A Novel Mutation of Aryl Hydrocarbon Receptor Interacting Protein Gene Associated with Familial Isolated Pituitary Adenoma Mediates Tumor Invasion and Growth Hormone Hypersecretion

Feng Cai[1], Yuan Hong[1], Jinghong Xu[2], Qun Wu[1], Cesar Reis[3,4], Wei Yan[1], Wei Wang[1], Jianmin Zhang[1]

■ BACKGROUND: **Germline mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene were identified in nearly 20% of families with familial isolated pituitary adenoma. Some variants of *AIP* have been confirmed to induce tumor cell proliferation and invasiveness; however, the mechanism is still unclear.**

■ METHODS: **A novel missense mutation (c.512C>T, p.T171I) was discovered in 3 patients from a Chinese family with familial isolated pituitary adenoma. In silico and multiplex ligation-dependent probe amplification analysis predicted the mutation to be pathogenic. GH3 and 293FT cell lines were used to verify the variant's effect on cell proliferation (Cell Counting Kit-8), invasiveness (Transwell) and growth hormone (GH) secretion (enzyme-linked immunosorbent assay) by transfection with different vectors: control, blank vector, wild-type *AIP*, p.T171I variant (experimental group), p.Q315* variant, and *AIP* small interfering RNA. Furthermore, Zac1, Sstr2, interleukin (IL)-6, and Stat3/phosphorylation-Stat3 expression (reverse transcription polymerase chain reaction, Western blot) in each group was also evaluated.**

■ RESULTS: **The experimental group, p.Q315* variant group, and *AIP* small interfering RNA-overexpressing group promoted cell proliferation at 24 and 48 hours, respectively (compared with the control group; $P < 0.01$ for both). Similarly, the cells in the experimental group manifested more invasion and GH secretion compared with the control group ($P < 0.01$ and $P < 0.05$, respectively). Furthermore, the experimental group cells expressed less Sstr2 (a prerequisite for the responsiveness to somatostatin analogues) and Zac1 (tumor suppressor gene), but more IL-6 and phosphorylated-Stat3 (GH-secretion related).**

■ CONCLUSIONS: **The novel *AIP* mutation c.512C>T (p.T171I) is a pathogenic variant that promoted cell proliferation, invasiveness, and GH secretion through regulation of Sstr2, Zac1, and IL-6/phosphorylated-Stat3 expression.**

INTRODUCTION

Pituitary adenomas (PAs) are commonly sporadic. However, some have been identified as familial adenomas and have been well described in the setting of an endocrine tumor syndrome, such as multiple endocrine neoplasia type 1 (MEN1), Carney complex (CNC), and familial isolated pituitary adenoma (FIPA). They account for 3%–5% of all cases.[1,2] In FIPA, only

Key words
- *AIP*
- Aryl hydrocarbon receptor interacting protein
- Drug resistance
- Familial isolated pituitary adenoma
- FIPA
- Hypersecretion
- Mutation
- Tumor invasiveness

Abbreviations and Acronyms
AIP: Aryl hydrocarbon receptor-interacting protein
cDNA: Complementary DNA
CNC: Carney complex
EDTA: Ethylenediaminetetraacetic acid
FBS: Fetal bovine serum
FIPA: Familial isolate pituitary adenoma
IGF-1: Insulin-like growth factor 1
MEN1: Multiple endocrine neoplasia type 1
MLPA: Multiplex ligation-dependent probe amplification
MRI: Magnetic resonance imaging
PA: Pituitary adenoma
PCR: Polymerase chain reaction
RT: Reverse transcription
siRNA: Small interfering RNA
SSA: Somatostatin analogue
TPR: Tetratricopeptide repeat

From the Departments of [1]Neurosurgery and [2]Pathology, the Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China; and Departments of [3]Preventive Medicine and [4]Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, California, USA

To whom correspondence should be addressed: Jianmin Zhang, M.D. [E-mail: zjm135@zju.edu.cn]

Feng Cai and Yuan Hong contributed equally to the present study.

Citation: World Neurosurg. (2019) 123:e45-e59.
https://doi.org/10.1016/j.wneu.2018.11.021

*Journal homepage: **www.journals.elsevier.com/world-neurosurgery***

*Available online: **www.sciencedirect.com***

1878-8750/$ - see front matter

pituitary tumors will occur in multiple members of a single family in the absence of MEN1 or CNC. Family members will have either the same or different types of PAs. Generally, PAs in FIPA members appear at a younger age and will be larger than sporadic PAs.

Mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene have been confirmed to be associated with the tumorigenesis of FIPA. *AIP* is localized at 11q13, near the *MEN1* gene region, and acts as a tumor suppressor gene. The AIP protein is composed of 330 amino acids, which have an N-terminal immunophilin-like domain and a C-terminal tetratricopeptide repeat (TPR) domain. The α-helices in the TPR domain mediate molecular interactions with many proteins, including heat shock proteins, aryl hydrocarbon receptor, estrogen receptor-α, phosphodiesterases (PDE4A5 and PDE2A3), glucocorticoid receptor, and G proteins. These proteins are involved in signaling processes in carcinogenesis, immunosuppression, and teratogenesis.[3-6] However, the exact mechanisms of tumorigenesis by *AIP* mutations are still poorly understood.

The first study of *AIP* mutations in the Chinese population discovered several novel mutations in both patients with FIPA and those with sporadic PA by analyzing *AIP* variants in silico; however, verification was not conducted.[7] In the present study, we investigated the involvement of a novel *AIP* mutation in a Chinese family with FIPA and growth hormone (GH)-secreting PAs. In addition, we examined the effect of the mutated *AIP* on tumor cell growth and invasiveness.

METHODS

Case Presentation

A 6-year-old boy was taken to the Second Affiliated Hospital of Zhejiang University School of Medicine with a chief complaint of bilateral visual disturbance lasting 3 years. On examination, he had diminished visual acuity and bitemporal hemianopsia. His medical history included a right femoral fracture and a left humeral fracture due to an accident. At the initial examination, his height and weight were 138 cm and 33 kg, respectively. Magnetic

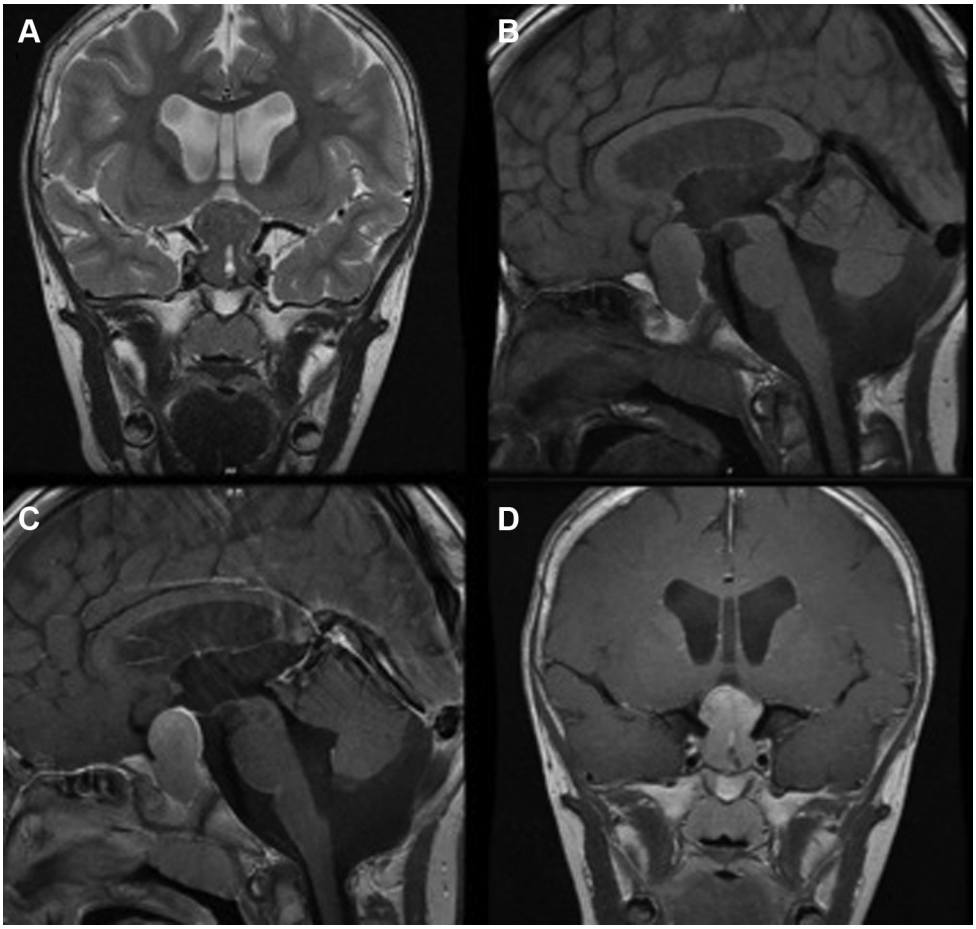


Figure 1. Magnetic resonance image of the sellar region of the patient showing a macroadenoma with suprasellar extension (31 × 24 ×16 mm), constricting the optic chiasma. (**A**) T2-weighted coronal image. (**B**) T1-weighted sagittal image. (**C**) T1-weighted contrast-enhanced sagittal image. (**D**) T1-weighted contrast-enhanced coronal image.

resonance imaging (MRI) of the sellar region displayed a macroadenoma with suprasellar extension (31 × 24 × 16 mm), which constricted the optic chiasma and enlarged the sella turcica (Figure 1).

Endocrine tests showed elevated levels of basal serum GH (78.3 ng/mL), insulin-like growth factor 1 (IGF-1; 433.0 ng/mL), and prolactin (12895.2 mIU/L). No other hormonal abnormalities were detected.

The patient underwent tumor resection with transsphenoidal surgery with an endoscope. The tumor was almost fully removed, with consequent resolution of the visual field defect concomitantly with a reduction in the GH (12.73 ng/mL), IGF-1 (290.0 ng/mL), and prolactin (185.5 mIU/L) levels. At 10 months postoperatively, no evidence of recurrence was demonstrated on the follow-up MRI. Further immunohistochemical analysis revealed the tumor was a mammosomatotroph adenoma with perinuclear positivity of cell adhesion molecules (CAM5.2) and <2% Ki-67 (Figure 2).

Family History

The patient's mother (at 33 years old) and older brother (at 6 years old) underwent PA resection via a transsphenoidal approach at another hospital in 2006. Their initial clinical presentation also involved visual field deficits. The tumor sample from the mother was pathologically confirmed as a GH-secreting PA, and that from the older brother was diagnosed as a mammosomatotroph adenoma. Neither of them showed evidence of tumor relapse on the follow-up MRI scans, although the mother still presented with acromegaly on the physical examination (Supplementary Figure 1). The family members also provided written informed consent, and additional family members were studied (Figure 3). All family members studied were clinically excluded from having MEN1 or CNC and did not carry *MEN1* mutations, as determined by conventional sequencing. No family members displayed acromegaly, except for the mother and her 2 sons, as described.

Standard Protocol Approval and Patient Consents

The study procedures were in strict accordance with the ethics standards of the committee on human experimentation and the 1975 Declaration of Helsinki. The institutional ethics committee of the Second Affiliated Hospital of Zhejiang University Medical School approved the protocols, in accordance with to the third edition of the Guidelines on the Practice of Ethical Committees in Medical Research issued by the Royal College of Physicians of London. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AIP Genetic Analysis

Genomic DNA isolation from ethylenediaminetetraacetic acid (EDTA) blood or tumor samples of patients and unaffected members of this family was performed as described previously.[7] The *AIP* gene and protein sequences were based on Ensembl sequences (ENST00000279146, ENSG00000110711, and ENSP00000279146). Polymerase chain reaction (PCR) of *AIP* exonic sequences was performed with 5 specific primer pairs (Supplementary Table 1), using AmpliTaq Gold DNA Polymerase (Qiagen, Shanghai, China), according to the manufacturer's instructions. Next, the PCR products were purified using the Ampure system (Agencourt Bioscience Corporation, Beverly, Massachusetts, USA) and sequenced using ABI3100 and BigDye Terminator, version 3.1, Technology (Applied Biosystems, Foster City, California, USA). The complete *AIP* coding region was sequenced from both ends, including the exon/intron junctions. Finally, the identified mutations were reamplified and resequenced from both ends.

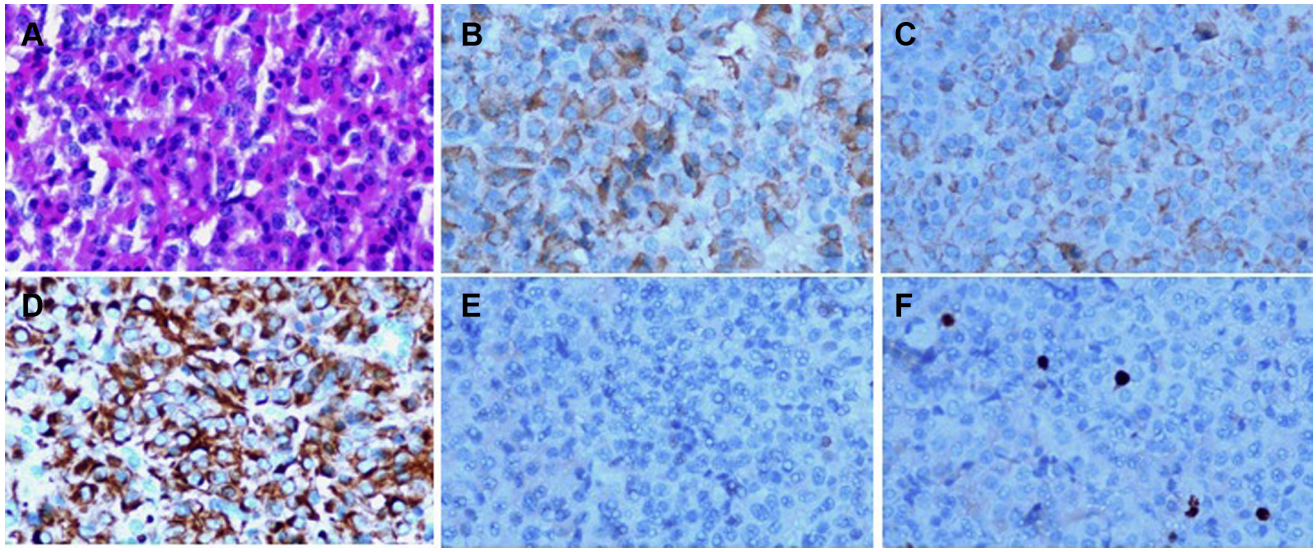


Figure 2. Immunostaining of the younger boy's pituitary adenoma tissue (400× amplification; scale bar, 100 μm): (**A**) hematoxylin and eosin &E staining, eosinophilic; (**B**) growth hormone staining and (**C**) prolactin staining, mammosomatotroph adenoma; (**D**) CAM5.2 staining showing perinuclear positivity; (**E**) p53 staining, with negative findings; and (**F**) Ki-67 staining showing <2%.

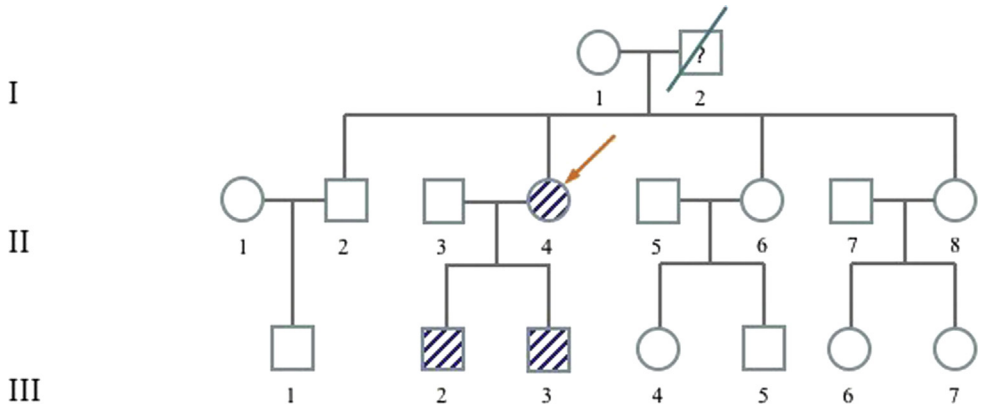


Figure 3. Pedigree of the family. The proband of the family is indicated by an *arrow*. *Squares* indicate male family members; *circles*, female family members; *diagonal lines*, deceased members; and *blue bias*, affected members. The "I2"member with the "?" symbol had died and was a foster son without any information of his family tree.

Heterozygous Deletion (Loss of Heterozygosity) Analysis

The only tumor sample in the present study was from the 6-year-old patient. Hence, loss of heterozygosity was performed using multiplex ligation-dependent probe amplification (MLPA) analysis with an MLPA kit (code no. P244-B1 AIP-MEN1; MRC Holland, Amsterdam, The Netherlands) on the patient's blood and tumor samples, in accordance with the manufacturer's instructions. The data were further analyzed using the Coffalyser MLPA data, version 8, which generated a relative ratio from a comparison of the tumor and normal tissues from the boy, normalized to the unrelated healthy blood. In the present analysis, a ratio between 0.65 and 0.85 was considered to indicate a suspected fragment deletion, and a ratio of <0.65 implied a possible loss of heterozygosity.

Bioinformatics Analysis

The effect of the newly detected *AIP* missense variant on protein structure or function was predicted in silico using PolyPhen-2 (available at: http://genetics.bwh.harvard.edu/pph2/) and SIFT (available at: http://sift.jcvi.org), which supply a prediction for an overall pathogenic score. Additionally, UCSC Genome Bioinformatics (available at: genome.ucsc.edu/cgibin/hgGateway), human single nucleotide polymorphism databases (available at: http://www.ncbi.nlm. nih.gov/SNP/snp.summary.cgi), and Exome Variant Server (available at: http://evs.gs.washington.edu/EVS/) were consulted.

Construction of Expression Vectors

Complementary DNA (cDNA) encoding full-length human AIP was generated by reverse transcription (RT) PCR of total RNA extracted from 293FT cells. The PCR products were cloned into the expression vector, pcDNA 3.1(+), containing a FLAG epitope, and construction of mutated *AIP* (R315*, T171I) expression vectors was performed using site-directed mutagenesis. Furthermore, wild-type *AIP* and *AIP*-small interfering RNA (siRNA) expression vectors were prepared for subcloning into the pCI-neoAIP-myc plasmid vector for the final transfections. Therefore, 6 experimental groups were included according to the subcloned vectors: control (no vector), blank vector, wild-type *AIP*, mutated *AIP* (Q315* or T171I), and *AIP*-siRNA.

Cell Proliferation Assay

293FT cells and the rat somatomammotroph pituitary cell line, GH3, were used in our experiment. The 293 FT cells were cultured in high-glucose Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS), and GH3 cells were cultured in Roswell Park Memorial Institute-1640 medium with 10% FBS. The culture media contained 100 U/mL penicillin G and 0.1 mg/mL streptomycin (Invitrogen, Shanghai, China). The cells were maintained in a humidified 5% carbon dioxide, 95% air incubator at 37°C and were passaged at ~80% confluence using trypsin-EDTA solution (0.05% trypsin, 0.5 mM EDTA). Transfections were performed using Effectene Reagent (Qiagen), as recommended by the manufacturer. Cell proliferation assays were performed using the Cell Counting Kit-8 (catalog no. CP002; SAB, College Park, Maryland, USA). The absorbance of cell supernatant aliquots at 450 nm was measured using an automatic plate analyzer (Bio-Rad Labs, Hercules, California, USA). We performed each experiment 3 times.

Transwell Invasion Assay

We coated 8-mm Transwell inserts in a 24-well plate with 150 μL of a mixture of culture medium and reconstituted Matrigel (BD Biosciences, Seattle, Washington, USA) at a ratio of 2:1. Cell suspension (300 μL) of 293FT or GH3 cells containing 5×10^5 cells/mL was loaded into the upper compartment and was allowed to invade the medium containing 10% FBS in the lower wells of the migration plate for 24 hours. Noninvading cells were removed mechanically using cotton swabs, and the membranes were stained with 1% crystal violet solution. The stained membranes were washed 3 times with distilled water before being photographed via a microscope. The crystal violet staining of the

membranes was dissolved in 33% ethylic acid, and 100 μL of the solution from each sample was transferred to a 96-well microtiter plate and measured at optical density 560 nm in a plate reader.

Enzyme-Linked Immunosorbent Assay

The levels of GH and interleukin (IL)-6 secreted into the culture medium by GH3 cells of each group were measured in 3 independent experiments using the rat GH and IL-6 enzyme-linked immunosorbent assay kit, respectively, according to the manufacturer's protocols.

Protein Extraction and Western Blot Analysis

The cells were harvested on ice using modified radioimmunoprecipitation assay buffer with complete protease inhibitor cocktail (Roche, Shanghai, China). The samples were incubated on ice for >1 hour with occasional gentle vortexing, and debris and insoluble materials were pelleted by centrifugation at 14,000*g* for 10 minutes. Total protein (20 μg) was loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, separated, and transferred onto a nitrocellulose membrane. The immunoblots were incubated in 3% bovine serum albumin, 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 0.1% Tween 20 at room temperature and probed with primary and appropriate secondary antibodies. The antibodies used included AIP (ab192024; Abcam, Cambridge, UK), STAT3 (9139; CST, Danvers, Massachusetts, USA), phosphorylated STAT3 (9145; CST), Sstr2 (ab134152; Abcam), Zac1 (ab90472; Abcam), and β-actin (4970; CST).

Real-Time PCR

To examine messenger RNA expression levels, 2×10^5 GH3 or 293FT cells of each group were seeded on 24-well plates, and total RNA was prepared after 48 hours of incubation using an RNeasy kit. cDNA was prepared from 1 μg of total RNA using the QuantiTect RT kit, and RT-PCR was performed using Light Cycler 2.0 and SYBR Premix Ex Taq II. The primers are listed in **Supplementary Table 1**. The PCR conditions are available on request.

Statistical Analysis

All experiments were performed ≥3 times. Statistical analysis was performed using 1-way analysis of variance with Tukey's post hoc test, as appropriate, using GraphPad Prism, version 5.0, software (GraphPad Software, La Jolla, California, USA). All data are expressed as the mean ± standard error of the mean. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Novel *AIP* Mutation in GH-Secreting FIPA

The *AIP* variant status in the germline of the 3 patients and their relatives was examined. We found a heterozygous missense mutation caused by a C to T nucleotide substitution in exon 4 (c.512 C>T) of the *AIP* gene in each patient but not in any normal relatives (**Figure 4A**) nor in any normal controls. This mutation resulted in replacement of a threonine codon with an isoleucine at amino acid position 171 (p.T171I), near the first TPR-containing domain (p.179-212) that were verified to be associated with a predisposition to PA.[8] To quantify the relative copy number of the *AIP* gene in the 11q13 region in the PA, we performed MLPA analysis on the sample from the younger boy. That analysis demonstrated an ~50% decrease in the copy number of the genes located in this region. In addition to the exons of the *AIP*, a somatic monoallelic deletion of 1 copy of other genes in these loci was observed in the tumor tissue, including *MEN1*, *SNX15*, *FAM89B*, *RELA*, *SART1*, *BRMS1*, and *CCND1* (**Figure 4B**).

Analyses of *AIP* Mutation In Silico

Multialignment analysis confirmed that the T171 residue in *AIP* is phylogenetically conserved (**Supplementary Figure 2**). The prediction software, PolyPhen-2, suggested that the substitution might be damaging to the AIP protein structure with high sensitivity and specificity (**Supplementary Figure 3A**). Another software program, SIFT, indicated that the substitution could be damaging, with high confidence prediction and a median conservation <3.25 (**Supplementary Figure 3B**). Therefore, combined with the MLPA results, we believe this *AIP* variant (c.512 C>T, p.T171I) contributes to the development of PA.

Inhibitory Effect of the *AIP* Variant on Cell Proliferation

First, the effect of this *AIP* variant on cell proliferation was examined in both 293FT and GH3 cell lines. When overexpressing wild-type *AIP*, both cell lines demonstrated significant inhibition of cell growth compared with cells transfected with the control vector (at 24, 48, and 72 hours). However, overexpression of p.T171I *AIP*, or the positive control p.Q315* or *AIP* siRNA, did not inhibit cell proliferation in either cell line (the inhibition ratios vs. the blank vector group were all negative), implying loss of the antineoplastic effect (**Figure 5A,B**).

Promoting Effect of the *AIP* Variant on GH Secretion

The culture medium of GH3 cells overexpressing wild-type *AIP* was examined and had a lower GH level than that of the control group. Furthermore, GH3 cells transfected with p.T171I *AIP*, or the positive control p.Q315* or *AIP* siRNA, secreted more GH than did the cells from the blank vector group. These results suggest that the novel variant promotes GH secretion (**Figure 6**).

Novel *AIP* Variant Promotes GH3 Cell Invasion

GH3 cells expressing p.T171I *AIP* invaded the medium in the lower wells more than did the control, similar to cells expressing p.Q315* or *AIP* siRNA. In contrast, cells transfected with wild-type *AIP* invaded the lower wells much less ($P < 0.05$; **Figure 7**). This result indicated that the novel variant stimulated tumor cell invasiveness.

Effect of Novel *AIP* Variant on Expression of IL-6/STAT3 Pathway

The IL-6 secretion levels from both cell lines with the novel *AIP* variant were greater than those from the control cells or wild-type *AIP*-expressing cells (**Figure 8A**). Moreover, the novel *AIP* variant-overexpressing cells expressed more phosphorylated STAT3 (Tyr705) but not total STAT3 (**Figure 8B**), which plays an important role in GH secretion.[9,10]

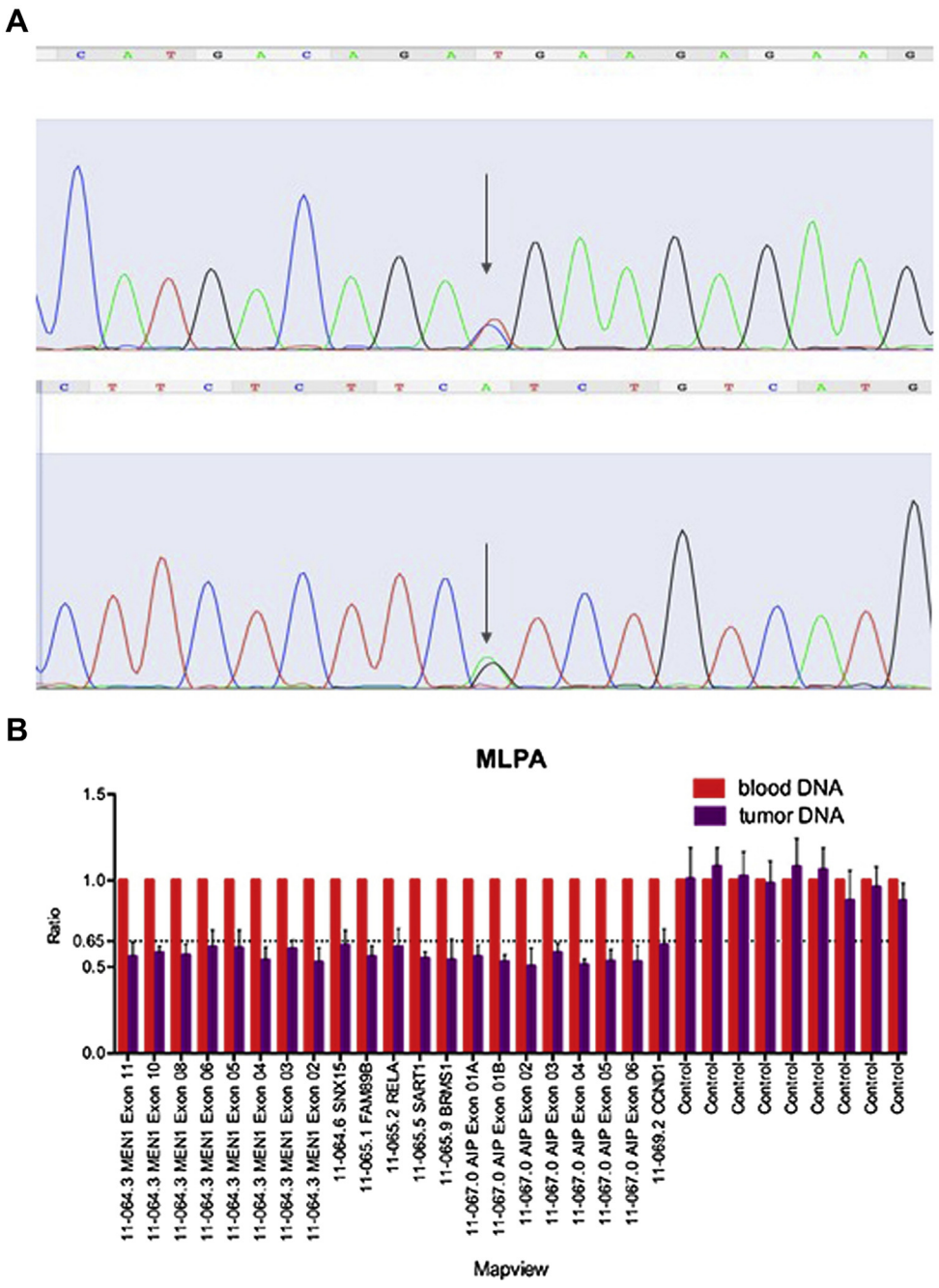


Figure 4. DNA sequencing and multiplex ligation-dependent probe amplification (MLPA) analysis. (**A**) The missense mutation, c.512 C>T (p. T171I), in exon 4 of the *AIP* gene was found in the genomic DNA of the 3 patients' blood samples. (*Top*) Graph showing forward sequencing results and (*Bottom*) graph showing reverse sequencing result for further confirmation. (**B**) Multiplex ligation-dependent probe amplification analysis of genomic DNA extracted from the younger boy's tumor sample. Probe signals from all exons of the *AIP* gene were significantly decreased (<0.65), as were those from other loci on 11q13, indicating the presence of a large deletion across these loci, at least in the tumor sample.

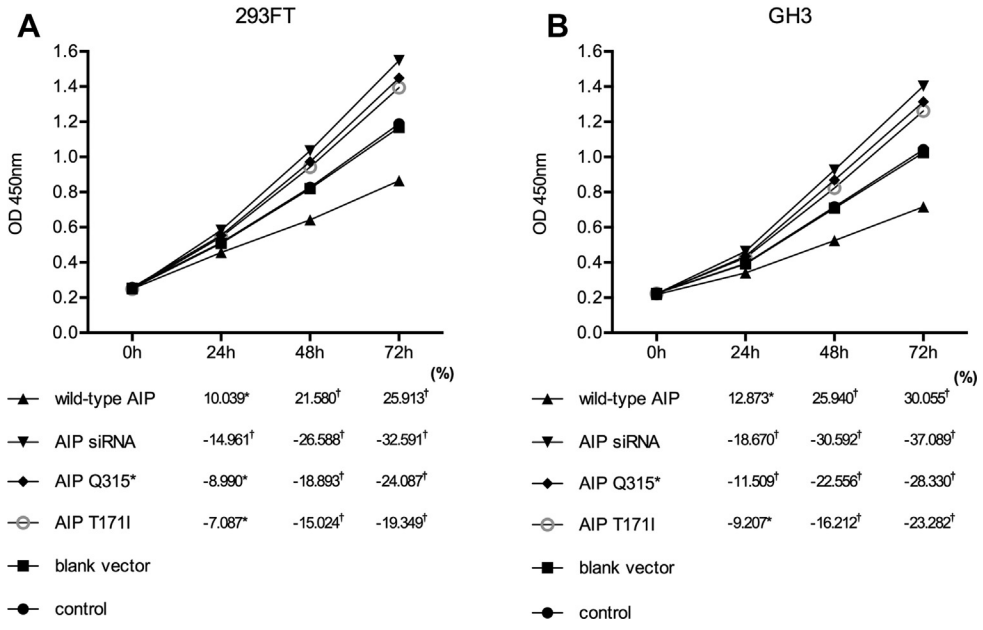


Figure 5. The effect of the mutation on cell proliferation. (**A**) 293FT and (**B**) GH3 cell lines were examined for verification of the effect of the new *AIP* mutant on cell proliferation using the Cell Counting Kit-8 method (at 24, 48, and 72 hours). (*Bottom*) The data represent the relative increased inhibition ratio of cell proliferation of each group versus the blank vector group at each time point. Negative numbers indicate that the proliferation of cells relative to the blank vector group was not inhibited but promoted. We performed analysis of variance and the Tukey post hoc test for the optical density (OD) of 450 nm of each group at each time point compared with the blank vector group (*$P <$ 0.05; †$P <$ 0.01). siRNA, small interfering RNA.

Expression of Zac1 and Sstr2 in GH3 Cells Expressing the Novel *AIP* Variant

We observed that the expression of the downstream antineoplastic gene, *Zac1*, in GH3 cells expressing the novel *AIP* variant was significantly lower than that in the control group or wild-type *AIP*-overexpressing group (Figure 9A). Additionally, the expression of the somatostatin analogue (SSA)-sensitive gene, *Sstr2*, in GH3 cells expressing the novel mutant was significantly reduced compared with the wild-type *AIP*-overexpressing or control group (Figure 9B). Moreover, Sstr2 expression in cells with *AIP* siRNA was increased significantly more than that in cells from the control or wild-type *AIP*-overexpressing group. These findings might explain why the tumors with *AIP* mutation or less AIP expression have a drug resistance phenotype.[11]

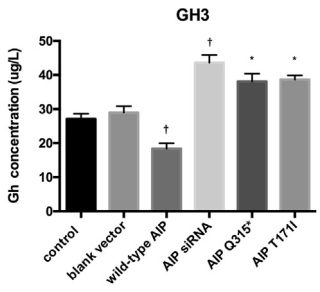


Figure 6. Effect of the *AIP* mutation on growth hormone (GH; Gh) secretion. The GH level in the culture medium from GH3 rat somatotropinoma cell line at 48 hours was examined using enzyme-linked immunosorbent assay. We performed analysis of variance and the Tukey post hoc test; data are presented as the mean ± standard error of the mean (*$P <$ 0.05; †$P <$ 0.01).

DISCUSSION

PAs are usually benign tumors in which classic oncogenes are seldom mutated. It has been identified that loss or gain of function of some genes leads to pituitary tumorigenesis.[12] Cases with a familial background account for 5% of PAs, either as part of another syndrome (i.e., MEN1 and MEN4, CNC, neurofibromatosis) or in FIPA, which only affects the pituitary gland.[1] FIPA is characterized by the manifestation of PAs in ≥2 members of the same family without other syndromic features. FIPA can be either homogeneous (affecting members with an identical tumor type) or heterogeneous (affecting members with different tumor types). In the present study, the case was a typical homogeneous FIPA with GH-secreting PA in all 3 affected members.

In the FIPA cases, 1 of the most important genes is *AIP* in which mutations and deletions are associated with FIPA

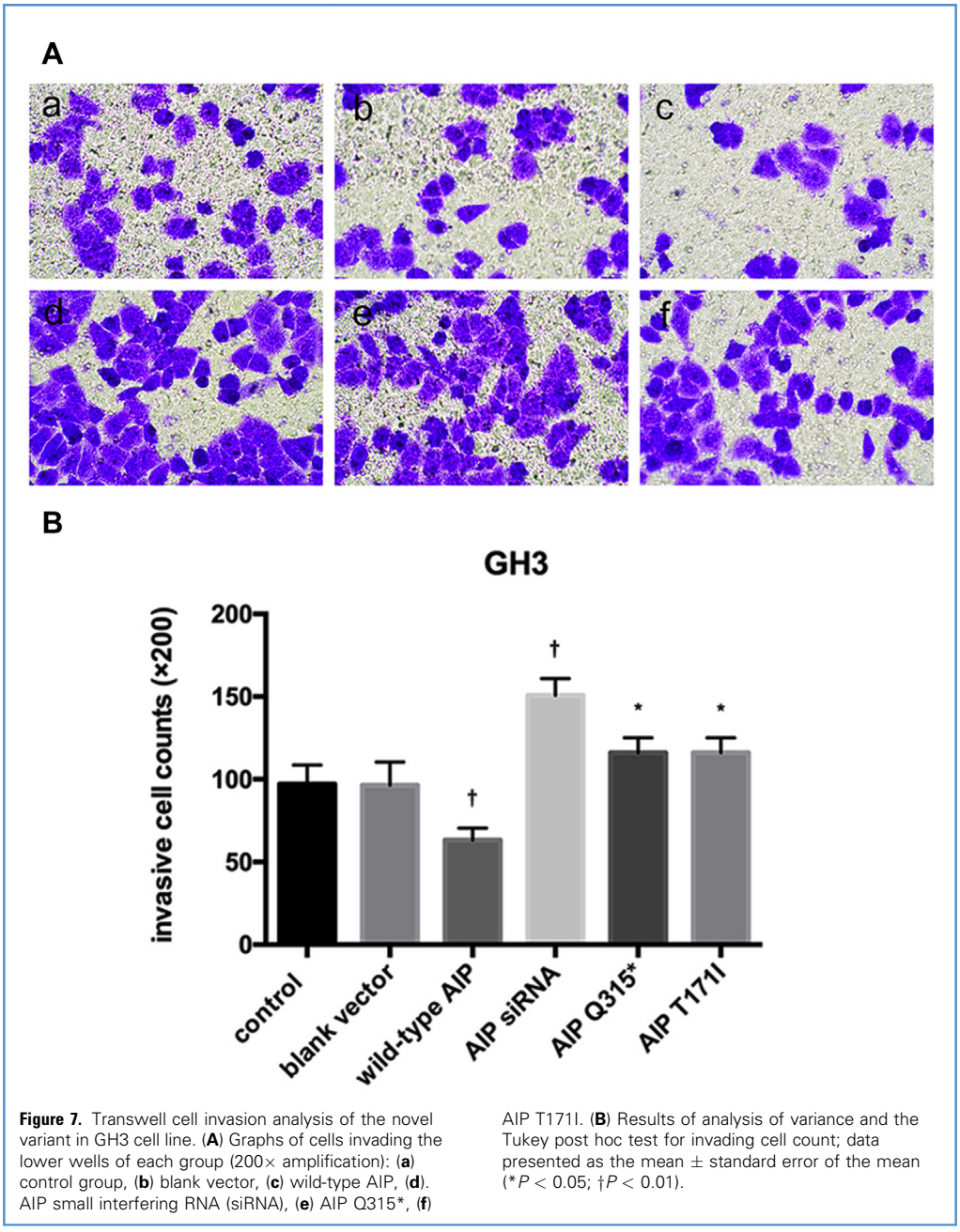


Figure 7. Transwell cell invasion analysis of the novel variant in GH3 cell line. (**A**) Graphs of cells invading the lower wells of each group (200× amplification): (**a**) control group, (**b**) blank vector, (**c**) wild-type AIP, (**d**). AIP small interfering RNA (siRNA), (**e**) AIP Q315*, (**f**) AIP T171I. (**B**) Results of analysis of variance and the Tukey post hoc test for invading cell count; data presented as the mean ± standard error of the mean (*$P < 0.05$; †$P < 0.01$).

tumorigenesis. Inactivation germline mutations in the *AIP* gene account for 17% of FIPA cases with low penetrance.[13,14] The human *AIP* gene encodes a 37-kDa protein of 330 amino acids that consists of an N-terminal immunophilin-like domain[15] and a highly conserved C-terminal with 3 TPR domains and an α-7 helix.[8] The conserved C-terminal motif was confirmed to mediate AIP interactions with a number of partners.[6] Because loss-of-function variants of *AIP* predispose to PAs, *AIP* is believed to be a tumor suppressor gene in the pituitary. Furthermore, sporadic GH-secreting pituitary tumors with lower *AIP* expression are aggressive and drug resistant.[16,17] The antiparallel α-helices are considered to be essential for AIP binding function; thus, damage to these structures might contribute to pituitary tumorigenesis.

In the present study, we report the case of a family with FIPA in which the mother and her 2 male children had developed a GH-secreting PA. The novel mutation, p.T171I, was identified in the germline of all 3 patients but was absent in the other healthy relatives. Additionally, MLPA analysis of the PA tissue from the younger boy showed at least a 2.5-Mb deletion in 11q13 containing the *AIP* locus. The loss of the wild-type allele in 11q13 from *MEN1* to *CCND1* in the PA was observed,[18-20] including 3

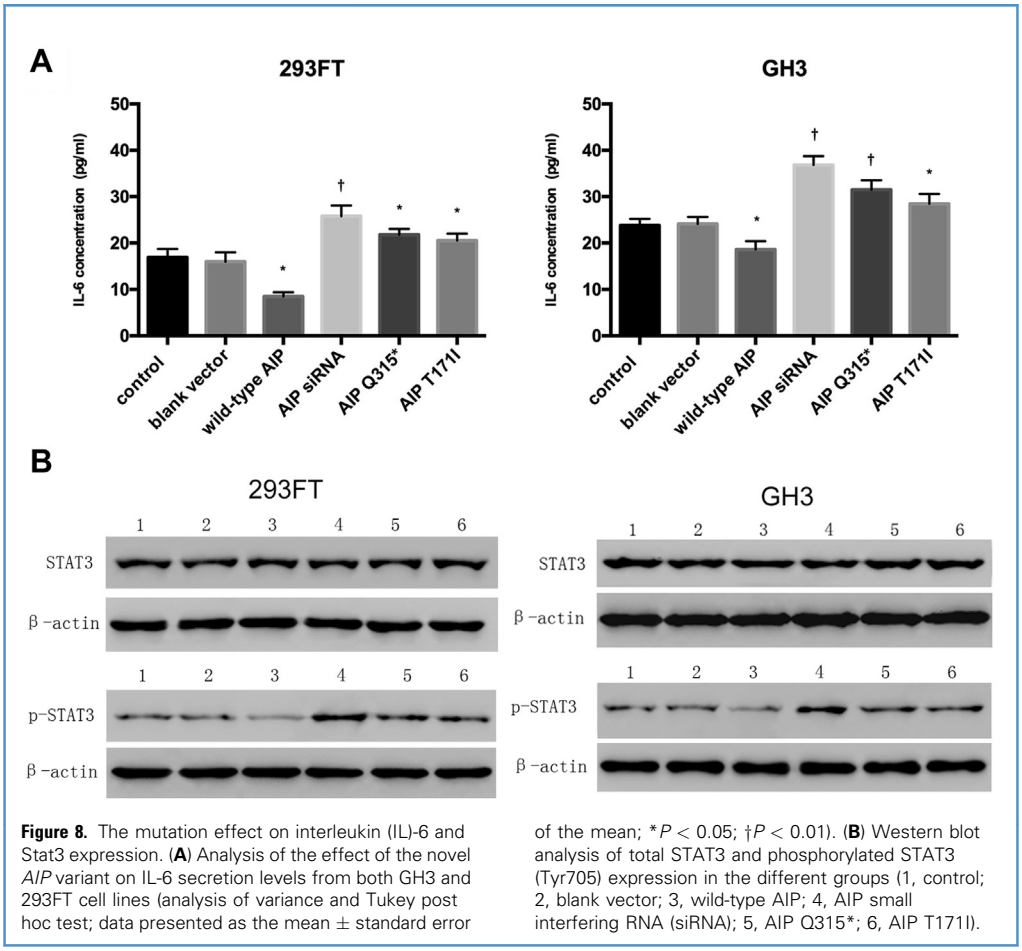


Figure 8. The mutation effect on interleukin (IL)-6 and Stat3 expression. (**A**) Analysis of the effect of the novel *AIP* variant on IL-6 secretion levels from both GH3 and 293FT cell lines (analysis of variance and Tukey post hoc test; data presented as the mean ± standard error of the mean; $*P < 0.05$; $\dagger P < 0.01$). (**B**) Western blot analysis of total STAT3 and phosphorylated STAT3 (Tyr705) expression in the different groups (1, control; 2, blank vector; 3, wild-type AIP; 4, AIP small interfering RNA (siRNA); 5, AIP Q315*; 6, AIP T171I).

Chinese patients with PA with previously reported *AIP* mutations.[7] In addition, deletion of the normal alleles of *AIP* alleles at the somatic level in tumor tissues usually spans a larger region than that at the germline level. The molecular mechanism is still unclear.

In silico analysis predicted that the p.T171I variant might have a damaging effect on the structure of the AIP protein. To date, >90 mutations of *AIP* have been identified as tumor causing (from Human Gene Mutation Database data).[21] Approximately 75% of *AIP* mutations will lead to an impaired C-terminal TPR domain and an α-helix, thus losing the ability to interact with other client proteins.[8,22] Although the novel mutation is not located within the TPR domain, although near the first repeat (179–212), considering the family history and the MLPA analysis results, it is likely pathogenic by affecting the motif secondary or tertiary structure, indirectly.

Hence, the following in vitro experiments were performed to verify the prediction. First, both 293FT and GH3 cells transfected with AIP p.T171I (experimental group) grew much faster than did the blank control (negative control) and the wild-type AIP-overexpressing groups but slower than the *AIP*-siRNA group (positive control). The cell proliferation rate of the experimental group was similar to that of the AIP p.Q315* group, which has been shown to influence pituitary tumor cell growth.[23] Furthermore, the experimental group cells appeared more aggressive, similar to the positive control and p.Q315* groups. As stated, the C-terminal domain of AIP protein is the main domain that interacts with other partners. By forming a complex with p23 and Hsp90 at its C-terminal domain, AIP is able to prevent the ubiquitin-mediated degradation of aryl hydrocarbon receptor,[24] which can act as a transcription factor of some genes, and its expression can be suppressed by low AIP expression or AIP mutation.[16,25] Moreover, recently, AIP was suggested to affect the Gαi-cyclic adenosine monophosphate pathway mediating somatotroph cell proliferation and somatostatin action.[26]

AIP has also been shown to regulate the cell cycle by influencing the expression of the tumor suppressor protein, ZAC1, whose activation can be triggered by SSAs via SSTR2. Clinically, ZAC1 expression was decreased when AIP was downregulated.[27,28] The membranous expression of SSTR2 is a prerequisite for the responsiveness to SSAs. Although previous studies have confirmed that tumors with reduced AIP expression were frequently resistant to first-generation SSAs, some showed no

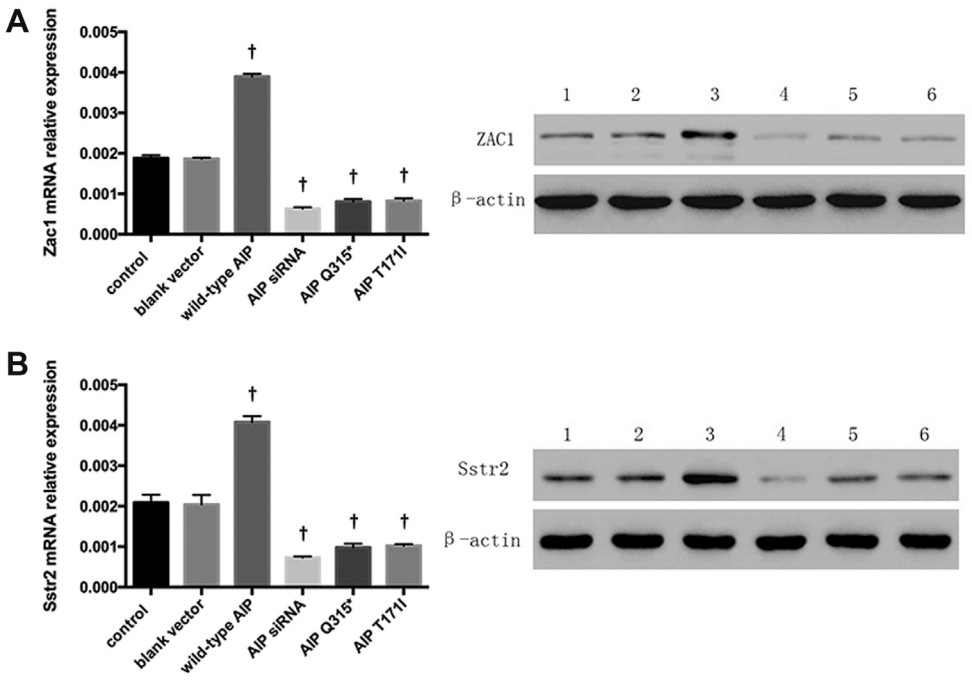


Figure 9. Analysis of the effect of the novel *AIP* variant on the (**A**) Zac1 and (**B**) Sstr2 expression (messenger RNA and protein levels) in GH3 cell lines (analysis of variance and Tukey post hoc test for messenger RNA expression; data presented as the mean ± standard error of the mean; $*P < 0.05$; $\dagger P < 0.01$).

difference in SSTR2 expression between sporadic somatotropinoma with low or conserved AIP expression[17] or AIP mutation-positive somatotropinoma and AIP mutation-negative cases.[27] Recently, using a more sensitive scoring system that accounts for the subcellular localization rather than the staining intensity, it has been observed that samples with low AIP expressed SSTR2 at a lower score compared with those with conserved AIP expression.[29] This implied that lower AIP expression or deficient AIP function could explain why some patients had tumors resistant to SSA treatment.[16,30-32] Therefore, we believe that AIP-SSTR2-ZAC1 signaling mediates the antitumor effect of SSAs on PAs. In the present study, we found that the novel AIP mutation reduced the expression of SSTR2 and ZAC1, partially explaining the tumors' invasiveness and the onset at a young age. Because all 3 patients had undergone surgery as first therapy, the tumors were almost totally resected, and it was unknown whether the patients with the novel AIP mutation were resistant to SSAs, SSA treatment was not suggested. Although low AIP expression or an AIP mutation is a risk factor for SSA resistance, patients with an AIP-deficient pituitary tumor might benefit from the multireceptor-targeted (SSTR1, SSTR2, SSTR 3, SSTR 5) SSA, pasireotide, which requires confirmation in a larger series of samples in the future.[29]

In addition to the mass effect, GH-secreting PAs can lead to acromegaly or gigantism, as well as other complications caused by IGF-1 directly. Thus, reducing the GH and IGF-1 levels became the target of GH-secreting PA therapy. In the present study, the 3 patients with FIPA had greater GH and IGF-1 levels in their serum preoperatively. In vitro, the novel p.T171I mutation discovered in the 3 patients promoted GH secretion. In the recently established novel somatotroph cell line, GH3-FTY, the endogenous *Aip* was completely disrupted using the CRISPR/Cas9 method. *Aip* inactivation in this cell line caused GH overproduction through IL-6/Stat3 pathway activation, which could be reversed by the exogenous expression of wild-type Aip.[9] However, in another study, GH induced Stat3 phosphorylation in GH3 cells, implying that an autoregulatory positive-feedback loop might exist between Stat3 and GH in somatotropic tumor cells.[10] In the present study, the cells with the novel mutation expressed increased IL-6 and phosphorylated Stat3 levels. It might be that AIP inactivation by the AIP mutation initiates a vicious circle between Stat3 phosphorylation and GH overproduction. Nevertheless, the relationship among AIP, GH, and IL-6/Stat3 should be studied further.

Although a novel *AIP* disease-causing mutation was identified in FIPA cases, *AIP* germline mutations account for only 17% of families with FIPA families.[13] Duplication of the *GPR101* gene in the Xq26.3 region has recently been discovered to be involved in the pathogenesis of FIPA X-linked acrogigantism.[33] However, it is unknown what genetic mutation most families with FIPA will harbor. In the present study, other tumorigenesis-related gene mutations might have been discovered if we had had the tumor samples from the mother and her older son.

CONCLUSION

The novel missense mutation in the *AIP* gene, p.T171I, identified in a Chinese GH-secreting FIPA case, is a pathogenic variant that stimulated tumor cell proliferation and invasiveness and GH hypersecretion. The AIP-SSTR2-ZAC1 and AIP-IL-6-STAT3 pathways might be involved in these cell processes. However, further research is required to elucidate the molecular mechanism.

ACKNOWLEDGMENTS

The authors thank Nurse Qinghua Huang, Dr. Jun Hu, and Dr. Ligen Shi for collection and storage of the patient samples and the patients and their family members for participating in the present study. We also thank Michal Bell, PhD, Liwen Bianji, Edanz Editing China (available at: www.liwenbianji.cn/ac), for editing the English text of a draft of our report.

REFERENCES

1. Beckers A, Daly AF. The clinical, pathological, and genetic features of familial isolated pituitary adenomas. *Eur J Endocrinol*. 2007;157:371-382.
2. Daly AF, Tichomirowa MA, Beckers A. The epidemiology and genetics of pituitary adenomas. *Best Pract Res Clin Endocrinol Metab*. 2009;23:543-554.
3. Bell DR, Poland A. Binding of aryl hydrocarbon receptor (AhR) to AhR-interacting protein: the role of hsp90. *J Biol Chem*. 2000;275:36407-36414.
4. Igreja S, Chahal HS, King P, Bolger GB, Srirangalingam U, Guasti L, et al. Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families. *Hum Mutat*. 2010;31:950-960.
5. Vargiolu M, Fusco D, Kurelac I, Dirnberger D, Baumeister R, Morra I, et al. The tyrosine kinase receptor RET interacts in vivo with aryl hydrocarbon receptor-interacting protein to alter survivin availability. *J Clin Endocrinol Metab*. 2009;94:2571-2578.
6. Trivellin G, Korbonits M. AIP and its interacting partners. *J Endocrinol*. 2011;210:137-155.
7. Cai F, Zhang YD, Zhao X, Yang YK, Ma SH, Dai CX, et al. Screening for AIP gene mutations in a Han Chinese pituitary adenoma cohort followed by LOH analysis. *Eur J Endocrinol*. 2013;169:867-884.
8. Morgan RM, Hernandez-Ramirez LC, Trivellin G, Zhou L, Roe SM, Korbonits M, et al. Structure of the TPR domain of AIP: lack of client protein interaction with the C-terminal alpha-7 helix of the TPR domain of AIP is sufficient for pituitary adenoma predisposition. *PLoS One*. 2012;7:e53339.
9. Fukuda T, Tanaka T, Hamaguchi Y, Kawanami T, Nomiyama T, Yanase T. Augmented growth hormone secretion and Stat3 phosphorylation in an aryl hydrocarbon receptor interacting protein (AIP)-disrupted somatotroph cell line. *PLoS One*. 2016;11:e0164131.
10. Zhou C, Jiao Y, Wang R, Ren SG, Wawrowsky K, Melmed S. STAT3 upregulation in pituitary somatotroph adenomas induces growth hormone hypersecretion. *J Clin Invest*. 2015;125:1692-1702.
11. Gadelha MR, Kasuki L, Korbonits M. Novel pathway for somatostatin analogs in patients with acromegaly. *Trends Endocrinol Metab*. 2013;24:238-246.
12. Caimari F, Korbonits M. Novel genetic causes of pituitary adenomas. *Clin Cancer Res*. 2016;22:5030-5042.
13. Hernandez-Ramirez LC, Gabrovska P, Denes J, Stals K, Trivellin G, Tilley D, et al. Landscape of familial isolated and young-onset pituitary adenomas: prospective diagnosis in AIP mutation carriers. *J Clin Endocrinol Metab*. 2015;100:E1242-E1254.
14. Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, Raitila A, et al. Pituitary adenoma predisposition caused by germline mutations in the AIP gene. *Science*. 2006;312:1228-1230.
15. Linnert M, Haupt K, Lin YJ, Kissing S, Paschke AK, Fischer G, et al. NMR assignments of the FKBP-type PPIase domain of the human aryl-hydrocarbon receptor-interacting protein (AIP). *Biomol NMR Assign*. 2012;6:209-212.
16. Jaffrain-Rea ML, Angelini M, Gargano D, Tichomirowa MA, Daly AF, Vanbellinghen JF, et al. Expression of aryl hydrocarbon receptor (AHR) and AHR-interacting protein in pituitary adenomas: pathological and clinical implications. *Endocr Relat Cancer*. 2009;16:1029-1043.
17. Kasuki L, Vieira Neto L, Wildemberg LE, Colli LM, de Castro M, Takiya CM, et al. AIP expression in sporadic somatotropinomas is a predictor of the response to octreotide LAR therapy independent of SSTR2 expression. *Endocr Relat Cancer*. 2012;19:L25-L29.
18. Guaraldi F, Corazzini V, Gallia GL, Grottoli S, Stals K, Dalantaeva N, et al. Genetic analysis in a patient presenting with meningioma and familial isolated pituitary adenoma (FIPA) reveals selective involvement of the R81X mutation of the AIP gene in the pathogenesis of the pituitary tumor. *Pituitary*. 2012;15(suppl 1):S61-S67.
19. Occhi G, Jaffrain-Rea ML, Trivellin G, Albiger N, Ceccato F, De Menis E, et al. The R304X mutation of the aryl hydrocarbon receptor interacting protein gene in familial isolated pituitary adenomas: mutational hot-spot or founder effect? *J Endocrinol Invest*. 2010;33:800-805.
20. Soares BS, Eguchi K, Frohman LA. Tumor deletion mapping on chromosome 11q13 in eight families with isolated familial somatotropinoma and in 15 sporadic somatotropinomas. *J Clin Endocrinol Metab*. 2005;90:6580-6587.
21. Cansu GB, Taskiran B, Trivellin G, Faucz FR, Stratakis CA. A novel truncating AIP mutation, p.W279*, in a familial isolated pituitary adenoma (FIPA) kindred. *Hormones (Athens)*. 2016;15:441-444.
22. Ozfirat Z, Korbonits M. AIP gene and familial isolated pituitary adenomas. *Mol Cell Endocrinol*. 2010;326:71-79.
23. Iwata T, Yamada S, Ito J, Inoshita N, Mizusawa N, Ono S, et al. A novel C-terminal nonsense mutation, Q315X, of the aryl hydrocarbon receptor-interacting protein gene in a Japanese familial isolated pituitary adenoma family. *Endocr Pathol*. 2014;25:273-281.
24. Kazlauskas A, Poellinger L, Pongratz I. The immunophilin-like protein XAP2 regulates ubiquitination and subcellular localization of the dioxin receptor. *J Biol Chem*. 2000;275:41317-41324.
25. Fujii-Kuriyama Y, Mimura J. Molecular mechanisms of AhR functions in the regulation of cytochrome P450 genes. *Biochem Biophys Res Commun*. 2005;338:311-317.
26. Ritvonen E, Pitkanen E, Karppinen A, Vehkavaara S, Demir H, Paetau A, et al. Impact of AIP and inhibitory G protein alpha 2 proteins on clinical features of sporadic GH-secreting pituitary adenomas. *Eur J Endocrinol*. 2017;176:243-252.
27. Chahal HS, Trivellin G, Leontiou CA, Alband N, Fowkes RC, Tahir A, et al. Somatostatin analogs modulate AIP in somatotroph adenomas: the role of the ZAC1 pathway. *J Clin Endocrinol Metab*. 2012;97:E1411-E1420.
28. Theodoropoulou M, Zhang J, Laupheimer S, Paez-Pereda M, Erneux C, Florio T, et al. Octreotide, a somatostatin analogue, mediates its antiproliferative action in pituitary tumor cells by altering phosphatidylinositol 3-kinase signaling and inducing Zac1 expression. *Cancer Res*. 2006;66:1576-1582.
29. Iacovazzo D, Carlsen E, Lugli F, Chiloiro S, Piacentini S, Bianchi A, et al. Factors predicting pasireotide responsiveness in somatotroph pituitary adenomas resistant to first-generation somatostatin analogues: an immunohistochemical study. *Eur J Endocrinol*. 2016;174:241-250.

30. Daly AF, Tichomirowa MA, Petrossians P, Heliovaara E, Jaffrain-Rea ML, Barlier A, et al. Clinical characteristics and therapeutic responses in patients with germ-line AIP mutations and pituitary adenomas: an international collaborative study. *J Clin Endocrinol Metab*. 2010; 95:E373-E383.

31. Leontiou CA, Gueorguiev M, van der Spuy J, Quinton R, Lolli F, Hassan S, et al. The role of the aryl hydrocarbon receptor-interacting protein gene in familial and sporadic pituitary adenomas. *J Clin Endocrinol Metab*. 2008;93: 2390-2401.

32. Oriola J, Lucas T, Halperin I, Mora M, Perales MJ, Alvarez-Escola C, et al. Germline mutations of AIP gene in somatotropinomas resistant to somatostatin analogues. *Eur J Endocrinol*. 2013;168:9-13.

33. Trivellin G, Daly AF, Faucz FR, Yuan B, Rostomyan L, Larco DO, et al. Gigantism and acromegaly due to Xq26 microduplications and GPR101 mutation. *N Engl J Med*. 2014;371: 2363-2374.

Conflict of interest statement: This work was supported by Medical and Health Research Project of Zhejiang Province (Grant number: 201482029), Natural Science Foundation of Zhejiang Province (Grant number: LQ15H070001, LY17H090012), Natural Science Foundation of China (Grant number: 81502321), Science and Technology Department of Zhejiang Province Project (Grant number: 2015C33192), and Grant WKJ-ZJ-1615(2016149634) from a major science and technology project in medical and health of Zhejiang Province (co-constructed project by the province and the ministry).

Received 23 June 2018; accepted 4 November 2018

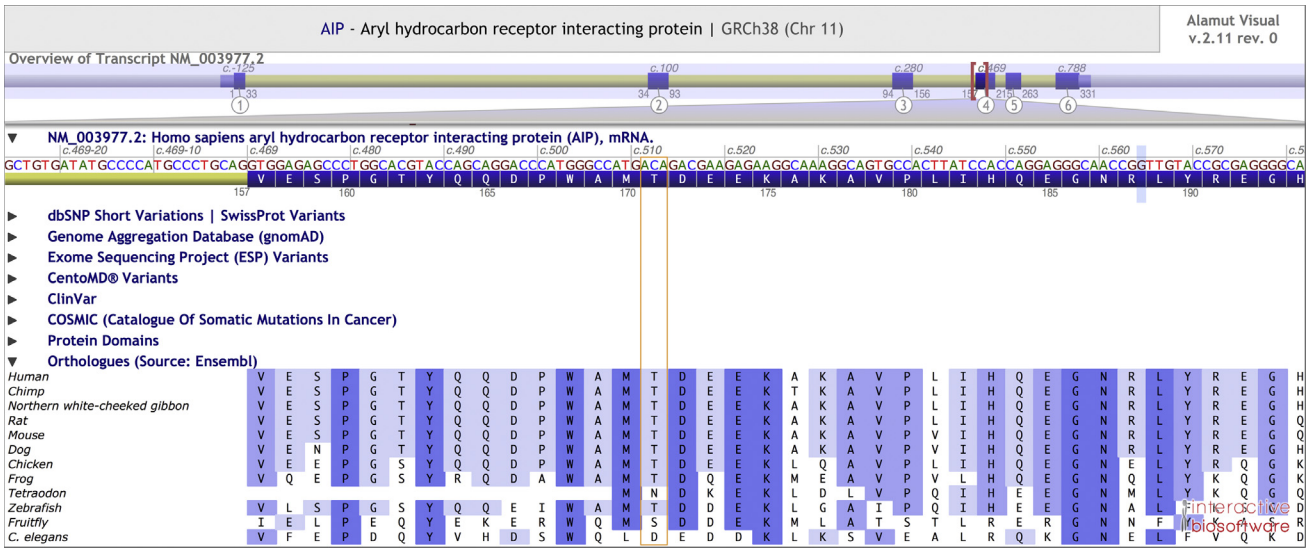
Citation: World Neurosurg. (2019) 123:e45-e59.
https://doi.org/10.1016/j.wneu.2018.11.021

Journal homepage: ***www.journals.elsevier.com/world-neurosurgery***

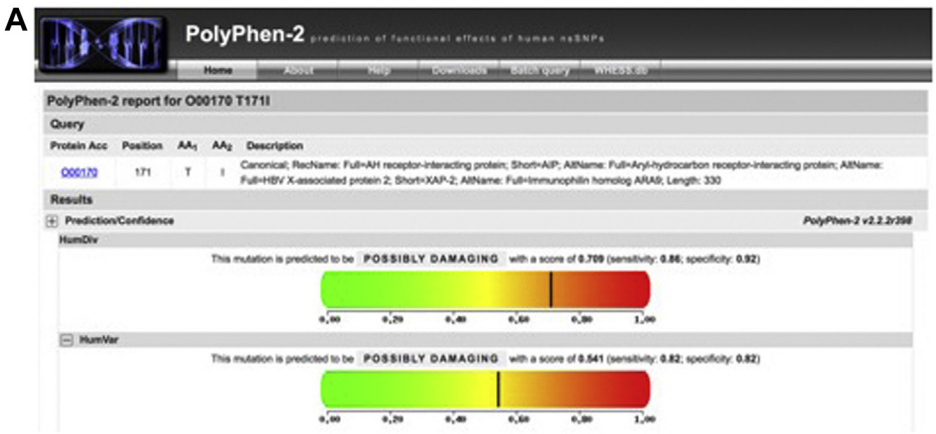
Available online: ***www.sciencedirect.com***

SUPPLEMENTARY DATA

Supplementary Figure 1. Family photograph of the 3 patients taken in 2016.



Supplementary Figure 2. Sequence alignment of aryl hydrocarbon receptor-interacting protein (AIP) protein in different species using Alamut software (Rouen, France). The number 171 at the top represents the related site in 12 different species at the location of the missense mutation in the present study. The darker the blue, the more conserved the residue of AIP protein.



B

User Input	ENSP	Pos	Ref	Subst	Prediction	SIFT Score	Median Information Content	# Seqs
ENSP00000279146, T171I	ENSP00000279146	171	T	I	DAMAGING	0.01	2.57	110

? - Indicates either ENSP or amino acid position not found.

*DAMAGING - Low confidence predictions with Median conservation above 3.25

Supplementary Figure 3. In silico prediction of the novel mutation. (**A**) PolyPhen-2, version 2.2.2r398: the mutation (protein access no. O00170 T171I) was predicted to be possibly damaging with greater scores. (**B**) SIFT prediction: ENSP00000279146, T171I was predicted to be damaging with high confidence prediction.

Supplementary Table 1. Primer Sequences for Reverse Transcription Polymerase Chain Reaction of Messenger RNAs Used in the Present Study

mRNA	Primer Sequence
IL-6	
Primer F	5′-ACCAGGAACGAAAGTCAACTC-3′
Primer R	5′-TGGCTGTCAACAACATCAGTC-3′
Zac1	
Primer F	5′-CTTGCTTGGCGTGTGGTTG-3′
Primer R	5′-CTAGGATGGCCGTCTTCTGTG-3′
GH	
Primer F	5′-AGTTCGAGCGTGCCTACATTCC-3′
Primer R	5′-CAGCGAGAAGCGAAGCAATTCC-3′
Sstr2	
Primer F	5′-GTGCTTGCCCGCTATGTAATC-3′
Primer R	5′-AGTCCTTGTCGTCAGGTTCAG-3′
Gapdh	
Primer F	5′-GTCGGTGTGAACGGATTTG-3′
Primer R	5′-TCCCATTCTCAGCCTTGAC-3′
IL-6	
Primer F	5′-GCACCTCAGATTGTTGTTG-3′
Primer R	5′-AGTGTCCTAACGCTCATAC-3′
SSTR2	
Primer F	5′-TCTGCCTTTCTTGGCTATG-3′
Primer R	5′-GCTGGTGAACTGATTGATG-3′
ZAC1	
Primer F	5′-ATCCTGCCTCATTTCCATC-3′
Primer R	5′-TCATCTCAAGCCAGTCATC-3′
GAPDH	
Primer F	5′-AATCCCATCACCATCTTC-3′
Primer R	5′-AGGCTGTTGTCATACTTC-3′

mRNA, messenger RNA; IL-6, interleukin-6; F, forward; R, reverse; GH, growth hormone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.