts in CRCs arising from a Japanese population. **a** RT-PCR analysis of cDNA derived from non-cancerous colorectal tissue (N) and colorectal cancerous tissue (T) for the detection of EIF3E-RSPO2 and PTPRK-RSPO3 fusion transcripts. RT-PCR products were subsequently subjected to agarose gel electrophoresis. The case no. is shown above the panel. M, size marker. **b** Sequencing analysis of the RSPO2 fusion transcripts in case no. 15. A sequencing electropherogram revealed a fusion occurring between exon 1 of EIF3E and exon 2 of RSPO2. **c** Sequencing analysis of the RSPO2 fusion transcripts in case no. 44. A sequencing electropherogram revealed exon 1 of EIF3E to be connected to exon 2 of RSPO2 by a 351-bp insertion. **d** Sequencing analysis of the RSPO3 fusion transcripts in case no. 73. A sequencing electropherogram revealed a fusion occurring between exon 1 of PTPRK and exon 2 of RSPO3

no. 15. Sequencing of the RT-PCR product of PTPRK-RSPO3, which was detected in case no. 73, revealed a fusion between exon 1 of PTPRK and exon 2 of RSPO3 (Fig. 1d). This fusion form was the same as that in a previous report [7], and the resultant chimeric protein was in-frame and retained the CR and TSR domains of RSPO3 [8]. All the CRCs containing an RSPO fusion were well to moderately differentiated adenocarcinomas that exhibited advanced growth (pT factor: pT2-pT4) (Table 2). Furthermore, two of the three cases exhibited lymph node metastasis. All the above findings suggest that RSPO fusion transcripts are expressed in a subset of CRCs, but not NSCLCs, in the Japanese population.

Next, to investigate the expression levels of RSPO2 and RSPO3 transcripts in CRC, we examined the mRNA expression of both RSPO2 and RSPO3 in 75 CRCs using a QRT-PCR analysis and calculated the ratio of the level of RSPO mRNA expression in the cancerous tissue to the level in the corresponding non-cancerous tissue (T/N ratio). Reduced RSPO2 and RSPO3 expression levels (T/N ratio <1) were observed in 72 (96.0 %) and 71 (94.7 %) of the 75 primary CRCs, respectively (Fig. 2a). Moreover, a significant difference was detected in the RSPO expression level between the cancerous tissue and the corresponding non-cancerous tissue using a statistical analysis ($P < 0.0000001$ for both RSPO2 and RSPO3 using the Wilcoxon matched pairs test). However, interestingly, an increased expression (T/N ratio >2) of RSPO2 was observed in two CRCs containing an RSPO2 fusion, and an increased expression of RSPO3 was observed in a CRC containing an RSPO3 fusion (Fig. 2a). Furthermore, an increased expression of RSPO2 protein was observed in a CRC containing an RSPO2 fusion transcript (case no. 15) (Fig. 2b), although the expression level of RSPO protein was not measured in the other two RSPO fusion-positive cases because of a lack of protein samples. These results suggested that RSPO mRNA expression is downregulated in most CRCs but is upregulated in CRCs containing RSPO fusion transcripts.

Table 2 Clinical profiles of the cases with CRC containing RSPO fusion transcripts and the pathological, immunohistochemical, and mutational statuses of the CRCs

Characteristic	Case no. 15	Case no. 44	Case no. 73
RSPO fusion	EIF3E-RSPO2	EIF3E-RSPO2	PTPRK-RSPO3
Age (years)	67	66	47
Sex	Male	Female	Female
Histology	W-M	W-M	W-M
Location	Rectum	Sigmoid colon	Sigmoid colon
pT factor	pT3	pT3	pT3
Lymph node metastasis	Positive	Negative	Positive
CDX2 expression	Positive	Positive	Positive
CK7 expression	Negative	Negative	Negative
CK20 expression	Positive	Positive	Positive
MLH1 expression	Positive	Positive	Positive
MSH2 expression	Positive	Positive	Positive
Somatic *APC* gene mutation	Wild-type	NE	Wild-type
Somatic *p53* gene mutation	Wild-type	NE	R175H mutation

W-M well to moderately differentiated adenocarcinoma, *NE* not examined

To characterize the CRCs containing RSPO fusion transcripts, we performed an immunohistochemical analysis for three RSPO fusion-positive CRCs. The analysis revealed that all the adenocarcinomas were positive for CDX2 and CK20 but were negative for CK7 (Fig. 3a–d; Table 2), meaning that the immunophenotype of these carcinomas was compatible with that of typical adenocarcinomas of colorectal origin. In addition, three adenocarcinomas were positive for MLH1 and MSH2 mismatch repair proteins (Fig. 3e, f; Table 2), suggesting that they did not contain defects in the mismatch repair system. These findings suggest that RSPO fusion-containing CRC has a CDX2 cell lineage and positive mismatch repair protein expression.

We next examined whether the CRCs containing the RSPO fusion transcripts also contained somatic *APC* and *p53* gene mutations, which are common in CRC [7, 10–12]. Since the genomic DNA of case no. 44 was not available for study, a mutational analysis of the entire coding region of *APC* and *p53* was only performed for the other two CRCs. No somatic *APC* mutations were detected in either of the CRCs, while a somatic c.524G>A mutation associated with an amino acid exchange from Arg to His at codon 175 (p.R175H) was detected in *p53* in case no. 73 (Fig. 4a; Table 2). The fact that the missense mutation was detected in the DNA binding region of the p53 protein suggested that the mutant p53 protein was stable in the cancer cells. We therefore performed immunohistochemical staining for p53 in case no. 73, and the results showed the nuclear accumulation of p53 exclusively in the cancer cells (Fig. 4b). These results suggested that a somatic *p53* mutation, but not an *APC* mutation, occurred in a subset of CRCs containing the RSPO fusion transcripts.

Finally, to determine whether RSPO fusion expression leads to an increase in the malignant potential of colorectal cells, we attempted to examine the functional effect of RSPO fusion proteins in colorectal cells. A RSPO2 protein and a fusion protein of PTPRK and RSPO3 are expected to be produced from EIF3E-RSPO2 and PTPRK-RSPO3 fusion transcripts, respectively. First, we established human DLD-1 colon cancer cells capable of inducibly expressing RSPO2 or PTPRK-RSPO3 with the FLAG tag at the C-terminus using the piggyBac transposon vector system [13]. In detail, DLD-1 cells were transfected with a piggyBac cumate switch inducible vector for the expression of RSPO2 or PTPRK-RSPO3 together with the piggyBac transposase vector; positively transposed cells were then selected using puromycin. We also transfected the cells using an empty (parental) piggyBac cumate switch inducible vector and transposase vector. The expression of RSPO2 and PTPRK-RSPO3 protein after cumate induction was examined using a western blot analysis (Fig. 5a). RSPO-FLAG proteins were abundantly expressed in RSPO-transposed cells after cumate induction, but not in empty vector-transposed cells. Next, the effect of RSPO fusion proteins on the growth of colorectal cells was compared between empty vector-transposed cells and RSPO-transposed cells. RSPO2- and PTPRK-RSPO3-transposed cells showed a statistically significant increase in cell proliferation, compared with the empty vector-transposed cells (Fig. 5b). This result suggests that RSPO2 and PTPRK-RSPO3 overexpression endowed colorectal cells with an increased growth ability.

Discussion

In this study, the expression of RSPO fusion transcripts was found in 3 (4.0 %) of 75 CRCs in a Japanese population, but no expression of RSPO fusion transcripts was detected in any of the 121 NSCLCs that were examined. Two CRCs contained EIF3E-RSPO2 fusion transcripts and another CRC contained PTPRK-RSPO3 fusion transcripts. In one of the two EIF3E-RSPO2 fusion-positive CRCs, a novel fusion form was detected: i.e., exon 1 of EIF3E was connected to exon 2 of RSPO2 by a 351-bp insertion. RSPO mRNA expression was upregulated in the three CRCs containing RSPO fusion transcripts, while it was downregulated in almost all the other CRCs. The RSPO fusion-containing CRC exhibited a CDX2 cell lineage, positive mismatch repair protein expression, and wild-type

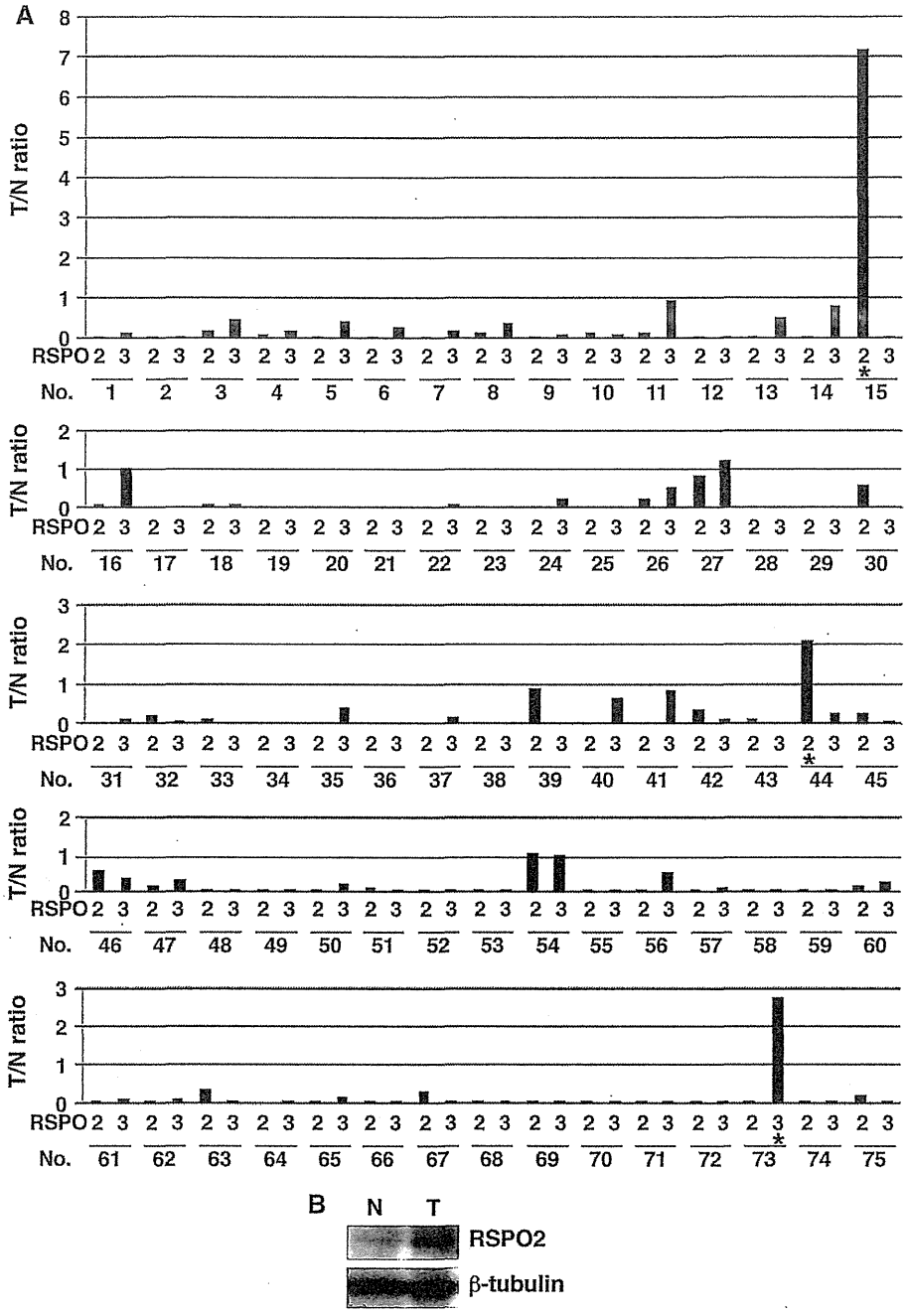

Fig. 2 Comparison of RSPO expression in primary CRCs. **a** Comparison between RSPO mRNA expression in cancerous tissues from 75 primary CRC and corresponding non-cancerous colorectal tissues, as determined using a QRT-PCR analysis. After normalizing the amounts of RSPO2 or RSPO3 transcripts to those of the GAPDH transcripts, the T/N values were calculated by dividing the amount of normalized transcripts in cancerous tissue by the amount in the corresponding non-cancerous tissue. RSPO fusion-positive CRCs are marked by asterisks. **b** Comparison of RSPO2 protein expression in a primary CRC containing the RSPO2 fusion mRNA transcript (case no. 15). The levels of RSPO2 protein expression in the cancerous tissue (T) and the corresponding non-cancerous tissue (N) were measured using a western blot analysis with anti-RSPO2 antibody. The expression of β-tubulin protein was analyzed as an internal control

APC allele. A somatic *p53* missense mutation status was not common among RSPO fusion-positive CRCs. The forced expression of RSPO2 and PTPRK-RSPO3 proteins derived from RSPO fusion transcripts endowed colorectal cells with an increased growth ability. All the above results suggested that the expression of RSPO fusion transcripts is related to a subset of CRCs in the Japanese population.

Seshagiri et al. [ ] reported the expression of RSPO fusion transcripts in 7 (10.3 %) out of 68 CRCs arising in the U.S. population, and in the present study, the expression of RSPO fusion transcripts were identified in 3 (4.0 %) out of 75 CRCs arising in the Japanese population. These results suggest that RSPO fusion is a recurrent event in CRCs in multiple populations. Regarding the difference in the incidence between the U.S. population and the Japanese population, the difference was not statistically significant, possibly because of the relatively small numbers of cases that were examined in both studies; this finding suggests an absence of apparent racial differences in the incidence. The absence of the expression of RSPO fusion transcripts in the NSCLCs that were studied suggests that RSPO fusions occur in an organ-specific manner. However,

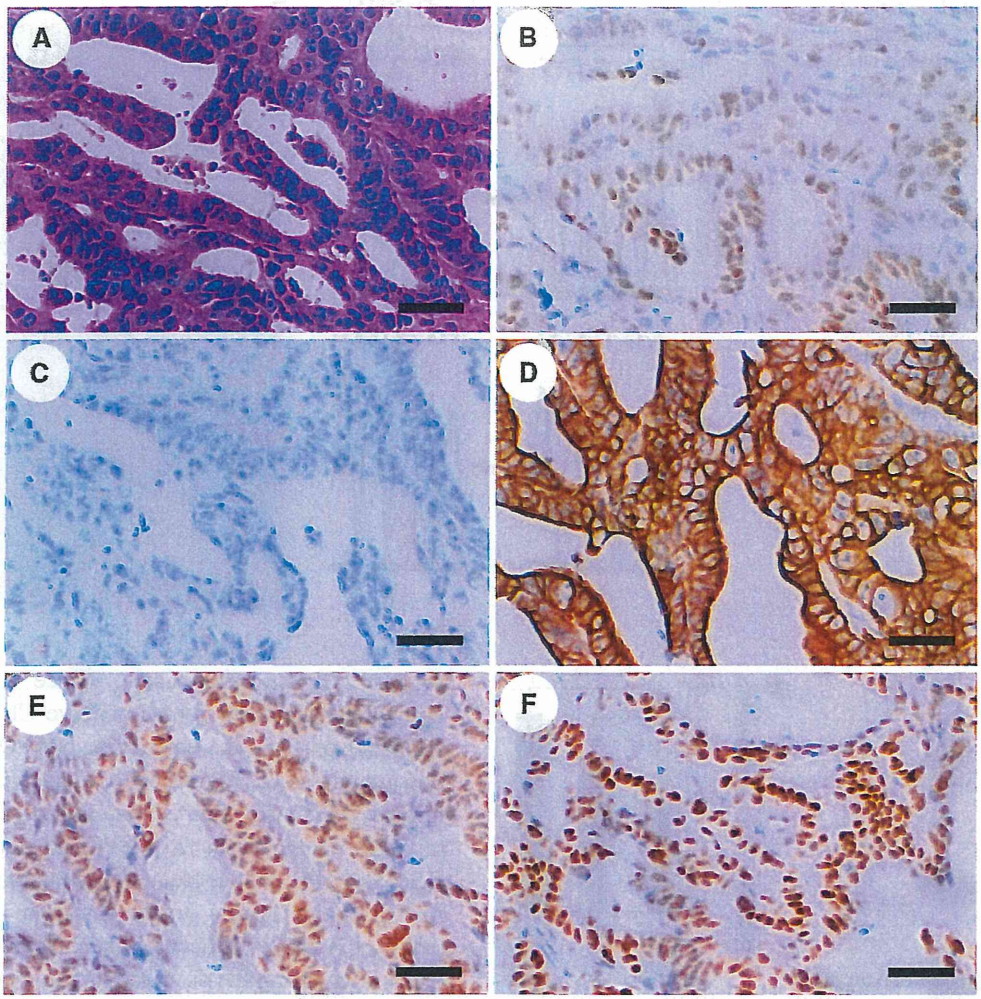

Fig. 3 Immunohistochemical analysis of CRC containing the RSPO fusion transcript. **a** Microscopic photo (H&E) of the CRC in case no. 15. The histological type of the CRC was moderately differentiated adenocarcinoma. **b–f** The adenocarcinoma was immunohistochemically positive for CDX2 (**b**), CK20 (**d**), MLH1 (**e**), and MSH2 (**f**) and immunohistochemically negative for CK7 (**c**). Scale bar, 50 μm

the absence of NSCLCs expressing RSPO fusion transcripts in our series might be due to the small number of examined cases, and a future study investigating a large sample is needed to clarify this point. RSPOs are a family of cysteine-rich secreted proteins containing a TSR domain that have roles in the activation of the Wnt signaling pathway, which is often aberrantly regulated in CRC [8, 14]. The results that the expression of RSPO fusion transcripts were mutually exclusive with somatic *APC* mutation in both our present report and the report by Seshagiri et al. [7] suggests that RSPO fusion may promote Wnt signaling in colorectal tumorigenesis. The upregulation of RSPO mRNA transcript in CRCs containing RSPO fusions and an increase in growth induced by the forced expression of RSPO fusion in colorectal cells in our data also support the hypothesis that RSPO fusion is related to a subset of CRCs. The RSPO upregulation implicates that RSPO fusion is a potential target for therapeutic intervention in RSPO fusion-positive CRC. Thus, it may be possible that CRC patients positive for RSPO fusion could receive some clinical benefit in the future.

In the present study, not only a known fusion between exon 1 of EIF3E and exon 2 of RSPO2 but also a novel fusion variant, in which exon 1 of EIF3E was connected to exon 2 of RSPO2 by a 351-bp insertion, was identified. Although the origin of the insertion sequence was unknown, the insertion of an unknown sequence at a fusion point was previously reported in an EML4-ALK fusion present in an NSCLC [15]. Since the first methionine of RSPO2 protein is located in exon 2 of the *RSPO2*, in both fusion forms, a full-length RSPO2 with CR and TSR domains, which is the same as wild-type RSPO2, is expected to be produced, resulting in no difference in the RSPO2 protein form between the two kinds of fusion transcripts. However, the distinct lengths of the 5′ untranslated regions between a known form and a novel

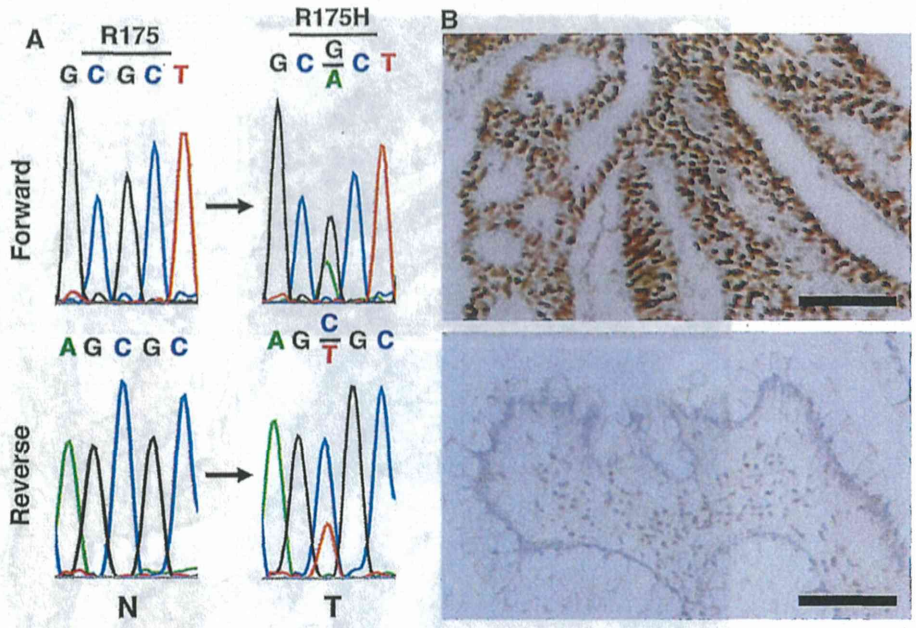

Fig. 4 Somatic *p53* mutation detected in a CRC containing RSPO fusion transcripts. **a** DNA sequence analysis of *p53* exon 10 in the non-cancerous colorectal tissue (N) and colorectal cancerous tissue (T) of case no. 73. A somatic R175H mutation of *p53* is shown in both the forward and reverse sequences. **b** Immunohistochemical detection of p53 accumulation in the carcinoma from case no. 73 (*upper panel*). Non-cancerous colorectal mucosa from the case is provided as a control (*lower panel*). Scale bar, 100 μm

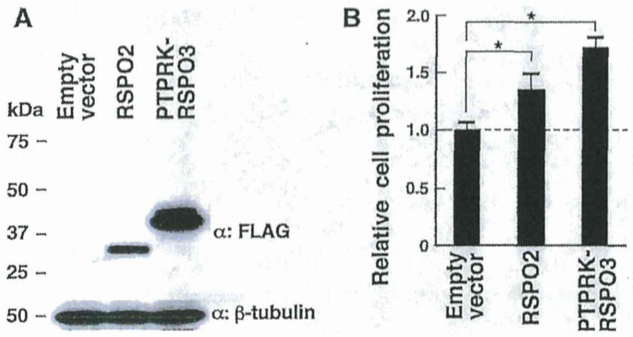

Fig. 5 Effect of RSPO2 and PTPRK-RSPO3 protein expression on the proliferation of colorectal cells. **a** Establishment of DLD-1 human colon cancer cell lines inducibly expressing RSPO2 or PTPRK-RSPO3 protein with the FLAG tag at the C-terminus. RSPO2 and PTPRK-RSPO3 proteins were detected in DLD-1 stable cell lines expressing RSPO-FLAG in the presence of cumate using a western blot analysis with an anti-FLAG antibody. β-Tubulin protein was also analyzed as an internal control. **b** Measurement of the proliferation of empty vector-transposed DLD-1 cells and RSPO2- or PTPRK-RSPO3-overexpressing cells, using a cellular proliferation assay with a Cell Counting Kit-8; 72 h after plating, the number of viable cells was counted by measuring the reduction in the tetrazolium monosodium salt WST-8. Data are shown as the means and standard deviation of three independent experiments. The average number of proliferating cells among the empty vector-transposed cells was set at 1.0. *P* values were calculated using Dunnett's multiple comparison test; *statistically significant

form might affect the level of RSPO2 expression. Regarding the difference between RSPO2 fusion-positive and negative situations, RSPO2 protein production is driven by the *EIF3E* promoter, but not the *RSPO2* promoter, in fusion-positive cancer.

In this study, reduced RSPO2 and RSPO3 expression were observed in 72 (96.0 %) and 71 (94.7 %) of the 75 primary CRCs, respectively. Since *RSPO* genes are upregulated in RSPO fusion-positive CRCs, if these cases are removed from the calculation, RSPO2 expression was downregulated in 72 (98.6 %) of the 73 RSPO2 fusion-negative CRCs and RSPO3 expression was downregulated in 71 (95.9 %) of the 74 RSPO3 fusion-negative CRCs. Since the *APC* gene is frequently mutated in CRC [16], the *APC* mutation leading to the activation of Wnt signaling pathway is likely to occur in such RSPO fusion-negative CRCs. Regarding the downregulation of RSPO expression, it is speculated that epigenetics, such as changes in the methylation of cytosines in the 5′ promoter region DNA, changes in the histone and chromatin structure, and alterations in microRNA expression [17], might be involved in it. Further study is needed regarding this point in the future. Regardless, the fact that the upregulation of RSPO expression is almost specific for RSPO fusion-positive CRCs suggests that screening for both the presence or absence of known RSPO fusion forms and the RSPO expression level leads to the detection of CRCs with no known RSPO fusion form and an increased RSPO expression level. The detailed examination of such cases might result in the identification of a novel fusion partner of RSPO.

RSPO fusion-positive CRCs showed positive mismatch repair protein expression in this study. Approximately 15 % of sporadic CRCs have defects in mismatch repair machinery, and a decreased expression of MLH1 and/or MSH2 because of epigenetic changes is a major cause of the defects in sporadic CRCs [18–20]. Our results suggest that RSPO fusions occur in mismatch repair-proficient CRC.

In summary, RSPO fusions including a novel variant form were found in three (4.0 %) of the 75 CRCs, but none of the 121 NSCLCs, in a Japanese population. RSPO fusion-positive CRCs showed the upregulation of RSPO mRNA expression, a CDX2 cell lineage, positive mismatch repair protein expression, and a wild-type *APC* allele. The forced expression of RSPO proteins based on the RSPO fusion transcripts led to an increase in the growth of colorectal cells. These results suggested that RSPO fusion is recurrent in CRCs in the Japanese population and might be involved in CRC via the activation of the Wnt signaling pathway.

Acknowledgments We acknowledge Dr. N. Kurabe and Ms. S. Izumo (Hamamatsu University School of Medicine) for their technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labour and Welfare (21-1), a Grant-in-Aid from the Japan Society for the Promotion of Science (25460476), a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (221S0001), and the Smoking Research Foundation.

References

1. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y, Mano H (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448:561–566. doi:10.1038/nature05945
2. Shinmura K, Kageyama S, Tao H, Bunai T, Suzuki M, Kamo T, Takamochi K, Suzuki K, Tanahashi M, Niwa H, Ogawa H, Sugimura H (2008) EML4-ALK fusion transcripts, but no NPM-, TPM3-, CLTC-, ATIC-, or TFG-ALK fusion transcripts, in non-small cell lung carcinomas. Lung Cancer 61:163–169. doi:10.1016/j.lungcan.2007.12.013
3. Shinmura K, Kageyama S, Igarashi H, Kamo T, Mochizuki T, Suzuki K, Tanahashi M, Niwa H, Ogawa H, Sugimura H (2010) EML4-ALK fusion transcripts in immunohistochemically ALK-positive non-small cell lung carcinomas. Exp Ther Med 1:271–275. doi:10.3892/etm_00000042
4. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, Asaka R, Hamanaka W, Ninomiya H, Uehara H, Lim Choi Y, Satoh Y, Okumura S, Nakagawa K, Mano H, Ishikawa Y (2012) RET, ROS1 and ALK fusions in lung cancer. Nat Med 18:378–381. doi:10.1038/nm.2658
5. Lipson D, Capelletti M, Yelensky R, Otto G, Parker A, Jarosz M, Curran JA, Balasubramanian S, Bloom T, Brennan KW, Donahue A, Downing SR, Frampton GM, Garcia L, Juhn F, Mitchell KC, White E, White J, Zwirko Z, Peretz T, Nechushtan H, Soussan-Gutman L, Kim J, Sasaki H, Kim HR, Park SI, Ercan D, Sheehan CE, Ross JS, Cronin MT, Jänne PA, Stephens PJ (2012) Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. Nat Med 18:382–384. doi:10.1038/nm.2673
6. Casaluce F, Sgambato A, Maione P, Rossi A, Ferrara C, Napolitano A, Palazzolo G, Ciardiello F, Gridelli C (2013) ALK inhibitors: a new targeted therapy in the treatment of advanced NSCLC. Target Oncol 8:55–67. doi:10.1007/s11523-012-0250-9
7. Seshagiri S, Stawiski EW, Durinck S, Modrusan Z, Storm EE, Conboy CB, Chaudhuri S, Guan Y, Janakiraman V, Jaiswal BS, Guillory J, Ha C, Dijkgraaf GJ, Stinson J, Gnad F, Huntley MA, Degenhardt JD, Haverty PM, Bourgon R, Wang W, Koeppen H, Gentleman R, Starr TK, Zhang Z, Largaespada DA, Wu TD, de Sauvage FJ (2012) Recurrent R-spondin fusions in colon cancer. Nature 488:660–664. doi:10.1038/nature11282
8. Yoon JK, Lee JS (2012) Cellular signaling and biological functions of R-spondins. Cell Signal 24:369–377. doi:10.1016/j.cellsig.2011.09.023
9. Shinmura K, Goto M, Suzuki M, Tao H, Yamada H, Igarashi H, Matsuura S, Maeda M, Konno H, Matsuda T, Sugimura H (2011) Reduced expression of MUTYH with suppressive activity against mutations caused by 8-hydroxyguanine is a novel predictor of a poor prognosis in human gastric cancer. J Pathol 225:414–423. doi:10.1002/path.2953
10. Wood LD, Parsons DW, Jones S, Lin J, Sjöblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, Silliman N, Szabo S, Dezso Z, Ustyanksky V, Nikolskaya T, Nikolsky Y, Karchin R, Wilson PA, Kaminker JS, Zhang Z, Croshaw R, Willis J, Dawson D, Shipitsin M, Willson JK, Sukumar S, Polyak K, Park BH, Pethiyagoda CL, Pant PV, Ballinger DG, Sparks AB, Hartigan J, Smith DR, Suh E, Papadopoulos N, Buckhaults P, Markowitz SD, Parmigiani G, Kinzler KW, Velculescu VE, Vogelstein B (2007) The genomic landscapes of human breast and colorectal cancers. Science 318:1108–1113. doi:10.1126/science.1145720
11. Bass AJ, Lawrence MS, Brace LE, Ramos AH, Drier Y, Cibulskis K, Sougnez C, Voet D, Saksena G, Sivachenko A, Jing R, Parkin M, Pugh T, Verhaak RG, Stransky N, Boutin AT, Barretina J, Solit DB, Vakiani E, Shao W, Mishina Y, Warmuth M, Jimenez J, Chiang DY, Signoretti S, Kaelin WG, Spardy N, Hahn WC, Hoshida Y, Ogino S, Depinho RA, Chin L, Garraway LA, Fuchs CS, Baselga J, Tabernero J, Gabriel S, Lander ES, Getz G, Meyerson M (2011) Genomic sequencing of colorectal adenocarcinomas identifies a recurrent VTI1A-TCF7L2 fusion. Nat Genet 43:964–968. doi:10.1038/ng.936
12. Zhou B, Yan H, Li Y, Wang R, Chen K, Zhou Z, Sun X (2012) PNAS-4 expression and its relationship to p53 in colorectal cancer. Mol Biol Rep 39:243–249. doi:10.1007/s11033-011-0732-3
13. Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T (2005) Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell 122:473–483. doi:10.1016/j.cell.2005.07.013
14. Najdi R, Holcombe RF, Waterman ML (2011) Wnt signaling and colon carcinogenesis: beyond APC. J Carcinog 10:5. doi:10.4103/1477-3163.78111
15. Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, Enomoto M, Takada S, Yamashita Y, Satoh Y, Okumura S, Nakagawa K, Ishikawa Y, Mano H (2008) Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 14:6618–6624. doi:10.1158/1078-0432.CCR-08-1018
16. Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama K, Utsunomiya J, Baba S, Hedge P (1991) Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 253:665–669. doi:10.1126/science.1651563
17. Goel A, Boland CR (2012) Epigenetics of colorectal cancer. Gastroenterology 143(1442–1460):e1. doi:10.1053/j.gastro.2012.09.032
18. Marcus VA, Madlensky L, Gryfe R, Kim H, So K, Millar A, Temple LK, Hsieh E, Hiruki T, Narod S, Bapat BV, Gallinger S, Redston M (1999) Immunohistochemistry for hMLH1 and hMSH2: a practical test for DNA mismatch repair-deficient tumors. Am J Surg Pathol 23:1248–1255. doi:10.1097/00000478-199910000-00010
19. Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Nakagawa H, Sotamaa K, Prior TW, Westman J, Panescu J, Fix D, Lockman J, Comeras I, de la Chapelle A (2005) Screening for the Lynch syndrome (hereditary nonpolyposis

colorectal cancer). N Engl J Med 352:1851–1860. doi:10.1056/NEJMoa043146

20. Yoon YS, Yu CS, Kim TW, Kim JH, Jang SJ, Cho DH, Roh SA, Kim JC (2011) Mismatch repair status in sporadic colorectal cancer: immunohistochemistry and microsatellite instability analyses. J Gastroenterol Hepatol 26:1733–1739. doi:10.1111/j.1440-1746.2011.06784.x

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2014, Article ID 617351, 12 pages
http://dx.doi.org/10.1155/2014/617351

Hindawi

Research Article

Impaired 8-Hydroxyguanine Repair Activity of MUTYH Variant p.Arg109Trp Found in a Japanese Patient with Early-Onset Colorectal Cancer

Kazuya Shinmura,[1] Masanori Goto,[1,2] Hong Tao,[1] Hisami Kato,[1] Rie Suzuki,[1] Satoki Nakamura,[1] Tomonari Matsuda,[3] Guang Yin,[4] Makiko Morita,[4] Suminori Kono,[4] and Haruhiko Sugimura[1]

[1] *Department of Tumor Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi Ward, Hamamatsu, Shizuoka 431-3192, Japan*
[2] *Division of Cancer Development System, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[3] *Research Center for Environmental Quality Management, Kyoto University, Shiga 520-0811, Japan*
[4] *Department of Preventive Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan*

Correspondence should be addressed to Kazuya Shinmura; kzshinmu@hama-med.ac.jp

Received 29 November 2013; Revised 5 February 2014; Accepted 11 February 2014; Published 23 March 2014

Academic Editor: Antonio Ayala

Purpose. The biallelic inactivation of the 8-hydroxyguanine repair gene *MUTYH* leads to MUTYH-associated polyposis (MAP), which is characterized by colorectal multiple polyps and carcinoma(s). However, only limited information regarding MAP in the Japanese population is presently available. Since early-onset colorectal cancer (CRC) is a characteristic of MAP and might be caused by the inactivation of another 8-hydroxyguanine repair gene, *OGG1*, we investigated whether germline *MUTYH* and *OGG1* mutations are involved in early-onset CRC in Japanese patients. *Methods.* Thirty-four Japanese patients with early-onset CRC were examined for germline *MUTYH* and *OGG1* mutations using sequencing. *Results.* Biallelic pathogenic mutations were not found in any of the patients; however, a heterozygous p.Arg19* *MUTYH* variant and a heterozygous p.Arg109Trp *MUTYH* variant were detected in one patient each. The p.Arg19* and p.Arg109Trp corresponded to p.Arg5* and p.Arg81Trp, respectively, in the type 2 nuclear-form protein. The defective DNA repair activity of p.Arg5* is apparent, while that of p.Arg81Trp has been demonstrated using DNA cleavage and *supF* forward mutation assays. *Conclusion.* These results suggest that biallelic *MUTYH* or *OGG1* pathogenic mutations are rare in Japanese patients with early-onset CRC; however, the p.Arg19* and p.Arg109Trp *MUTYH* variants are associated with functional impairments.

1. Introduction

8-Hydroxyguanine (8OHG) is an oxidized form of guanine, and the formation of 8OHG in DNA causes a G:C to T:A mutation, since 8OHG can pair with adenine as well as cytosine [1, 2]. To prevent such mutations in human cells, MUTYH (MIM #604933) and OGG1 (MIM #601982) proteins are involved in DNA glycosylase-initiating base excision repair, which is a component of the human DNA repair system [3–8]. MUTYH catalyzes the removal of adenine mispaired with 8OHG in double-stranded DNA, and OGG1 catalyzes the removal of 8OHG at the 8OHG:C site. Regarding MUTYH, multiple forms, including two major forms (type 1 mitochondrial form and type 2 nuclear form), are expressed in human cells [3–6].

Clinically, biallelic germline inactivating *MUTYH* mutations are known to predispose an individual to MUTYH-associated polyposis (MAP: MIM #608456), a hereditary disorder characterized by multiple colorectal polyps and carcinoma(s) [9–11]. Various pathogenic *MUTYH* mutations, including two major mutations (p.Tyr179Cys and p.Gly396Asp), have been detected in MAP patients in several

ethnic populations [12]; however, neither the p.Tyr179Cys nor the p.Gly396Asp mutation has been found in the Japanese population [13–15], and which *MUTYH* variations are the major pathogenic mutations in the Japanese population remains unclear. In accordance with the notion that early-onset cancer is likely to be associated with the germline abnormality of certain gene(s) [16], early-onset colorectal cancer (CRC) is one of the characteristics of MAP [12], and early-onset CRC is thought to occur in patients with biallelic-inactivating mutations of another 8OHG repair gene, *OGG1*. Therefore, we hypothesized that biallelic inactivating mutations of *MUTYH* or *OGG1* might lead to early-onset CRC in Japanese patients. To test this hypothesis, we examined 34 Japanese patients with early-onset CRC for germline *MUTYH* and *OGG1* mutations. Then, we investigated whether the detected variants were associated with defective 8OHG repair activity.

2. Materials and Methods

2.1. Clinical Samples and Cell Line. Blood specimens from 685 CRC cases and 778 controls were collected in a previous study [17]. Written informed consent was obtained from each individual patient [17]. The characteristics of the cases and the controls have been described previously [17, 18]. Briefly, the cases were composed of a consecutive series of patients with histologically confirmed incident colorectal adenocarcinomas, and the controls were composed of individuals that had no diagnosis of CRC. Additionally, the cases had no prior history of removal of the colorectum, familial adenomatous polyposis (FAP: MIM #175100), or inflammatory bowel disease (IBD). All the cases and the controls had been previously genotyped for c.36+11C>T, c.504+35A>G, c.934−2A>G, and c.1014G>C *MUTYH* polymorphisms [19], but none had been sequenced for the whole coding exons of the *MUTYH* and *OGG1* genes. A human cancer cell line, H1299, was obtained from the American Type Culture Collection (Manassas, VA). The H1299 cells and their derivatives were maintained at 37°C in RPMI1640 medium supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX) and penicillin/streptomycin under a 5% CO_2 atmosphere. The study design was approved by the institutional review boards.

2.2. PCR-Sequencing Analysis. Genomic DNA was extracted as described previously [17]. All the coding exons of the *MUTYH* and *OGG1* genes and their boundary regions were amplified using PCR with HotStarTaq DNA polymerase (Qiagen, Valencia, CA). The PCR primer sequences of *MUTYH* and *OGG1* are summarized in Supplementary Tables S1 and S2 in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/617351, respectively. The PCR-amplified products were directly sequenced with a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and an ABI 3130 Genetic Analyzer (Applied Biosystems).

2.3. PCR with Confronting Two-Pair Primers (PCR-CTPP) Analysis. Genotyping of the c.55C>T and c.325C>T variants was performed using a PCR-CTPP analysis, as described previously [20]. The PCR primer sequences are summarized in Supplementary Table S3. The PCR products were fractionated by electrophoresis on a 2.0% agarose gel and were stained with ethidium bromide.

2.4. Construction of Expression Plasmid. Plasmid vectors for the expression of human wild-type (WT) MUTYH type 2 in *Escherichia coli* (*E. coli*) and human cells were previously constructed by inserting the cDNA into a pET25b(+) vector (Novagen, Darmstadt, Germany) and a piggyBac cumate switch inducible vector (System Biosciences, Mountain View, CA), respectively [21, 22]. Expression vectors for the MUTYH variants were generated using site-directed mutagenesis with a QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA).

2.5. Preparation of Recombinant Protein. *E. coli* BL21-CodonPlus (DE3)-RP competent cells (Stratagene) were transformed with the MUTYH-pET25b vector and cultured at 37°C until an A_{600} of 0.6. After incubation with 0.1 mM IPTG at 15°C for 12 h, MUTYH-His_6 protein was purified with TALON metal affinity resins (Clontech, Palo Alto, CA) and a TALON 2 mL disposable gravity column (Clontech). The protein was then dialyzed against buffer containing 10 mM sodium phosphate (pH 7.6), 50 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.5 mM PMSF, 2 μg/mL pepstatin, 2 μg/mL leupeptin, 50 μM chymostatin, and 10% glycerol. The quality and concentration of MUTYH proteins were determined by resolving the proteins with SDS-polyacrylamide gel electrophoresis (PAGE) and staining them with Coomassie Brilliant Blue (CBB); Image J software (National Institutes of Health, Bethesda, MD) was then used for quantification.

2.6. Western Blot Analysis. A Western blot analysis using a mouse anti-MUTYH monoclonal antibody (4D10; Abnova, Taipei, Taiwan) or an anti-β-tubulin monoclonal antibody (clone 2-28-33; Sigma-Aldrich, St. Louis, MO) was performed as described previously [22, 23].

2.7. DNA Cleavage Assay. First, 30-mer oligonucleotides containing or not containing a single 8OHG (5′-CTG GTG GCC TGA C[8OHG or T]C ATT CCC CAA CTA GTG-3′) were chemically synthesized and purified using PAGE (Japan Bio Services, Saitama, Japan). Complementary oligonucleotides containing an adenine opposite the 8OHG or T were ^{32}P-labeled at the 5′ terminus with a MEGALABEL kit (Takara, Osaka, Japan) and [γ-^{32}P]ATP (PerkinElmer, Tokyo, Japan), and these oligonucleotides were then annealed to oligonucleotides containing a single 8OHG or T. A reaction mixture containing 20 mM sodium phosphate (pH 7.6), 100 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 5 μM $ZnCl_2$, 1.5% glycerol, 2.5 nM labeled oligonucleotide, 50 μg/mL BSA, and 90 fmoles of MUTYH protein was then incubated at 37°C for 15 min, and the mixture was treated with 0.1 M NaOH. After the mixture was denatured, it was subjected to 20% PAGE. A

^{32}P-labeled marker oligonucleotide was used as a size marker for the cleavage products. The radioactivities of the intact and cleaved oligonucleotides were quantified using an FLA-3000 fluoroimage analyzer (Fuji Film, Tokyo, Japan) and ImageGauge software (Fuji Film).

2.8. Establishment of Stable Inducible Cell Lines. H1299 cells were transfected with the piggyBac 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). Since the inducible piggyBac vector features a tight cumate switch combined with an EF1-CymR repressor-T2A-Puro cassette to establish stable cell lines, the addition of cumate solution (System Biosciences) to the puromycin-selected cells leads to the induction of MUTYH expression.

2.9. Indirect Immunofluorescence Analysis. Cells were fixed with 10% formalin at room temperature, permeabilized with 1% Nonidet P-40, blocked with 10% normal goat serum, and probed with mouse anti-MUTYH monoclonal antibody (4D10; Abnova). Indirect immunofluorescence labeling was performed by exposure to an Alexa Fluor 594-conjugated secondary antibody (Molecular Probes, Eugene, OR), and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The slides were promptly 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 images captured were digitized and stored in the image analysis program (MetaMorph; Molecular Devices, Palo Alto, CA).

2.10. supF Forward Mutation Assay. A single 8OHG:C mispair was introduced at nucleotide position 159 of the bacterial suppressor tRNA (*supF*) gene of the shuttle vector plasmid pMY189, as described previously [22–24]. A *supF* forward mutation assay for H1299-derived cells was performed using the 8OHG-containing pMY189 plasmid and the KS40/pKY241 indicator *E. coli* strain, as described previously [22, 23]. The mutation frequencies were calculated as the number of *E. coli supF* mutants per total number of *E. coli* transformants. The mutations in the *supF* gene were then analyzed as described previously [22].

2.11. Computational Analysis for Variants. The functional effects of nonsynonymous variants were predicted by the online software tools PolyPhen-2 [25], SIFT [26], and PROVEAN [27]. The allele frequencies of variants in a large number of Japanese individuals were examined using a reference database of genetic variations in the Japanese population (http://www.genome.med.kyoto-u.ac.jp/SnpDB/).

2.12. Statistical Analysis. The statistical analyses were performed using an unpaired *t*-test, Dunnett test, or Fisher exact test. JMP version 9 software (SAS Institute, Cary, NC) was used for all the statistical analyses. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Identification of p.Arg19 and p.Arg109Trp MUTYH Variants in Japanese Patients with Early-Onset CRC.* To investigate whether germline mutations of the DNA glycosylase genes *MUTYH* and *OGG1* are involved in early-onset CRC in Japanese patients, we attempted to utilize a population of 685 Japanese CRC patients. Among them, we selected 34 CRC patients with the lowest ages of onset (corresponding to 5% of the total patient population) (Table 1). All 34 CRC patients were less than 43 years old and had no history of FAP or IBD. We then examined the 34 CRC patients for germline *MUTYH* and *OGG1* mutations by sequencing every coding exon of both genes. As a result, 9 *MUTYH* variants and 7 *OGG1* variants were found (Table 2, Figure 1(a)), and the genotype and allele frequencies of the nucleotide variations are summarized in Table 2 and the characteristics of the variations are summarized in Table 3. Genotypes of the variations in the coding region and splice-site region in 34 patients are also summarized in Supplementary Tables S4 and S5. Among the 9 *MUTYH* variants, although c.36+11C>T, c.504+35A>G, c.934−2A>G, c.1014G>C (p.Gln338His), c.1118C>T (p.Ala373Val), c.1431G>C (p.Thr477Thr), and c.1477−40C>G are not considered to be MAP pathogenic alleles, the remaining two variants of c.55C >T (p.Arg19*) and c.325C>T (p.Arg109Trp) were identical to variants previously found in one patient each with colorectal multiple adenomas and a carcinoma [28]. In our study, the p.Arg19* variant was detected in one patient as a heterozygous status for the wild-type and variant alleles, and the p.Arg109Trp variant was also detected heterozygously in one patient. Moreover, the p.Arg19* variant encodes a truncating protein, and the p.Arg109Trp variant encodes a protein with the substitution of a highly conserved amino acid, p.Arg109 (National Center for Biotechnology Information, Bethesda, MD) (Figure 1(b)); the missense protein was predicted to be functionally damaged by the PolyPhen-2, SIFT, and PROVEAN software programs (Table 3). Therefore, we considered the two variants to be candidate MAP pathogenic alleles. Regarding the 7 *OGG1* variants, c.949−89G>T and c.966C>T (p.Asp322Asp) were novel (Supplementary Figure S1); however, since the former exists in an intron and the latter is a synonymous variation, these variants were not considered to be pathogenic mutations. Among the other 5 *OGG1* variants, c.−18G>T and c.977C>G (p.Ser326Cys) have been reported as low-penetrance cancer susceptibility variants [29, 30], while the remaining three were untranslated, synonymous, or intronic variations, meaning that all of them are not highly pathogenic mutations. Therefore, none of the patients were thought to have biallelic pathogenic *MUTYH* or *OGG1* mutations in the presently studied early-onset CRC group. However, we decided to further investigate the p.Arg19* and p.Arg109Trp *MUTYH* variants as candidate pathogenic alleles.

Table 1: Clinicopathological profiles of 34 Japanese patients with early-onset CRC.

Case number	Age	Sex	Tumor site	Tumor histology	Tumor stage
1	27	F	Distal colon	Mucinous adenocarcinoma	II
2	29	F	Proximal colon	WD adenocarcinoma[a]	II
3	34	F	Distal colon	WD adenocarcinoma	I
4	36	M	Distal colon	WD adenocarcinoma	IIIa
5	39	F	Rectum	MD adenocarcinoma[b]	IIIa
6	39	F	Distal colon	WD adenocarcinoma	IV
7	38	M	Proximal colon	MD adenocarcinoma	II
8	36	F	Distal colon	MD adenocarcinoma	IIIb
9	39	F	Rectum	WD adenocarcinoma	IIIa
10	39	M	Proximal colon	WD adenocarcinoma	IIIb
11	37	F	Proximal colon	WD adenocarcinoma	I
12	33	F	Proximal colon	WD adenocarcinoma	IIIa
13	36	M	Rectum	WD adenocarcinoma	I
14	35	M	Distal colon	MD adenocarcinoma	II
15	37	M	Proximal colon	MD adenocarcinoma	IIIb
16	38	M	Rectum	WD adenocarcinoma	II
17	43	M	Rectum	WD adenocarcinoma	I
18	41	F	Distal colon	WD adenocarcinoma	II
19	41	M	Distal colon	MD adenocarcinoma	IIIb
20	42	M	Rectum	WD adenocarcinoma	IIIb
21	40	M	Proximal colon	WD adenocarcinoma	I
22	43	F	Proximal colon	MD adenocarcinoma	II
23	42	M	Rectum	MD adenocarcinoma	IIIa
24	42	M	Proximal colon	MD adenocarcinoma	I
25	42	M	Rectum	WD adenocarcinoma	I
26	43	M	Rectum	PD adenocarcinoma[c]	IV
27	43	M	Distal colon	WD adenocarcinoma	IIIa
28	41	M	Rectum	MD adenocarcinoma	II
29	42	M	Rectum	WD adenocarcinoma	IIIa
30	40	M	Rectum	MD adenocarcinoma	II
31	43	M	Distal colon	WD adenocarcinoma	I
32	43	M	Distal colon	WD adenocarcinoma	II
33	42	M	Proximal colon	WD adenocarcinoma	II
34	40	M	Rectum	MD adenocarcinoma	IIIb

[a]Well-differentiated adenocarcinoma. [b]Moderately differentiated adenocarcinoma. [c]Poorly differentiated adenocarcinoma.

3.2. Absence of p.Arg19 and p.Arg109Trp MUTYH Variants in Japanese Individuals without CRC.* To determine the frequency of the p.Arg19* and p.Arg109Trp *MUTYH* variants in Japanese individuals without CRC, we genotyped both nucleotide variations using PCR-CTPP in as many as 100 individuals randomly selected from the control group (Figures 1(c) and 1(d)). Since the above-described cases with a p.Arg19* or p.Arg109Trp variant exhibited a heterozygous pattern of the WT and variant alleles, these cases were used as positive controls in the PCR-CTPP analysis. The results showed that none of the controls exhibited p.Arg19* or p.Arg109Trp variants, suggesting that p.Arg19* and p.Arg109Trp are rare variants in the Japanese population.

3.3. Defective DNA Glycosylase Activity of MUTYH Type 2 p.Arg81Trp Variant. To conclude whether a *MUTYH* variant allele is pathogenic, an impairment in the repair activity of the protein based on the nucleotide variant must be evident. p.Arg19* and p.Arg109Trp correspond to p.Arg5* and p.Arg81Trp, respectively, in the type 2 nuclear-form protein, which is the major MUTYH form in human cells. Since p.Arg5* has a length of only 5 amino acids, compared with 521 amino acids in the full-length type 2 protein, the DNA repair activity of p.Arg5* is most likely defective. On the other hand, p.Arg81Trp yields a missense form, and the repair activity of the mutant protein has not yet been analyzed. Thus, we planned to evaluate the DNA glycosylase activity of the p.Arg81Trp protein by comparing the cleavage activity of the mutant with that of the WT protein in the presence of an A:8OHG mismatch-containing DNA substrate. We also planned to utilize the p.Asp208Asn mutant as a negative control in the comparison [21]. First, the WT, p.Arg81Trp,

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

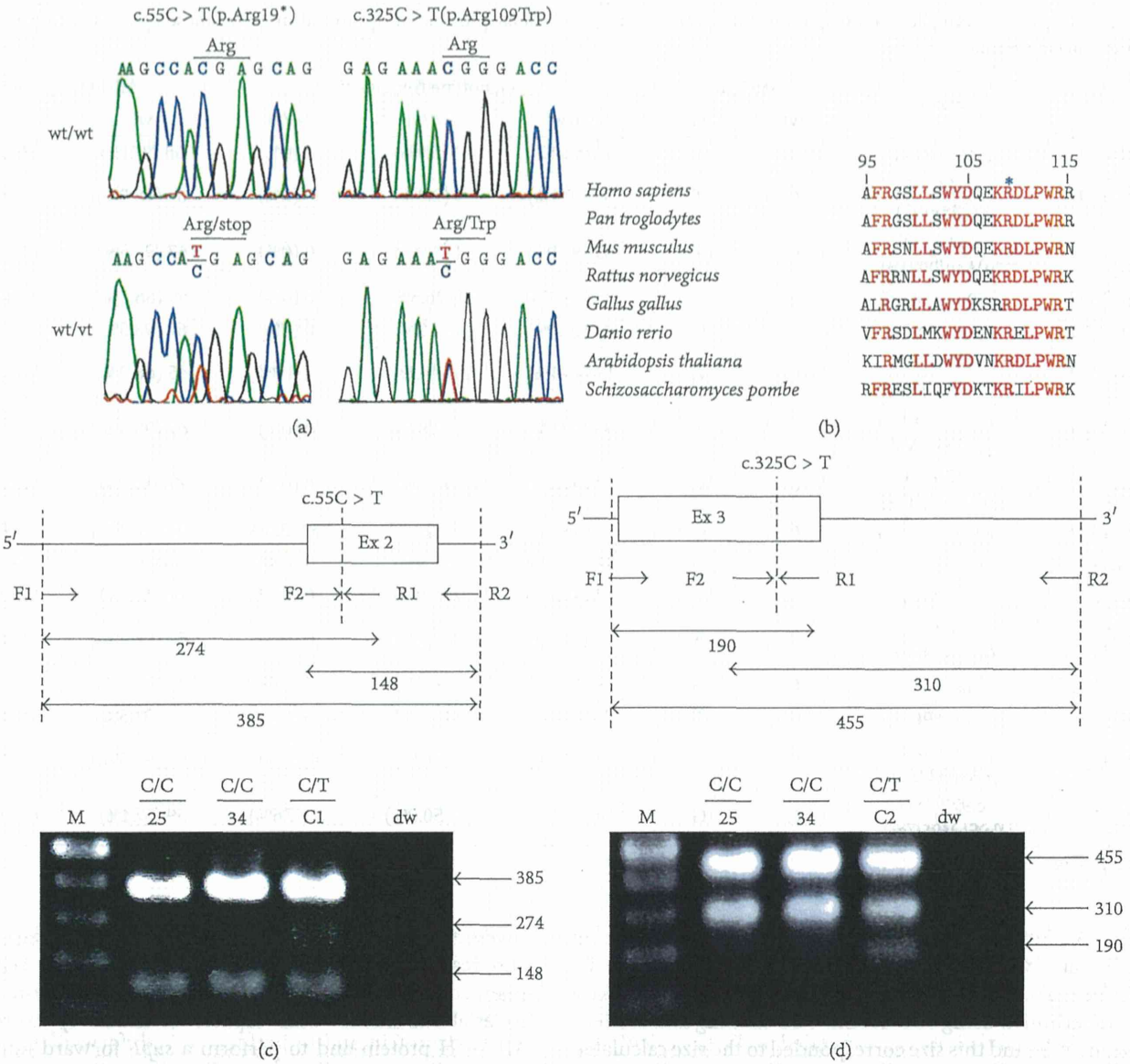

FIGURE 1: Identification and genotyping of c.55C>T (p.Arg19*) and c.325C>T (p.Arg109Trp) variants of the *MUTYH* gene in the Japanese population. (a) Identification of c.55C>T and c.325C>T variants of the *MUTYH* gene in Japanese patients with early-onset CRC. Sequencing electropherograms show a C to 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

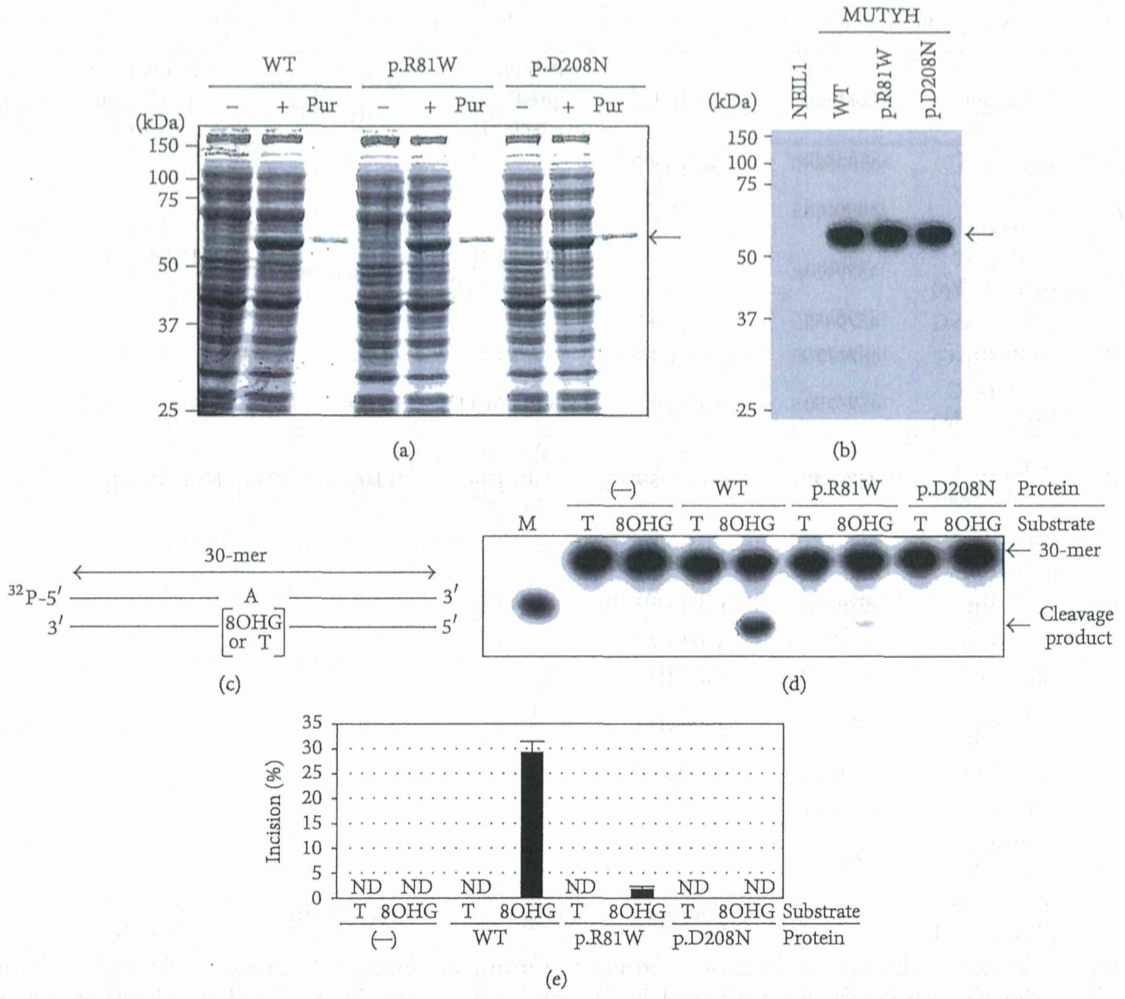

FIGURE 2: Comparison of the DNA glycosylase activity of WT MUTYH and p.Arg81Trp variant MUTYH protein using a DNA cleavage assay. (a) Purification of WT, p.Arg81Trp, and p.Asp208Asn MUTYH type 2 recombinant proteins. The MUTYH proteins were overexpressed and purified using the pET system and TALON metal 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

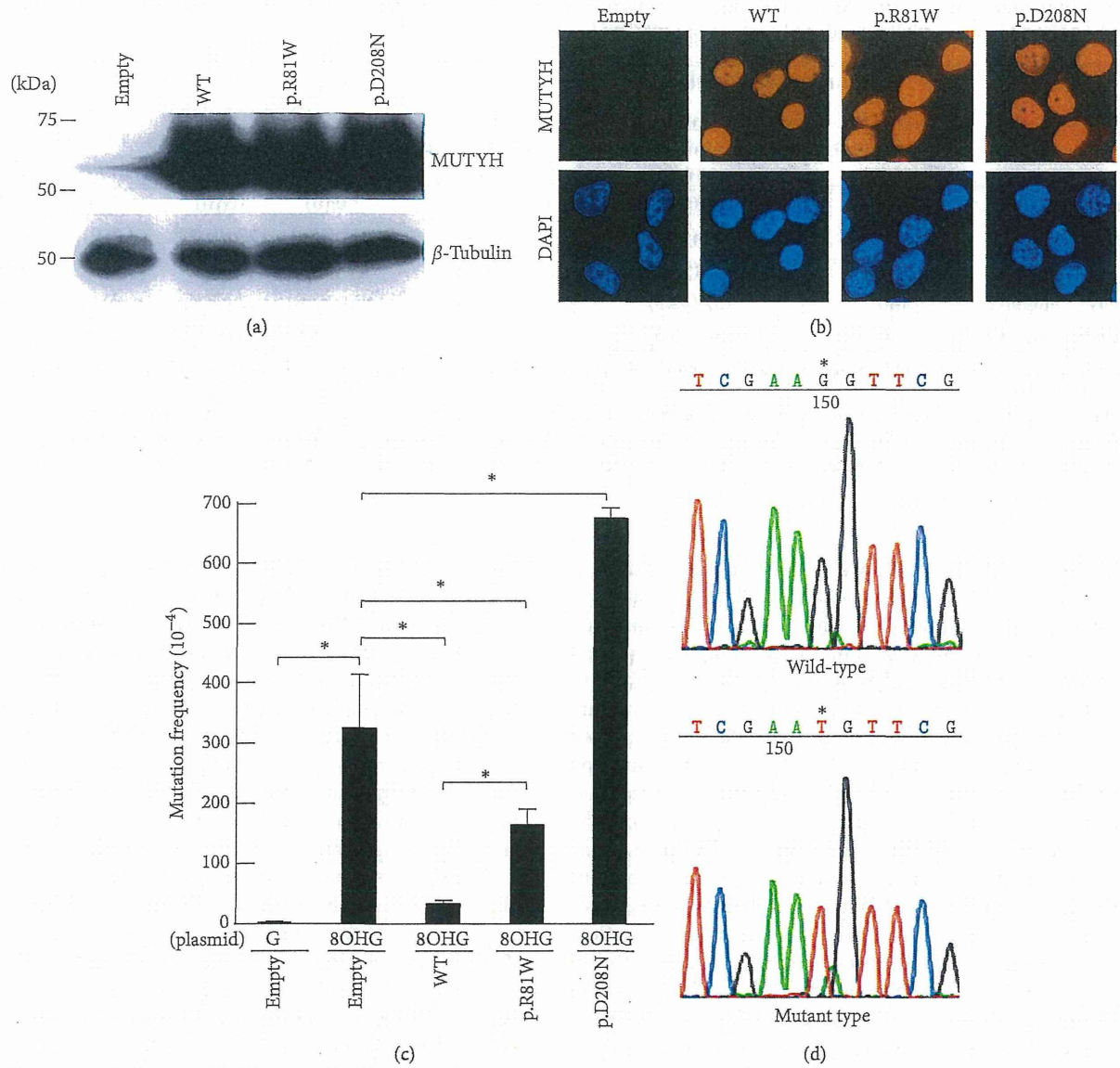

FIGURE 3: Comparison of the activity to suppress the mutation caused by 8OHG between H1299 human cell lines inducibly expressing WT MUTYH and p.Arg81Trp variant MUTYH protein using a *supF* forward mutation assay. (a) Detection of MUTYH proteins in cumate-inducible stable cell lines expressing MUTYH 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.

Acknowledgments

This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labour, and Welfare (21-1), the Japan Society for the Promotion of Science (25460476), the Ministry of Education, Culture, Sports, Science and Technology (221S0001), the Takeda Science Foundation, the Hamamatsu Foundation for Science and Technology Promotion, and Japan Science and Technology Agency (Center of Innovation Program).

References

[1] H. Kasai and S. Nishimura, "Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents," *Nucleic Acids Research*, vol. 12, no. 4, pp. 2137–2145, 1984.

[2] S. Shibutani, M. Takeshita, and A. P. Grollman, "Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG," *Nature*, vol. 349, no. 6308, pp. 431–434, 1991.

[3] M. M. Slupska, W. M. Luther, J.-H. Chiang, H. Yang, and J. H. Miller, "Functional expression of hMYH, a human homolog of the *Escherichia coli* MutY protein," *Journal of Bacteriology*, vol. 181, no. 19, pp. 6210–6213, 1999.

[4] M. Takao, Q.-M. Zhang, S. Yonei, and A. Yasui, "Differential subcellular localization of human MutY homolog (hMYH) and the functional activity of adenine:8-oxoguanine DNA glycosylase," *Nucleic Acids Research*, vol. 27, no. 18, pp. 3638–3644, 1999.

[5] K. Shinmura, S. Yamaguchi, T. Saitoh et al., "Adenine excisional repair function of MYH protein on the adenine:8-hydroxyguanine base pair in double-stranded DNA," *Nucleic Acids Research*, vol. 28, no. 24, pp. 4912–4918, 2000.

[6] T. Ohtsubo, K. Nishioka, Y. Imaiso et al., "Identification of human MutY homolog (hMYH) as a repair enzyme for 2-hydroxyadenine in DNA and detection of multiple forms of hMYH located in nuclei and mitochondria," *Nucleic Acids Research*, vol. 28, no. 6, pp. 1355–1364, 2000.

[7] S. S. David, V. L. O'Shea, and S. Kundu, "Base-excision repair of oxidative DNA damage," *Nature*, vol. 447, no. 7147, pp. 941–950, 2007.

[8] Z. Cai, H. Chen, J. Tao et al., "Association of base excision repair gene polymorphisms with ESRD risk in a Chinese population," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 928421, 10 pages, 2012.

[9] N. Al-Tassan, N. H. Chmiel, J. Maynard et al., "Inherited variants of *MYH* associated with somatic G : C → T : A mutations in colorectal tumors," *Nature Genetics*, vol. 30, no. 2, pp. 227–232, 2002.

[10] S. Jones, P. Emmerson, J. Maynard et al., "Biallelic germline mutations in *MYH* predispose to multiple colorectal adenoma and somatic G : C → T : A mutations," *Human Molecular Genetics*, vol. 11, no. 23, pp. 2961–2967, 2002.

[11] O. M. Sieber, L. Lipton, M. Crabtree et al., "Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in *MYH*," *New England Journal of Medicine*, vol. 348, no. 9, pp. 791–799, 2003.

[12] M. Nielsen, H. Morreau, H. F. Vasen, and F. J. Hes, "*MUTYH*-associated polyposis (MAP)," *Critical Reviews in Oncology/Hematology*, vol. 79, no. 1, pp. 1–16, 2011.

[13] M. Miyaki, T. Iijima, T. Yamaguchi et al., "Germline mutations of the *MYH* gene in Japanese patients with multiple colorectal adenomas," *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 578, no. 1-2, pp. 430–433, 2005.

[14] R. Yanaru-Fujisawa, T. Matsumoto, Y. Ushijima et al., "Genomic and functional analyses of *MUTYH* in Japanese patients with adenomatous polyposis," *Clinical Genetics*, vol. 73, no. 6, pp. 545–553, 2008.

[15] T. Kuno, N. Matsubara, S. Tsuda et al., "Alterations of the base excision repair gene *MUTYH* in sporadic colorectal cancer," *Oncology Reports*, vol. 28, pp. 473–480, 2012.

[16] A. Bleyer, R. Barr, B. Hayes-Lattin et al., "The distinctive biology of cancer in adolescents and young adults," *Nature Reviews Cancer*, vol. 8, no. 4, pp. 288–298, 2008.

[17] S. Kono, K. Toyomura, G. Yin, J. Nagano, and T. Mizoue, "A case-control study of colorectal cancer in relation to lifestyle factors and genetic polymorphisms: design and conduct of the fukuoka colorectal cancer study," *Asian Pacific Journal of Cancer Prevention*, vol. 5, no. 4, pp. 393–400, 2004.

[18] T. Hagiwara, S. Kono, G. Yin et al., "Genetic polymorphism in cytochrome P450 7A1 and risk of colorectal cancer: the Fukuoka colorectal cancer study," *Cancer Research*, vol. 65, no. 7, pp. 2979–2982, 2005.

[19] H. Tao, K. Shinmura, M. Suzuki et al., "Association between genetic polymorphisms of the base excision repair gene *MUTYH* and increased colorectal cancer risk in a Japanese population," *Cancer Science*, vol. 99, no. 2, pp. 355–360, 2008.

[20] H. Tao, K. Shinmura, T. Hanaoka et al., "A novel splice-site variant of the base excision repair gene *MYH* is associated with production of an aberrant mRNA transcript encoding a truncated *MYH* protein not localized in the nucleus," *Carcinogenesis*, vol. 25, no. 10, pp. 1859–1866, 2004.

[21] M. Goto, K. Shinmura, Y. Nakabeppu et al., "Adenine DNA glycosylase activity of 14 Human MutY homolog (*MUTYH*) variant proteins found in patients with colorectal polyposis and cancer," *Human Mutation*, vol. 31, no. 11, pp. E1861–E1874, 2010.

[22] K. Shinmura, M. Goto, H. Tao, S. Matsuura, T. Matsuda, and H. Sugimura, "Impaired suppressive activities of human *MUTYH* variant proteins against oxidative mutagenesis," *World Journal of Gastroenterology*, vol. 18, no. 47, pp. 6935–6942, 2012.

[23] K. Shinmura, M. Goto, M. Suzuki et al., "Reduced expression of *MUTYH* with suppressive activity against mutations caused by 8-hydroxyguanine is a novel predictor of a poor prognosis in human gastric cancer," *Journal of Pathology*, vol. 225, no. 3, pp. 414–423, 2011.

[24] T. Matsuda, T. Yagi, M. Kawanishi, S. Matsui, and H. Takebe, "Molecular analysis of mutations induced by 2-chloroacetaldehyde, the ultimate carcinogenic form of vinyl chloride, in human cells using shuttle vectors," *Carcinogenesis*, vol. 16, no. 10, pp. 2389–2394, 1995.

[25] I. A. Adzhubei, S. Schmidt, L. Peshkin et al., "A method and server for predicting damaging missense mutations," *Nature Methods*, vol. 7, no. 4, pp. 248–249, 2010.

[26] P. Kumar, S. Henikoff, and P. C. Ng, "Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm," *Nature Protocols*, vol. 4, no. 7, pp. 1073–1081, 2009.

[27] Y. Choi, G. E. Sims, S. Murphy, J. R. Miller, and A. P. Chan, "Predicting the functional effect of amino acid substitutions and indels," *PLoS ONE*, vol. 7, no. 10, Article ID e46688, 2012.

[28] S. Vogt, N. Jones, D. Christian et al., "Expanded extracolonic tumor spectrum in *MUTYH*-associated polyposis," *Gastroenterology*, vol. 137, no. 6, pp. 1976–1985, 2009.

[29] T. Ishida, R. Takashima, M. Fukayama et al., "New DNA polymorphisms of human *MMH/OGG1* gene: prevalence of one polymorphism among lung-adenocarcinoma patients in Japanese," *International Journal of Cancer*, vol. 80, pp. 18–21, 1999.

[30] Z. Xu, L. Yu, and X. Zhang, "Association between the hOGG1 Ser326Cys polymorphism and lung cancer susceptibility: a meta-analysis based on 22, 475 subjects," *Diagnostic Pathology*, vol. 8, article 144, 2013.

[31] S. Ding, X. Wu, G. Li, M. Han, Y. Zhuang, and T. Xu, "Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice," *Cell*, vol. 122, no. 3, pp. 473–483, 2005.

[32] S. Aretz, M. Genuardi, and F. J. Hes, "Clinical utility gene card for: *MUTYH*-associated polyposis (MAP), autosomal recessive colorectal adenomatous polyposis, multiple colorectal adenomas, multiple adenomatous polyps (MAP)—update 2012," *European Journal of Human Genetics*, vol. 21, 2013.

[33] L. Lipton, S. E. Halford, V. Johnson et al., "Carcinogenesis in *MYH*-associated polyposis follows a distinct genetic pathway," *Cancer Research*, vol. 63, no. 22, pp. 7595–7599, 2003.

[34] H. Bai, S. Jones, X. Guan et al., "Functional characterization of two human MutY homolog (hMYH) missense mutations (R227W and V232F) that lie within the putative hMSH6 binding domain and are associated with hMYH polyposis," *Nucleic Acids Research*, vol. 33, no. 2, pp. 597–604, 2005.

[35] V. G. D'Agostino, A. Minoprio, P. Torreri et al., "Functional analysis of *MUTYH* mutated proteins associated with familial adenomatous polyposis," *DNA Repair*, vol. 9, no. 6, pp. 700–707, 2010.

[36] Y. Bromberg and B. Rost, "SNAP: predict effect of non-synonymous polymorphisms on function," *Nucleic Acids Research*, vol. 35, no. 11, pp. 3823–3835, 2007.

[37] K. Shinmura, M. Goto, H. Tao, and H. Sugimura, "Role of base excision repair enzyme *MUTYH* in the repair of 8-hydroxyguanine and *MUTYH*-associated polyposis (MAP)," *Hereditary Genetics*, vol. 1, article 111, 2012.

[38] K. Shinmura, H. Tao, M. Goto et al., "Inactivating mutations of the human base excision repair gene *NEIL1* in gastric cancer," *Carcinogenesis*, vol. 25, no. 12, pp. 2311–2317, 2004.