

Reduced Expression of Mismatch Repair Genes *MSH6/MSH2* Directly Promotes Pituitary Tumor Growth via the ATR–Chk1 Pathway

Shinsuke Uraki,¹ Hiroyuki Ariyasu,¹ Asako Doi,¹ Shintaro Kawai,¹ Ken Takeshima,¹ Shuhei Morita,¹ Junya Fukai,² Koji Fujita,² Hiroto Furuta,¹ Masahiro Nishi,¹ Kokichi Sugano,³ Naoko Inoshita,⁴ Naoyuki Nakao,² Shozo Yamada,⁵ and Takashi Akamizu¹

¹First Department of Internal Medicine, Wakayama Medical University, Wakayama 641-8509, Japan;

²Department of Neurologic Surgery, Wakayama Medical University, Wakayama 641-8509, Japan;

³Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, Tochigi 320-0834, Japan; ⁴Department of Pathology, Toranomon Hospital, Tokyo 105-8470, Japan; and

⁵Department of Hypothalamic and Pituitary Surgery, Toranomon Hospital, Tokyo 105-8470, Japan

Context: The mechanisms of pituitary adenoma (PA) pathogenesis and proliferation remain largely unknown.

Objectives: To clarify the role of mismatch repair (MMR) genes in the molecular mechanism of PA proliferation.

Design: We performed quantitative analyses by real-time polymerase chain reaction and immunohistochemistry to detect MMR gene and protein expression in human PAs (n = 47). We also performed correlation analyses of expression levels and tumor volume doubling time (TVDT; n = 31). Specifically, correlation analyses were performed between genes with significant correlation and ataxiatelangiectasia and Rad3-related (*ATR*) expression in cell-cycle regulatory mechanism ATR–checkpoint kinase 1 (Chk1) pathway (n = 93). We investigated the effect of reduced gene expression on cell proliferation and *ATR* gene expression in AtT-20ins cells and primary cultures of human PAs.

Results: Expression of mutS homologs 6 and 2 (*MSH6* and *MSH2*) was positively associated with TVDT (R = 0.52, P = 0.003, and R = 0.44, P = 0.01), as were the corresponding protein levels. Gene expression was positively associated with *ATR* expression (R = 0.47, P < 0.00001, and R = 0.49, P < 0.00001). In AtT-20ins, the reduction of *MSH6* and/or *MSH2* expression by small interfering RNA significantly promoted cell proliferation by decreasing *ATR* expression. This effect was also observed in primary culture.

Conclusion: Reduction of *MSH6* and *MSH2* expression at the messenger RNA and protein levels could be involved in direct PA proliferation by promoting cell-cycle progression or decreasing the rate of apoptosis through interference with the function of the ATR–Chk1 pathway. (*J Clin Endocrinol Metab* 103: 1171–1179, 2018)

ISSN Print 0021-972X ISSN Online 1945-7197

Printed in USA

Copyright © 2018 Endocrine Society

Received 24 October 2017. Accepted 9 January 2018.

First Published Online 12 January 2018

Abbreviations: ANOVA, analysis of variance; ATR–Chk1, ataxiatelangiectasia and Rad3-related–checkpoint kinase 1; DMEM, Dulbecco's modified Eagle medium; EPCAM, epithelial cell adhesion molecule; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LI, labeling index; MLH1/3, mutL homolog 1/3; MMR, mismatch repair; MRI, magnetic resonance images; mRNA, messenger RNA; MSH2/3/6, mutS homolog 2/3/6; PA, pituitary adenoma; PCR, polymerase chain reaction; PMS1/2, PMS1 homolog 1/2; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA; TV, tumor volume; TVDT, tumor volume doubling time.

Pituitary adenomas (PAs) are common intracranial tumors, accounting for 15% to 20% of all brain neoplasms (1). Although PAs are predominantly benign lesions arising from adenohypophysis, they include highly proliferative and invasive, aggressive PAs and pituitary carcinoma (2, 3). When considering clinically aggressive adenomas, oncologists are strongly recommended to take into account the 2017 World Health Organization classification of tumors of the pituitary gland, accurate histological tumor subtyping, assessment tumor proliferative potential by mitotic count and Ki-67 index, and other clinical parameters, such as tumor invasion (3). In addition, the expression levels of mutS homolog 6 (MSH6) and O-6-methylguanine-DNA methyltransferase have been used to predict the therapeutic effect of temozolomide on aggressive PAs and pituitary carcinomas (4). Some pituitary tumors with high proliferative potential, however, do not meet the criteria for aggressive PA. Therefore, it is clinically important to elucidate the mechanism of development and proliferation of pituitary tumors.

The mismatch repair (MMR) mechanism corrects errors resulting from nucleotide misincorporation by DNA polymerase (5–8). Genes involved in the MMR pathway include mutL homolog 1 (*MLH1*), PMS1 homolog 2 (*PMS2*), mutS homolog 2 (*MSH2*), *MSH6*, mutL homolog 3 (*MLH3*), mutS homolog 3 (*MSH3*), PMS1 homolog 1 (*PMS1*), and epithelial cell adhesion molecule (*EPCAM*). Germline mutations of the MMR genes cause an inherited disease, called Lynch syndrome, in which tumors, such as colorectal, endometrial, and ovarian cancer, occur at high frequency (9, 10). In colorectal cancer, *MLH1* expression is reduced as a result of methylation of the promoter region, even if germline gene mutations are absent (11). In addition to the prevention of genetic mutations related to tumor development (5), MMR proteins can sense against DNA damage and directly suppress cell proliferation via a cell-cycle regulatory mechanism known as the ataxiatelangiectasia and Rad3-related-checkpoint kinase 1 (*ATR-Chk1*) pathway (12, 13).

Regarding the relationship between MMR genes and pituitary tumors, one study found that *MSH6* immunorepression was reduced in four out of 13 cases of highly proliferative pituitary tumors (4). We previously reported an aggressive PA that exhibited both loss of heterozygosity of an *MLH1* germline mutation (Lynch syndrome) and somatic mutation of *MSH6* (14). In addition, Bengtsson *et al.* (15) reported that a corticotroph pituitary carcinoma developed in a patient with Lynch syndrome, and they also showed the high prevalence of PAs in patients with Lynch syndrome from a nation-wide survey in Sweden.

Based on these findings, we hypothesize that MMR genes might affect pituitary tumor growth, development,

and proliferation. In this study, we sought to analyze the correlation between MMR gene-expression levels and clinical factors related to tumor proliferation, using tissue from human PAs. We also sought to clarify the relationship between MMR genes and the molecular mechanisms underlying pituitary tumor proliferation.

Materials and Methods

Ethics statement

Informed consent was obtained from all patients for the collection, analysis, and publication of personal and clinical data and for the use of pituitary tumor specimens in gene-expression and immunohistochemical studies. This study was performed with approval of the Wakayama Medical University Ethics Committee.

Patient samples

Between September 2013 and September 2016, 47 patients with PA consented to participate in our study at Wakayama Medical University Hospital. Clinical, imageological, and histopathological characteristics of these patients are shown in Table 1 and Supplemental Table 1A. In addition, between February and September 2016, 46 patients with non-functioning PA consented to participate in this study at Toranomon Hospital. Clinical, imageological, and histopathological characteristics of these patients are shown in Table 2 and Supplemental Table 1B. The 46 nonfunctioning PA samples were used only for detection of messenger RNA (mRNA) levels [*MSH2*, *MSH6*, *ATR*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)].

Measurement of tumor volume and tumor volume doubling time

Serial magnetic resonance images (MRI) were used to establish tumor volume (TV) and growth rate. The Centricity® PACS Universal Viewer web client, version 6.0 (GE Healthcare, New York, NY) was used for the MRI analysis. The tumor region of interest on enhanced T1-weighted images was manually defined and used to measure the TV. The pituitary tumor was manually outlined on each section using the free-hand region of interest measuring option, and the outlined area was automatically calculated. The product of the pituitary tumor area and the section thickness represented the volume of the pituitary tumor in each single section. Total volume was computed by summing the entire section volume. To estimate the tumor growth rate, we used a previously established formula for measuring TV doubling time (TVDT): $TVDT = t \log_2 2 / \log_2 (V_f / V_0)$, where t is the time interval between the first and second MRI examinations, V_0 is the initial TV, and V_f is the final TV (16, 17). We calculated preoperative TV for all 47 pituitary tumor cases. Of the 47, there were 31 cases in which the first and second MRI examinations were performed postoperatively, and TVDT could be calculated. The median follow-up period was 15.7 months (3.5 to 53.6 months), and the median MRI interval time was 12.9 months (1.8 to 50.2 months; Table 1). Preoperative TV, TVDT, and MRI interval time of each case are shown in Supplemental Table 1A.

Table 1. Clinical, Imageological, and Histopathological Characteristics of Patients With PA at Wakayama Medical University Hospital

Clinical Phenotype	Number	Pathological Phenotype	Number	Summarized Data	
Nonfunctioning PA	34	Gonadotroph adenoma	24	Male/female	20/27
		Silent somatotroph adenoma	5	Age	55.9 ± 13.8 years ^a
		Silent thyrotroph adenoma	3	Micro/macro	2/45
		Silent lactotroph adenoma	2	TV, cm ³	9.5 cm ^{3b} (0.4–49.2)
		Somatotroph adenoma	6	TVDT, mo (n = 31)	29.4 mo ^b (1.9–91.3)
Lactotroph adenoma	3	Lactotroph adenoma	3	Follow-up period, mo (n = 31)	15.7 mo ^b (3.5–53.6)
Corticotroph adenoma	2	Corticotroph adenoma	2	Time interval of MRI, mo (n = 31)	12.9 mo ^b (1.8–50.2)
Thyrotroph adenoma	1	Thyrotroph adenoma	1	MIB-1 LI, %	2.4% ^b (0.2–20.0)
Plurihormonal (ACTH, PRL, GH) adenoma	1	Plurihormonal (ACTH, PRL, GH) adenoma	1		<3%: 34
Total	47				≥3%: 13
(Recurrence)	14				

Abbreviations: ACTH, adrenocorticotropic hormone; GH, growth hormone; LI, labeling index; PRL, prolactin.

^aMeans ± standard deviation.

^bMedian.

Measurement of MMR gene and ATR gene mRNA expression

Total RNA was extracted using the TRIzol reagent. cDNA was synthesized using the TaqMan™ Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses were carried out to detect mRNA levels (*MLH1*, *PMS2*, *MSH2*, *MSH6*, *MLH3*, *MSH3*, *PMS1*, *EPCAM*, and *ATR*) using SYBR Green Real-Time PCR Master Mixes (Applied Biosystems). *GAPDH* was used as an internal control. Primers for qRT-PCR are shown in Supplemental Table 2A.

Immunohistochemistry and quantitative analysis

Formalin-fixed paraffin-embedded tissues sections of pituitary tumors were examined histopathologically and immunohistochemically using the following antibodies: MSH6 (M3646; Dako, Tokyo, Japan), MSH2 (#2017; Cell Signaling Technology, Danvers, MA), and Ki-67 (MIB-1; 790-4286; Ventana, Tucson, AZ). Immunohistochemistry was performed using the BenchMark Ultra automated system (Ventana, Tucson, AZ), as previously described (14). Next, we quantitatively analyzed the immunohistochemical results for MSH6 and MSH2 using the

BZ-H3C application (Keyence, Osaka, Japan). In this analysis, protein expression was quantified by calculating intensity from the degree of staining. Intensity had a value between zero (black) and 255 (white). When the degree of staining was high, indicating high protein expression, the intensity was low, so we converted the intensity by black/white reversal (Supplemental Fig. 1). The calculated value was then corrected by subtracting the value of unstained normal pituitary, and the corrected value was defined as protein expression in each tissue (Supplemental Fig. 1).

Cell culture and treatment schedule

The mouse pituitary tumor cell line AtT-20ins, obtained from the American Type Culture Collection (Manassas, VA), was cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA), supplemented with 10% fetal calf serum (FCS). AtT-20ins cells were transfected with 5, 10, or 20 nM small interfering RNA (siRNA) targeting MSH6, MSH2, or ATR (QIAGEN, Hilden, Germany), using the HiPerFect transfection reagent (QIAGEN, Hilden, Germany), and cultured them for 48 hours. As a negative control, the cells were transfected with 5 nM siRNA of AF488 (QIAGEN) as control.

Table 2. Clinical, Imageological, and Histopathological Characteristics of Patients With PA at Toranomon Hospital

Clinical Phenotype	Number	Pathological Phenotype	Number	Summarized Data	
Nonfunctioning PA	46	Gonadotroph adenoma	27	Male/female	20/26
		Silent corticotroph adenoma	13	Age	51.6 ± 15.5 y ^a
		Silent somatotroph adenoma	1	Micro/macro	0/46
		Silent thyrotroph adenoma	1	MIB-1 LI, %	1.8% ^b (0.3–4.1)
		Silent lactotroph adenoma	1		<3%: 39
		Silent plurihormonal (PRL, GH) adenoma	1		≥3%: 7
		Null cell adenoma	2		
Total	46				
(Recurrence)	5				

^aMeans ± standard deviation.

^bMedian.

After 48 hours of incubation in 24- or 96-well plates under standard conditions (37°C, 5% CO₂), total RNA was isolated from AtT-20ins cells in a 24-well plate using the RNeasy Mini Kit (QIAGEN), and cDNA was synthesized with the TaqMan™ Reverse Transcription Reagents (Applied Biosystems). qRT-PCR analyses, to detect mRNA levels (*MSH6*, *MSH2*, and *ATR*), were carried out using SYBR Green Real-Time PCR Master Mixes (Applied Biosystems), and *GAPDH* was used as an internal control. Primers for qRT-PCR are shown in Supplemental Table 2B. Cell proliferation assays on AtT-20ins were performed in a 96-well plate using the Cell Counting Kit-8 (Dojindo, Rockville, MD).

To establish PA cells in culture, two fresh, nonfunctioning PAs were obtained from the Operating Room in Wakayama Medical University Hospital. The tumors were mechanically dissociated in DMEM, supplemented with 10% FCS. Enzymatic digestion buffer consisted of HAM-F2 Medium (Life Technologies, Carlsbad, CA) and 1 mg/mL collagenase type IA (Sigma Chemical, St. Louis, MO). Tissue suspensions were transferred to a vial containing 5 mL digestion buffer and incubated at 37°C for 45 minutes with rotation. Tumor suspensions were then washed with 5% FCS in DMEM and passed through a 70- μ m cell strainer (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). Cell suspensions were then pelleted and plated on Matrigel-coated 24- or 96-well plates at 3.5×10^5 viable cells/mL in complete DMEM. To establish Matrigel-coated plates, 24-well plates were coated with 95 μ L Matrigel (BD Biosciences, San Jose, CA) per well, and 96-well plates were coated with 16 μ L Matrigel per well. The primary culture method was described previously (18). Once established, these human PA cells were transfected with 5, 10, or 20 nM siRNA targeting *MSH6* (QIAGEN), using the HiPerFect transfection reagent and cultured for 48 hours. Next, qRT-PCR analyses were carried out to measure mRNA levels (*MSH6* and *ATR*); *GAPDH* was used as an internal control. Cell proliferation assays were also performed with these human PA cells.

Statistical analysis

Data were statistically analyzed using simple linear regression analysis, unpaired *t* test, and one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons. Values of *P* < 0.05 were considered statistically significant. Analyses were performed using a commercially available statistical package (JMP Pro version 13.0; SAS Institute, Cary, NC).

Results

Expression of *MSH6* and *MSH2* is positively associated with Log₂ TVDT

To investigate how MMR genes are involved in pituitary tumor growth, we calculated TVDT, an index of tumor growth, and performed simple linear regression analysis on the relationship between MMR gene expression and TVDT (*n* = 31). Expression of *MSH6* and *MSH2* was positively associated with Log₂ TVDT (respectively, *R* = 0.52, *P* = 0.003, and *R* = 0.44, *P* = 0.01; Fig. 1), suggesting that reduction of *MSH6* and *MSH2* expression promotes pituitary tumor growth by shortening TVDT. However, expression of other MMR genes

(*MLH1*, *PMS2*, *MLH3*, *MSH3*, *PMS1*, and *EPCAM*) was not associated with Log₂ TVDT (Supplemental Fig. 2).

To investigate the association between MMR gene expression and preoperative TV and MIB-1 labeling index (LI), used as an indicator of pituitary tumor growth, we performed another simple linear regression analysis. However, we observed no significant correlation between MMR gene expression and preoperative TV or MIB-1 LI (*n* = 47; Supplemental Table 3). In addition, we performed simple linear regression analysis of the relationship between MMR gene expression and preoperative TV in pituitary tumor cases, taking into account the influence of recurrence vs nonrecurrence and functional vs nonfunctional pituitary tumors. Specifically, we excluded recurrent cases (*n* = 33), nonfunctional pituitary tumors (including recurrent cases; *n* = 34), and nonfunctional (but not recurrent) pituitary tumors (*n* = 20). We observed no significant correlation between MMR gene expression and preoperative TV (Supplemental Table 3).

MSH6 and MSH2 protein levels are positively associated with Log₂ TVDT

Based on the positive association between Log₂ TVDT and gene expression, we next quantified the levels of *MSH6* and *MSH2* to determine whether protein expression was associated with tumor growth. For this purpose, we performed simple linear regression analysis of the relationship between *MSH6* and *MSH2* protein levels and TVDT (*n* = 31). We found that *MSH6* and *MSH2* protein levels were positively associated with Log₂ TVDT, similar to the correlation between gene expression and TVDT (Fig. 1). Taken together, our findings suggest that the reduction of *MSH6* and *MSH2* mRNA and protein expression could promote pituitary tumor growth by shortening TVDT.

Expression of *MSH6* and *MSH2* is positively associated with *ATR* gene expression

In HeLa cells, *MSH6* and *MSH2* form a complex, called Mut α , which senses DNA damage and induces cell-cycle regulation via the ATR–Chk1 pathway (12). The association between *MSH6* and *MSH2* and the ATR–Chk1 pathway in pituitary tumors, however, remains unknown. To address this question, we performed a simple linear regression analysis on the relationship between *MSH6* and *MSH2* expression and *ATR* expression in pituitary tumors (*n* = 96). *MSH6* and *MSH2* expression was positively associated with *ATR* expression (respectively, *R* = 0.47, *P* < 0.00001, and *R* = 0.49, *P* < 0.00001; Fig. 2). These results suggest that reduction of *MSH6* and *MSH2* expressions may result in reduction of *ATR* expression and may promote cell proliferation by diminishing the function of the ATR–Chk1 pathway.

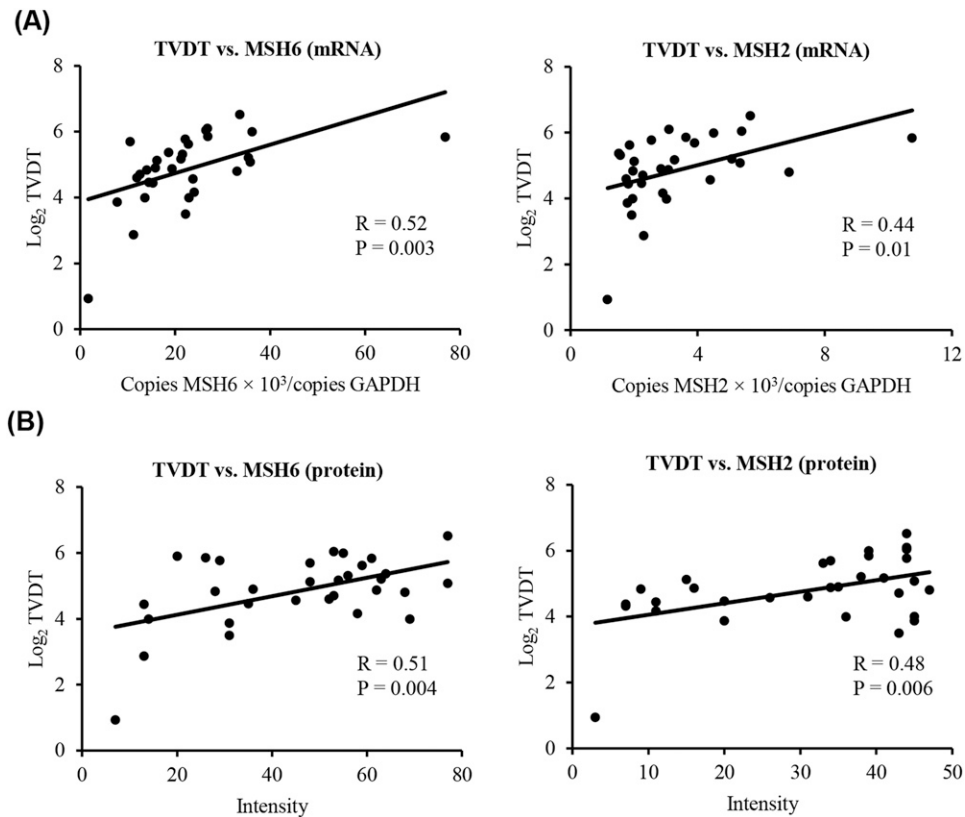


Figure 1. Correlation between *MSH6* and *MSH2* (A) mRNA/(B) protein expression and Log_2 TVDT. The relationship between MMR gene expression in PAs and Log_2 TVDT was examined using simple linear regression analysis. *MSH6* and *MSH2* expression was positively associated with Log_2 TVDT (respectively, $R = 0.52$, $P = 0.003$, and $R = 0.44$, $P = 0.01$). Likewise, *MSH6* and *MSH2* protein levels were positively associated with Log_2 TVDT (respectively, $R = 0.51$, $P = 0.004$, and $R = 0.48$, $P = 0.006$).

Reduction of *MSH6* and/or *MSH2* expression by siRNA promotes cell proliferation by decreasing *ATR* expression in AtT-20ins

Next, to determine whether *MSH6* and *MSH2* and the ATR–Chk1 pathway affect pituitary tumor growth, we investigated the effect of *MSH6* or *MSH2* knockdown on *ATR* expression and cell proliferation in AtT-20ins.

First, we examined the effect of reduction of *MSH6* or *MSH2* expression on cell proliferation. When *MSH6*

expression was gradually decreased by siRNA, the rate of cell proliferation increased significantly [Fig. 3(A)]. Similar results were obtained with *MSH2* knockdown [Fig. 3(B)]. Moreover, in double knockdowns, cell proliferation was increased further compared with either single knockdown [Fig. 3(C)]. Furthermore, although *ATR* expression was only slightly decreased in the single knockdowns, a substantial reduction of *ATR* expression was observed in the double knockdown

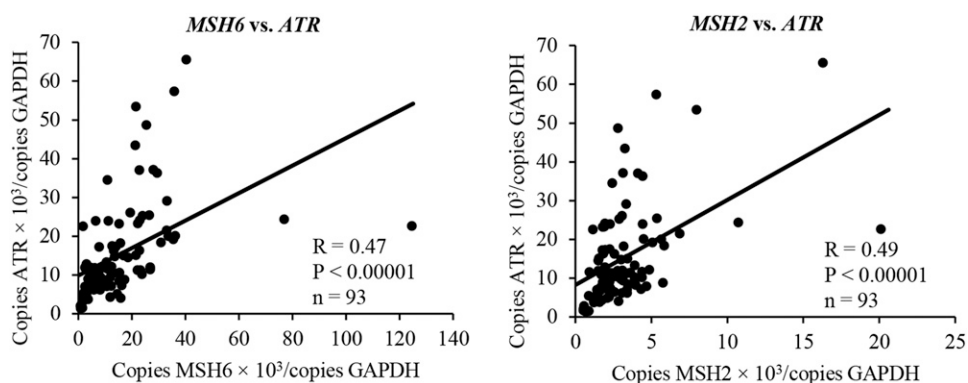


Figure 2. Correlation between *MSH6* and *MSH2* expression and *ATR* expression. The relationship between *MSH6* and *MSH2* expressions and *ATR* expression in PAs was examined using simple linear regression analysis. *MSH6* and *MSH2* expression was positively associated with *ATR* expression (respectively, $R = 0.47$, $P < 0.00001$, and $R = 0.49$, $P < 0.00001$).

[Fig. 3(C)]. Likewise, knockdown of *ATR* substantially promoted cell proliferation, although it did not substantially affect expression of *MSH6* or *MSH2* [Fig. 3(D)].

These results showed that the reduction of *MSH6* and *MSH2* gene expressions *in vitro* led to reduction of *ATR* gene expression and promoted cell proliferation. This suggests that the reduction of *MSH6* and *MSH2* gene expressions may reduce the function of the cell regulatory mechanism *ATR*–*Chk1* pathway through the reduction of *ATR* gene expression and promote cell proliferation by promoting cell-cycle progression and decreasing the rate of apoptosis in pituitary tumors.

Knockdown of *MSH6* promotes cell proliferation by decreasing *ATR* expression in primary cultures of human nonfunctioning PAs

In primary cultures of human nonfunctional PAs, siRNA-mediated knockdown of *MSH6* significantly promoted cell proliferation by decreasing *ATR* expression (Fig. 4). This suggests that in human pituitary tumors, reduced *MSH6* expression decreases *ATR* expression, thereby attenuating the function of the *ATR*–*Chk1* pathway and permitting unregulated proliferation.

Discussion

The MMR mechanism, which is governed by the MMR genes (*MLH1*, *PMS2*, *MSH2*, *MSH6*, *MLH3*, *MSH3*, *PMS1*, and *EPCAM*), corrects DNA polymerase misincorporation errors that arise during DNA replication or recombination. It also repairs some forms of DNA damage (5–8). In addition, these genes contribute to the effectiveness of immune checkpoint inhibitors against colon cancer (19). Although the roles of MMR genes have been studied extensively, the detailed mechanisms underlying their functions remain unknown. In this study, we showed that MMR genes *MSH6* and *MSH2* are directly involved in proliferation of pituitary

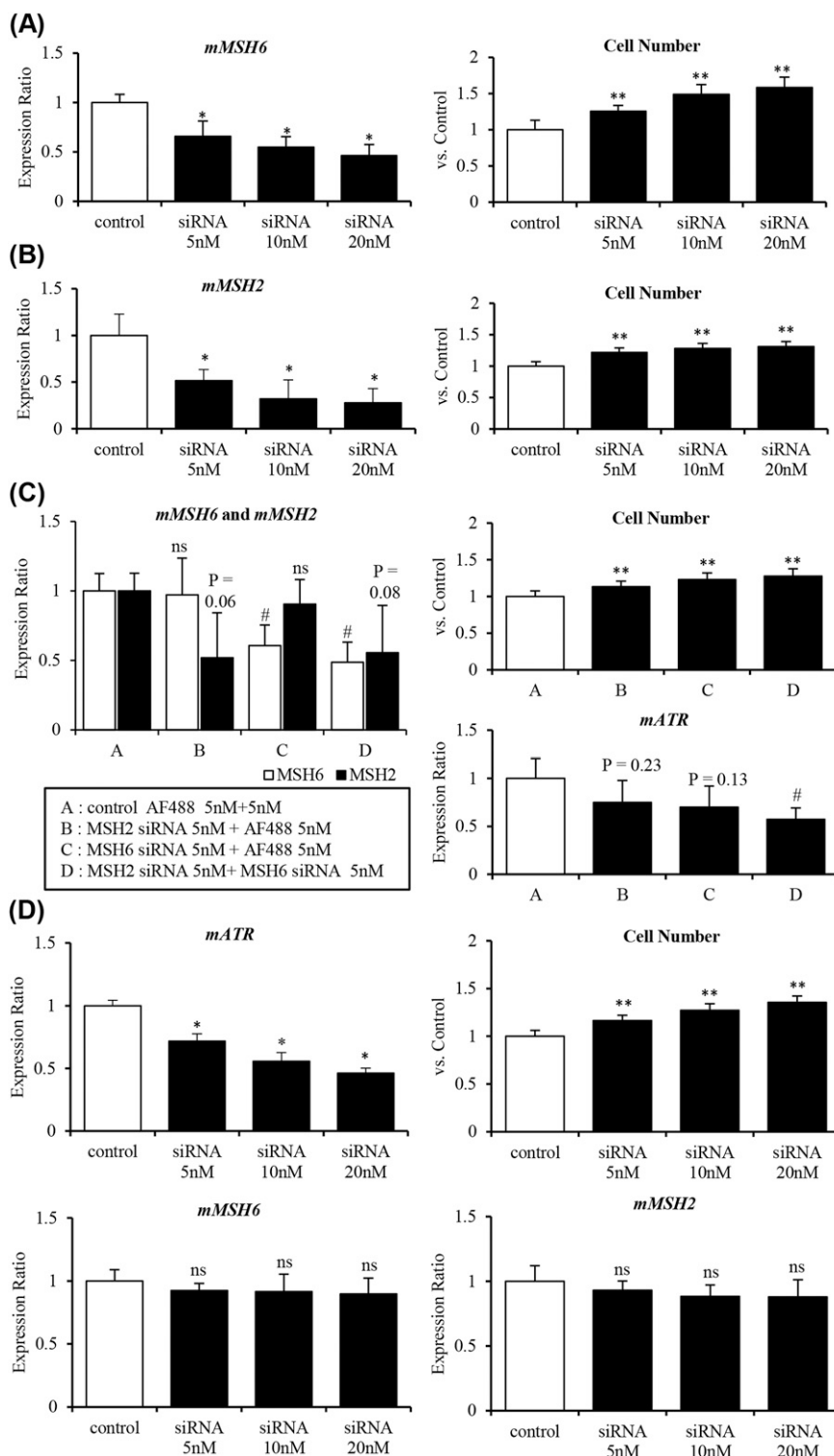


Figure 3. Reduction of *MSH6* and/or *MSH2* expression by siRNA promotes cell proliferation by decreasing *ATR* expression in AtT-20ins cells. (A) Knockdown of *MSH6* promoted cell proliferation in AtT-20ins treated with various concentrations of siRNA targeting *MSH6* (0 to 20 nM). (B) Knockdown of *MSH2* promoted cell proliferation in AtT-20ins treated with various concentrations of siRNA targeting *MSH2* (0 to 20 nM). (C) Knockdown of both *MSH6* and *MSH2* significantly decreased *ATR* expression and promoted cell proliferation to a greater extent than either single knockdown. (D) Knockdown of *ATR* expression promoted cell proliferation without significantly changing in *MSH6* and *MSH2* expressions in AtT-20ins treated with various concentrations of siRNA targeting *ATR* (0 to 20 nM). * $P < 0.01$ vs control (ANOVA Dunnett's test); ** $P < 0.001$ vs control (ANOVA Dunnett's test); # $P < 0.05$ vs control (ANOVA Dunnett's test); ns, not significant vs control (ANOVA Dunnett's test). m, mouse.

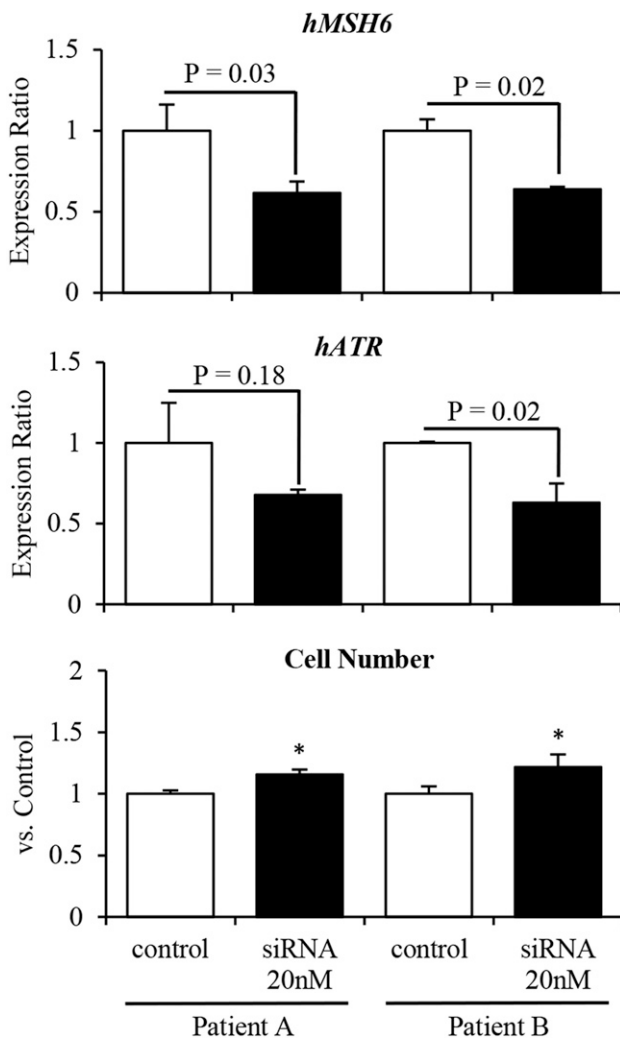


Figure 4. Knockdown of *MSH6* expression promotes cell proliferation by decreasing *ATR* expression in primary cultures of two human (h) nonfunctioning PAs. siRNA-mediated knockdown of *MSH6* decreased *ATR* expression and significantly promoted cell proliferation in primary cultures of two human nonfunctioning PAs.

tumors. Specifically, we found that reduction of *MSH6* and *MSH2* expression at the mRNA and protein levels promoted cell proliferation via the ATR–Chk1 pathway, a cell-cycle regulatory mechanism, in both human pituitary tumors and the pituitary tumor cell line AtT-20ins.

First, to determine whether differences in MMR gene expression (*MLH1*, *PMS2*, *MSH2*, *MSH6*, *MLH3*, *MSH3*, *PMS1*, and *EPCAM*) are involved in pituitary tumor proliferation, we investigated the correlation between MMR gene expression in human PAs and preoperative TV, MIB-1 LI, and TVDT, used as indicators of tumor proliferation. Among the MMR genes, only *MSH6* and *MSH2* exhibited a substantial positive correlation with TVDT. *MSH6* and *MSH2* protein levels also showed a similar correlation. However, preoperative TV and MIB-1 LI did not significantly correlate with *MSH6* or *MSH2* expression. We speculate that this is because preoperative TV is a value calculated in the pituitary

tumor having different timing of tumor occurrence, whereas MIB-1 LI is a value calculated in a hot spot of a pituitary tumor; consequently, preoperative TV and MIB-1 LI may not accurately represent pituitary tumor proliferation. In the context of MMR, *MSH6* and *MSH2* assemble to form a complex called MutS α (5). Accordingly, we presumed that *MSH6* and *MSH2* also work cooperatively in pituitary tumors and are involved in tumor proliferation. We observed no correlation between the expression of other MMR genes and tumor proliferation, suggesting that *MSH6* and *MSH2* are directly involved in tumor proliferation.

MutS α , a complex of *MSH6* and *MSH2*, acts as a sensor of DNA damage and induces the ATR–Chk1 pathway in HeLa cells (12). In response to a DNA damage signal, ATR is activated via phosphorylation by binding to the activator, ATR-interacting protein, and the signal is transmitted to downstream proteins Cdc25A, Cdc25C, and p53 via phosphorylation of Chk1 to induce cell-cycle arrest in S or G2 phase and cell apoptosis (13). We found that expression of *MSH6* and *MSH2* was significantly, positively correlated with *ATR* expression. Even in human pituitary tumor cells, reduction of *MSH6* and *MSH2* expression at the mRNA and protein level might result in dysfunction of the ATR–Chk1 pathway and promote tumor proliferation, because cell-cycle arrest and apoptosis cannot be induced efficiently.

We investigated whether the reduction in *MSH6* and *MSH2* expressions directly promotes cell proliferation using AtT-20ins cells. After a short incubation (48 hours), the reduction in *MSH6* or *MSH2* expression promoted cell proliferation. Interestingly, knockdown of both *MSH6* and *MSH2* promoted cell proliferation more than either single knockdown alone, suggesting that reduction of *MSH6* and *MSH2* expression in AtT-20ins promotes cell proliferation cooperatively.

We next investigated whether reduction of *MSH6* and *MSH2* gene expression affects cell-cycle regulation by the ATR–Chk1 pathway, as well as whether it is involved in cell proliferation in AtT-20ins. The reduction in *MSH6* and *MSH2* expression resulted in reduction of *ATR* expression via the ATR–Chk1 pathway and promoted cell proliferation. Furthermore, when *ATR* expression was decreased directly, cell proliferation was promoted in the same manner as when *MSH6* and *MSH2* were knocked down, although the expression of neither gene was decreased. This is in agreement with a previous report that *MSH6* and *MSH2* function as MutS α to induce the ATR–Chk1 pathway (12). Reduction of *MSH6* and *MSH2* expressions could thus promote cell proliferation by disrupting the function of the ATR–Chk1 pathway.

Finally, to determine whether similar mechanisms are preserved in humans, we examined the mechanism

involved in cell proliferations observed in AtT-20ins in primary cultures of human nonfunctional PAs. As a result of limited primary cell culture, this was examined only when *MSH6* expression was reduced, but as in the case of AtT-20ins, promotion of cell proliferation was accompanied by a decrease in *ATR* expression. This suggests that reduction of *MSH6* and *MSH2* expression could promote the proliferation of human pituitary tumors. As mentioned above, the *ATR*–*Chk1* pathway induces cell-cycle arrest via various mechanisms and induces p53-mediated apoptosis (13). Our findings suggest that cell-cycle arrest and apoptosis via the *ATR*–*Chk1* pathway might also influence the mechanism of pituitary tumor growth and specifically, that reduction of *MSH6* and *MSH2* expression could promote pituitary tumor cell proliferation.

In summary, we demonstrated that reduction of *MSH6* and *MSH2* expression at the mRNA or protein level in human pituitary tumor tissue promotes tumor proliferation by shortening TVDT. An *in vitro* investigation revealed that the reduction of *MSH6* and *MSH2* expression in pituitary tumor cells results in *ATR*–*Chk1* pathway dysfunction, cell-cycle arrest, and inhibition of apoptosis, thereby promoting pituitary tumor proliferation (Fig. 5).

On the other hand, mutation in an exon or promoter region or epigenetic alteration as a result of promoter methylation or microRNA, could decrease *MSH6* and *MSH2* expression (5, 20). We looked for mutations in the exon or promoter region, as well as methylation in the promoter region, but found no association between these features and gene expression (data not shown). We were unable to examine the relationship between gene expression and microRNAs. Nonetheless, it seems clear that some mechanism involved in regulating *MSH6* and

MSH2 expression functions in pituitary tumors, and this issue should be addressed in future studies.

It is very important to clarify whether *MSH6* and *MSH2* affect the proliferation of functional and non-functional pituitary tumors in different ways. However, a limitation of this study is that the number of samples of functional pituitary tumors was small, making it impossible to examine the relationship between *MSH6* and *MSH2* and each clinical or histopathological type of pituitary tumor. Another limitation was the short (48-hour) *in vitro* incubation time; the reduction of *MSH6* and *MSH2* gene expressions simultaneously decrease the function of MMR mechanism, resulting in mutations of other genes involved in tumor proliferation. We cannot exclude the possibility that these indirect mechanisms might be involved in tumor growth.

In conclusion, the reduction of *MSH6* and *MSH2* expression, as well as reduced expression of the encoded proteins, could be involved in both tumor development and proliferation. These effects could be mediated indirectly by the accumulation of genetic abnormalities caused by dysfunction of MMR, as well as directly via cell-cycle promotion or apoptosis reduction caused by dysfunction of the *ATR*–*Chk1* pathway in pituitary tumors. This study partially reveals the mechanisms underlying the development and proliferation of pituitary tumors. Our findings suggest that in pituitary tumors, especially those that are aggressive, expression of *MSH6* and *MSH2* could be used as a predictor of proliferation; currently, few such predictive biomarkers are available. Further studies of this mechanism are necessary, especially in pituitary tumors with high proliferative ability.

Acknowledgments

Financial Support: This work was supported by a Grant-in-Aid from the Takeda Science Foundation.

Correspondence and Reprint Requests: Hiroyuki Ariyasu, MD, First Department of Internal Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan. E-mail: ariyasu@wakayama-med.ac.jp.

Disclosure Summary: The authors have nothing to disclose.

References

- Daly AF, Tichomirowa MA, Beckers A. The epidemiology and genetics of pituitary adenomas. *Best Pract Res Clin Endocrinol Metab.* 2009;23(5):543–554.
- Ezzat S, Asa SL. Mechanisms of disease: the pathogenesis of pituitary tumors. *Nat Clin Pract Endocrinol Metab.* 2006;2(4):220–230.
- Mete O, Lopes MB. Overview of the 2017 WHO Classification of Pituitary Tumors. *Endocr Pathol.* 2017;28(3):228–243.
- Hirohata T, Asano K, Ogawa Y, Takano S, Amano K, Isozaki O, Iwai Y, Sakata K, Fukuhara N, Nishioka H, Yamada S, Fujio S, Arita K, Takano K, Tominaga A, Hizuka N, Ikeda H, Osamura RY, Tahara S, Ishii Y, Kawamata T, Shimatsu A, Teramoto A, Matsuno A.

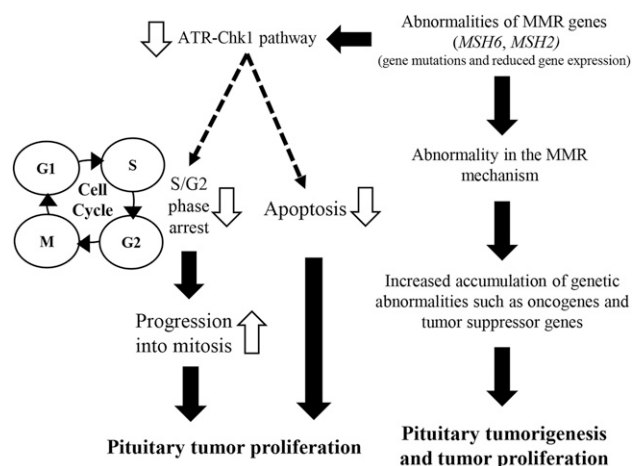


Figure 5. Schematic model of pituitary tumor proliferation associated with abnormal *MSH6* and *MSH2* expression.

- DNA mismatch repair protein (MSH6) correlated with the responses of atypical pituitary adenomas and pituitary carcinomas to temozolomide: the national cooperative study by the Japan Society for Hypothalamic and Pituitary Tumors. *J Clin Endocrinol Metab.* 2013;**98**(3):1130–1136.
5. Hewish M, Lord CJ, Martin SA, Cunningham D, Ashworth A. Mismatch repair deficient colorectal cancer in the era of personalized treatment. *Nat Rev Clin Oncol.* 2010;**7**(4):197–208.
 6. Martin SA, Lord CJ, Ashworth A. Therapeutic targeting of the DNA mismatch repair pathway. *Clin Cancer Res.* 2010;**16**(21):5107–5113.
 7. Peña-Díaz J, Jiricny J. Mammalian mismatch repair: error-free or error-prone? *Trends Biochem Sci.* 2012;**37**(5):206–214.
 8. Martín-López JV, Fishel R. The mechanism of mismatch repair and the functional analysis of mismatch repair defects in Lynch syndrome. *Fam Cancer.* 2013;**12**(2):159–168.
 9. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. *N Engl J Med.* 2003;**348**(10):919–932.
 10. Tiwari AK, Roy HK, Lynch HT. Lynch syndrome in the 21st century: clinical perspectives. *QJM.* 2016;**109**(3):151–158.
 11. Imai K, Yamamoto H. Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. *Carcinogenesis.* 2008;**29**(4):673–680.
 12. Yoshioka K, Yoshioka Y, Hsieh P. ATR kinase activation mediated by MutSalpha and MutLalpha in response to cytotoxic O6-methylguanine adducts. *Mol Cell.* 2006;**22**(4):501–510.
 13. Wagner JM, Kaufmann SH. Prospects for the use of ATR inhibitors to treat cancer. *Pharmaceuticals (Basel).* 2010;**3**(5):1311–1334.
 14. Uraki S, Ariyasu H, Doi A, Furuta H, Nishi M, Sugano K, Inoshita N, Nakao N, Yamada S, Akamizu T. Atypical pituitary adenoma with MEN1 somatic mutation associated with abnormalities of DNA mismatch repair genes; MLH1 germline mutation and MSH6 somatic mutation. *Endocr J.* 2017;**64**(9):895–906.
 15. Bengtsson D, Joost P, Aravidis C, Askmal Stenmark M, Backman AS, Melin B, von Salomé J, Zagoras T, Gebre-Medhin S, Burman P. Corticotroph pituitary carcinoma in a patient with Lynch syndrome (LS) and pituitary tumors in a nationwide LS cohort. *J Clin Endocrinol Metab.* 2017;**102**(11):3928–3932.
 16. Nakaguchi H, Fujimaki T, Matsuno A, Matsuura R, Asai A, Suzuki I, Sasaki T, Kirino T. Postoperative residual tumor growth of meningioma can be predicted by MIB-1 immunohistochemistry. *Cancer.* 1999;**85**(10):2249–2254.
 17. Ebinu JO, Lwu S, Monsalves E, Arayee M, Chung C, Laperriere NJ, Kulkarni AV, Goetz P, Zadeh G. Gamma knife radiosurgery for the treatment of cystic cerebral metastases. *Int J Radiat Oncol Biol Phys.* 2013;**85**(3):667–671.
 18. Yagnik G, Jahangiri A, Chen R, Wagner JR, Aghi MK. Role of a p53 polymorphism in the development of nonfunctional pituitary adenomas. *Mol Cell Endocrinol.* 2017;**446**:81–90.
 19. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Duffy SM, Goldberg RM, de la Chapelle A, Koshiji M, Bhajee F, Huebner T, Hruban RH, Wood LD, Cuka N, Pardoll DM, Papadopoulos N, Kinzler KW, Zhou S, Cornish TC, Taube JM, Anders RA, Eshleman JR, Vogelstein B, Diaz LA Jr. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med.* 2015;**372**(26):2509–2520.
 20. Valeri N, Gasparini P, Fabbri M, Braconi C, Veronese A, Lovat F, Adair B, Vannini I, Fanini F, Bottoni A, Costinean S, Sandhu SK, Nuovo GJ, Alder H, Gafa R, Calore F, Ferracin M, Lanza G, Volinia S, Negrini M, McIlhatton MA, Amadori D, Fishel R, Croce CM. Modulation of mismatch repair and genomic stability by miR-155. *Proc Natl Acad Sci USA.* 2010;**107**(15):6982–6987.