Title:

A novel C-terminal nonsense mutation, Q315X, of the aryl hydrocarbon receptor-interacting protein gene

in a Japanese familial isolated pituitary adenoma family

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Abstract

Although the cause of familial isolated pituitary adenoma (FIPA) remains unknown in many cases, germ-line mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene were identified in approximately 20% of families with FIPA. We investigated the *AIP* gene mutation by a standard sequencing method in 12 members of a Japanese two-generation FIPA family, which includes 3 patients with early-onset acromegaly. Multiplex ligation-dependent probe amplification analysis in a tumor sample was attempted to examine the loss of heterozygosity (LOH) in the locus. The effect of the detected mutation on cell proliferation was investigated. A germ-line mutation of c.943C>T (p.Q315X) generating an AIP protein with the C-terminal end deleted was found in the FIPA family. Biallelic inactivation of AIP by a combination of the germ-line mutation and LOH at 11q13 was confirmed in the tumor. The nonsense mutation disrupted the ability to inhibit cell proliferation. We conclude that p.Q315X mutation in the *AIP* gene is a pathogenic variant and the C-terminal region of AIP plays an important role in the predisposition to pituitary adenomas.

Key words: familial isolated pituitary adenoma, acromegaly, aryl hydrocarbon receptor-interacting protein, loss of heterozygosity

Introduction

Pituitary adenomas that are relatively common in the general population are usually sporadic; however, familial adenomas have been identified in 3-5% of all cases [1, 2]. Multiple endocrine neoplasia type 1 (MEN1) and Carney complex are well-characterized familial syndromes forming multiple endocrine neoplasia including anterior pituitary tumors. In MEN1, germ-line mutations in the *MEN1* gene have been found in most patients [3, 4]. In 60% of patients with Carney complex, germ-line mutations of the *PRKAR1A* gene encoding the R1α regulatory subunit of cAMP-dependent protein kinase A on 17q22-24 were detected [5]. Familial isolated pituitary adenoma (FIPA) is defined as the occurrence of two or more related members of the pituitary adenomas outside of the setting of MEN1 or Carney complex in a kindred. In FIPA, pituitary adenomas present homogeneously or heterogeneously within the same family [6]. Mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene located on 11q13 are reported to be associated with pituitary adenoma predisposition [7]. The *AIP* mutations occur in 15-20% of FIPA and in 3-5% of sporadic pituitary adenomas, especially GH-secreting adenomas and prolactinomas, and are associated with the occurrence of large pituitary adenomas at a young age [8]. Gigantism is a particular feature of *AIP* mutations and occurs in more than 30% of affected GH-secreting adenoma patients.

The human *AIP* gene encodes a 37-kDa protein composed of 330 amino acids that has an N-terminal immunophilin-like domain and a C-terminal tetratricopeptide repeat (TPR) domain [9, 10]. The TPR domains consist of three sets of a consensus sequence of 34 amino acids forming two α-helices. AIP has been reported to interact with various proteins such as chaperone proteins (heat shock protein 90 (HSP90), HSP70, and translocase of outer mitochondrial membrane 20), client proteins including nuclear

receptors (aryl hydrocarbon receptor (AhR), estrogen receptor-α, glucocorticoid receptor, peroxisome proliferator-activated receptor-α, and thyroid hormone receptor-β1), phosphodiesterase (PDE4A5 and PDE2A3), survivin, G proteins, RET, and Epstein-Barr virus-encoded nuclear antigen 3 [11].   Of note, a final C-terminal α-7 helix (Cα-7h) mediates molecular interactions with many proteins including a co-chaperone of HSP90 and AhR [12].   Approximately 75% of *AIP* mutations completely disrupt the C-terminal TPR domain and/or the Cα-7h [6, 11], suggesting that these domains have an important role in the function of AIP as a tumor suppressor.   However, the exact mechanisms of tumor suppression by AIP are poorly understood.

We investigated the involvement of the *AIP* mutation in a Japanese FIPA family with pituitary adenomas and found a novel *AIP* nonsense mutation at the C-terminus.   An effect of the mutated AIP on cell growth was also examined.   In addition, a region of loss of heterozygosity (LOH) on 11q13 in *AIP* or *MEN1*-related pituitary adenomas is discussed.

Subject and methods

Case report

The index case was a 16-year-old female who was taken to hospital because of headache and visual disturbance in 2012. She showed diminished visual acuity and bitemporal hemianopsia by visual field and displayed a slightly enlarged nose. Her medical history included nothing notable. Her height and weight were 170 cm and 64 kg, respectively.

Magnetic resonance imaging (MRI) of the brain showed a pituitary adenoma with suprasellar extension (23 mm x 19 mm x 15 mm) (Figure 1).

Endocrine studies showed elevated serum basal GH level (47.1 ng/mL, normal range 0.28-1.64 ng/mL) and IGF-1 level (1,050 ng/mL, normal range for sex and age 262-510). The nadir GH level following a 75 g oral glucose tolerance test (OGTT) was 18.2 ng/mL, which was not suppressed. The plasma glucose was 152 mg/dL 120 min after the OGTT. After subcutaneous administration of 100 mg of octreotide, the serum GH level was moderately suppressed from 21.4 ng/mL to 12.9 ng/mL. The serum GH level upon oral administration of bromocriptine was suppressed from 23.9 ng/mL to 9.3 ng/mL. Serum PRL level was 58.5 ng/mL (normal range 4.9-29.3). No other hormonal abnormalities were present.

The patient underwent tumor resection through a transsphenoidal approach; the tumor was fully excised. The defect in the visual field disappeared, accompanied by normalization of GH, IGF-1, and PRL levels. A follow-up MRI showed no evidence of recurrence. After the operation, the nadir GH level after OGTT decreased to 0.2 ng/mL.

Family history

The father (subject II-2 in Figure 2C) of the index case underwent transsphenoidal surgery due to acromegaly at the age of 20, and re-surgery due to recurrence at the age of 35 at another institution. Postoperatively, he has received GH replacement therapy.   Her paternal uncle (subject II-5) had a past history of transsphenoidal resection for the treatment of acromegaly at the age of 20 at another institution. His recent serum GH and IGF-1 levels were 0.15 ng/mL and 165 ng/mL (normal range for sex and age 67-318), respectively.

In addition, other family members, namely, paternal grandfather (subject I-1), paternal grandmother (subject I-2), paternal aunt (subject II-3), sister (subject III-2), and paternal cousins (subjects III-3, III-4, and III-5), were also studied with their informed consent.   Blood samples were taken around 0900 h after an overnight fasting.   Serum levels of anterior pituitary hormones and IGF-1 did not indicate the presence of pituitary adenomas.   No family members showed acromegaly except for the father (subject II-2) and the paternal uncle (subject II-5) described above.   This study was approved by the ethics committees of Toranomon Hospital and the University of Tokushima.

Immunohistochemical study

Adenoma tissues were fixed in 10% formaldehyde, embedded in paraffin, and cut into 3-μm-thick sections for hematoxylin-eosin and immunohistochemical staining.   Immunohistochemistry for paraffin-embedded tumor samples was performed by the avidin-biotin-peroxidase method.   Sections were incubated with the following antibodies: anti-GH (Dako, Carpinteria, CA; A0570), anti-PRL (Dako; A0569), anti-adrenocorticotropic hormone (Dako; A0571), antibodies against each β-subunit of thyroid-stimulating hormone (Kyowa Medex Co., Ltd., Tokyo, Japan), follicle-stimulating hormone

(BioGenex, San Ramon, CA; MU026-UC), and luteinizing hormone (Nichirei Biosciences Inc., Tokyo, Japan), anti-cytokeratin CAM 5.2 (Becton Dickinson, San Jose, CA), and anti-Ki-67 clone MIB-1 (Dako; M7240).

Gene mutation analysis

Gene mutation analysis using PCR and sequencing was performed as described previously [13]. Briefly, genomic DNA isolated from leukocytes and a pituitary adenoma was subjected to 35 cycles of PCR using TaKaRa Ex Taq^{TM} Polymerase (TaKaRa, Shiga, Japan) with each *AIP* exon primer set. PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH) and then subjected to direct sequencing in sense and antisense directions using an ABI PRISM BigDye^{TM} terminator v3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3500xL sequencing analyzer (Applied Biosystems).

Multiplex ligation-dependent probe amplification (MLPA) analysis

MLPA analysis was performed using the SALSA MLPA probemix kit P244-B1 (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's instructions. Briefly, 50 ng of genomic DNA obtained from tumor tissues was denatured and hybridized with the SALSA probe-mix, containing probes for each *AIP* and *MEN1* exon and 6 other genes in the 11q13 region. After treatment with Ligase-65 at 54°C for 15 min, PCR amplification was performed using each primer set attached to the kit. The PCR products were run on an ABI 3500 DNA sequencing analyzer (Applied Biosystems) together with Genescan-500LIZ size standard. The data were analyzed with the GeneMapper software (Applied Biosystems). For data normalization, relative peak areas for each probe were calculated as fractions of

the total sum of peak areas in each sample and then the fraction of each peak was divided by the average

peak fractions of the corresponding probe in control normal male or female DNA (Promega, Madison,

WI).

Construction of expression vectors

cDNA encoding full-length human *AIP* was generated by reverse-transcription PCR from total

RNA extracted from 293FT cells. The PCR products were cloned into the expression vector

pcDNA^{TM}3.1(+) containing a FLAG epitope at the N- or C-terminus. Construction of mutated AIP

(Q315X)-expressing vector was carried out by standard PCR-based site-directed mutagenesis.

Cell proliferation assay

293FT cells were cultured in Dulbecco's modified Eagle's medium (WAKO, Tokyo, Japan)

supplemented with 10% fetal calf serum and antibiotic reagent (Sigma, St. Louis, MO) in an atmosphere

of 5% CO_2 at 37˚C. Transfections were carried out using Effectene^{TM} Reagent (Qiagen, Chatsworth,

CA) as recommended by the manufacturer. Expression of transfected AIP was confirmed by Western

blot analysis with antibodies against FLAG (Sigma) and β-actin (Sigma). Cell proliferation assays were

carried out using a Cell Counting Kit-8 (Dojindo Labs, Kumamoto, Japan) according to the

manufacturer's protocol. The absorbance at 450 nm of aliquots of cell supernatants was measured using

an automatic plate analyzer (Bio-Rad Laboratories, Hercules, CA). Each experiment was performed

three times in triplicate. Results are expressed as the mean ± SE. Two-sided Student's t-test was used

for statistical comparisons. A *P*-value <0.05 was considered statistically significant.

Results

Immunohistochemical study

The resected tumor specimen showed a chromophobe-type adenoma. Immunohistochemical analysis revealed that the tumor consisted of possible double adenomas, which were not supported by findings in MRI and surgery. One was GH-PRL-secreting adenoma with a perinuclear pattern of cytokeratin. The other constituting a small fraction of the tumor was GH-secreting adenoma with a dot-like pattern of cytokeratin. The two adenomas were clearly separated in sections. Ki-67 labeling indexes were 1.5% and 3.5% in each GH-PRL-secreting and GH-secreting adenoma, respectively.

Nonsense mutation of the AIP gene in an FIPA family

Mutations of the *AIP* gene were screened for 5 overlapping PCR products with the corresponding primer sets covering the entire coding region and splice junctions. Direct sequencing of leukocyte genomic DNA from the index case (subject III-1 in Figure 2C) revealed a heterozygous nonsense mutation caused by a C to T nucleotide substitution in exon 6 (c.945C>T) of the *AIP* gene (Figure 2A). This mutation resulted in the replacement of a glutamine codon (CAG) with a stop codon (TAG) at amino acid position 315 (p.Q315X) in the Cα-7h region (Figure 2B). As shown in Figure 2C, the mutation was found in not only affected members (subjects II-2, II-5, and III-1), but also unaffected ones (subjects I-2, III-2, III-4, and III-5). Although her 72-year-old grandmother (I-2) has a mutated *AI*P, she was asymptomatic with normal serum GH and IGF-1 levels and declined MRI. Subjects II-2, III-4, and III-5, ranging from 14 to 2 years of age, were clinically and biochemically normal. The mutation was negative in subjects I-1, II-1, II-3, II-6, and III-3.

Sequencing of genomic DNA from a GH-secreting adenoma resected from the index case revealed that a peak of the wild-type allele C was lower than the peak in her leukocytes (Figure 3A). To quantify the relative copy number of the *AIP* gene in the 11q13 region in the pituitary adenoma, we performed MLPA analysis. It showed an approximately 40% decrease of copy numbers of genes located in the 11q13 region. A somatic monoallelic deletion of one copy of these loci, such as *MEN1*, *SNX15*, *FAM89B*, *RELA*, *SART1*, *BRMS1*, *AIP*, and *CCND1*, was observed in the pituitary adenoma (Figure 3B).

The mutation nullified the inhibitory effect of AIP on cell proliferation

The p.Q315X AIP protein shows a shortage of 16 amino acid residues at the C-terminus compared with wild-type AIP. To investigate whether the mutation contributes to the development of pituitary adenoma, the effect of the mutation on cell proliferation was examined. 293FT cells were transiently transfected with the expression vector inserted with cDNA encoding mutated AIP (p.Q315X) and wild-type AIP proteins tagged with FLAG peptide at the N- or C-terminus. Each expression was confirmed by Western blot analysis (Figure 4A), indicating that the mutant was not subjected to accelerated degradation of mRNA or protein. As shown in Figure 4B, 293FT cells overexpressing wild-type AIP with FLAG at the C-terminus showed significant inhibition of cell growth compared with cells transduced with the control vector. Overexpression of AIP with FLAG at the N-terminus showed the same result (data not shown). On the other hand, overexpression of p.Q315X AIP with FLAG peptide at the N- or C-terminus did not inhibit cell proliferation, suggesting loss of the property to inhibit cell proliferation.

Discussion

We found an AIP p.Q315X mutation within the Cα-7h at the C-terminus of AIP in a Japanese FIPA family. This nonsense mutation has not been described before in FIPA families or cases with sporadic pituitary adenomas and is the nearest to the C-terminus of the AIP protein among the reported *AIP* nonsense mutations.

Besides the p.Q315X mutation, a nonsense mutation of p.R304X [7, 14-21] and missense mutations of p.R304Q [15, 17, 20, 22, 23], p.E319K [24], p.R323W [24], p.R325Q [25, 26], and p.G326R [24] at the Cα-7h of AIP have been reported (Table 1). The p.R304 residue of AIP is a hotspot for truncating mutation (c.910C>T) and missense mutation (c.911G>A), owing to it being a CpG site; several families with these mutations have been described. p.R304Q, which was shown to destabilize slightly the PDE4A5 interaction, has been considered to be pathogenic [17]. The missense variants may affect the three-dimensional structure of Cα-7h, which is involved in protein interactions. p.E319K, p.R323W, and p.G326R found in Chinese patients with sporadic pituitary adenomas were considered to be pathogenic by Cai *et al.* [24]. However, the effect of missense mutations on tumorigenesis is difficult to predict. *In vitro* studies such as on the effect of an AIP mutant on cell growth will reveal the functional role of the missense variants of AIP. Furthermore, deletion of the last 5 amino acids from the C-terminus of AIP (AIP-325) abolishes AhR-AIP binding, whereas AIP-325 binds HSP90 *in vitro*, while its effect on cell growth was not shown [27]. Accordingly, p.Q315X mutation should affect the interaction with AhR and may lead to the loss of inhibition of cell growth.

Loss of the wild-type allele on chromosome 11 spanning at least from *MEN1* to *CCND1* in the GH-secreting adenoma of the present case was observed, which has been reported in other *AIP-* or

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MEN1-related pituitary adenomas (Table 2). According to our previous LOH analysis using microsatellite markers, loss of the wild-type allele spanning at least from *PYGM* to *D11S527* in pituitary adenomas with p.V96PfsX32 of *AIP* [4, 28], from *D11S1883* to *D11S1889* in the pituitary adenoma with p.P71PfsX46 of *MEN1* [4], and from *D11S480* to *D11S527* in the pituitary adenoma with somatic p.P71PfsX46 mutation of *MEN1* [29] was observed. At least a 2 Mb deletion on 11q13 in pituitary adenomas with *AIP* mutations has also been reported [18, 30-32]. Furthermore, MLPA analysis on pituitary adenomas with *AIP* mutations showed at least a 2.5 Mb deletion on 11q13 in a Japanese pituitary adenoma (unpublished result) and in 3 out of 4 Chinese pituitary adenomas [24]. Table 2 shows that concomitant deletions of normal *AIP* and *MEN1* alleles were observed in most *AIP*- or *MEN1*-related pituitary adenomas. Furthermore, all parathyroid adenomas with deletion at the *MEN1* gene showed deletion of the gene *AIP* [33]. In these adenomas, the possibility of loss of the whole of chromosome 11 as a result of mitotic nondisjunction remains to be elucidated.

A number of single-exon and partial/whole-gene deletions have been detected in the *MEN1* and *AIP* genes at the germ-line level. Large germ-line deletions of 1.5 kb, 5.8 kb, and the whole locus in the *AIP* gene [17, 34] and 312 bp [35], 1,453 bp [33], approximately 5 kb [36], 29 kb, and 68 kb spanning the whole locus in the *MEN1* gene [37] have been reported. Thus, deletion of the normal alleles of *MEN1* and *AIP* at the somatic level in tumors seems to span a larger region than deletion at the germ-line level. However, the underlying molecular mechanism of such large somatic deletions is unknown.

Results of immunohistochemistry showed the possibility of double adenomas consisting of GH-PRL and GH adenomas, however, two tumors in the pituitary were not demonstrated by the brain MRI and operative findings. Although heterozygous *Aip* mice develop multiple pituitary adenomas [38], double pituitary adenomas have not been reported in human pituitary adenomas with *AIP* mutation.

MLPA analysis showed the approximately 40% decrease, rather than 50%, of signals at the *AIP* loci (Figure 3B), suggesting existence of cells without LOH in the tumor. However, we could not demonstrate whether the cells without LOH were derived from another adenoma with biallelic retention of *AIP* or from normal tissue contaminated in the tumor sample.

In conclusion, p.Q315X nonsense mutation in the *AIP* gene is a pathogenic variant and the present study reinforces the importance of the C-terminal region of AIP for pituitary tumorigenesis.

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Figure legends

Figure 1.　Preoperative coronal T1-weighted enhanced MRI of the index case showed a macroadenoma with a suprasellar extension.

Figure 2.　An *AIP* germ-line mutation found in a family.　A. A nonsense mutation, c.945C>T (p.Q315X), in exon 6 of the *AIP* gene was found in genomic DNA extracted from a blood sample.　B. Schematic description of the position of the nonsense mutation in AIP.　C. A pedigree of the family with pituitary adenomas.　The index case is indicated by an arrow.　Family members are indicated by generation (Roman numerals) and individuals (Arabic numerals).　Individuals are represented as male (squares) and female (circles).　Filled symbols denote patients with pituitary adenoma.　Sequencing of the *AIP* gene showed the presence of a mutation (mut).　wt, wild type; nd, not determined.

Figure 3.　Loss of wild-type allele of the *AIP* in a pituitary adenoma.　A. The wild-type allele (C) at c.915 in a pituitary tumor showed reduced signal compared with a peak in her leukocytes.　B. MLPA analysis in genomic DNA extracted from a tumor sample.　Probe signals from exons 1 to 6 of the *AIP* gene were significantly decreased, indicating the presence of deletion across those exons.　Furthermore, deletion of other loci on 11q13 was also observed in the tumor.

Figure 4.　Effect of overexpression of mutated AIP (p.Q315X) on cell proliferation.　A. A representative image of Western blotting of 293FT cell lysates overexpressing each indicated plasmid vector.　β-actin was used as an internal control.　B.　The proliferation of cultured cells transiently

transfected with each indicated vector was assayed. The graph is representative of three independent experiments. Each value represents the mean ± SE (n=3). The asterisk indicates significant difference ($P<0.05$) compared with data from cells overexpressing the control vector pcDNA^{TM}3.1(+).

Table 1   Germ-line mutations at the final C-terminal α-7 helix region of the *AIP* gene

Mutations	Identified in familial, sporadic or both	Reduced ability of mutant AIP to inhibit cell growth
p.R304X	both	observed
p.R304Q	both	NA
p.Q315X	familial	observed
p.E319K	sporadic, GH-secreting adenoma	NA
p.R323W	sporadic, nonfunctioning adenoma	NA
p.R325Q	both	NA
p.G326R	sporadic, GH-secreting adenoma	NA

NA, not analyzed

Table 2. Region of allelic loss on 11q12.1-13.5 in AIP- or MEN1-related pituitary adenomas

Markers	Genomic position	Pituitary adenoma																				
		1A	1B	2A	2B	3A	3B	3C	3D	3E	4A	4B	5	6	7	8	9	10	11	12	13	14
D11S956	58.49	NA	NA	NA	NA			NO	NA			NA	NI	NA	NA	NA	NA	NA	NA	NA	NA	NA
D11S4076	61.12	NA	NA	NA	NA			NI	NI		NI	NI		NI	NA	NA	NA	NA	NA	NA	NA	NA
D11S1765	62.47	NA	NA	NA	NA			NI	NA		NI			NA	NA	NA	NA	NA	NA	NA	NA	NA
D11S1883	63.13	NO	NO	NI	NI				NA		NI				NA	NA	NA	NA	NA	NA		NI
D11S480	63.06	NI	NI						NI		NO				NA	NA	NA	NA	NA	NA		
Chr11-64-A-C110	64.26	NA	NA						NA		NA				NA	NA	NA	NA	NA	NA		
PYGM	64.27										NA			NA	NA	NA	NA	NA	NA	NA		
MEN1	64.57										NA				NO							
SNX15	64.79										NA				NO							
Chr11-64-TG-110	64.97										NA				NA							
D11S4941	65.02														NA							
D11S4191	65.02														NA							
FAM89B	65.34														NO							
RELA	65.41														NO							
SART1	65.73														NO							
D11S913	65.9														NA							
BRMS1	66.11														NO							
D11S1249	67.12														NA							
AIP	67.25																					
D11S1889	67.31															NA	NA	NA				
D11S987	68.1											NI				NA	NA	NA				NA
D11S1337	68.14											NA	NI			NA	NA	NA				NA
D11S4905	68.97											NO	NI			NA	NA	NA				NA
CCND1	69.46			NA				NA	NA			NA	NI		NO	NO	NO					NA
D11S4136	71.81			NA				NA	NA				NI			NA	NA	NA	NA			NA
D11S534	75.19			NA				NA	NA	NA	NA	NA	NI	NA		NA	NA	NA	NA			NA
D11S527	76.42			NA				NA	NA	NA	NA		NI	NA		NA	NA	NA	NA			NA
Germ-line mutation		AIP: p.V96PfsX32		AIP: p.C238Y		AIP: p.E24X					AIP: p.R81X		AIP: p.R304X	AIP: p.R81X	AIP: p.D30E VUS	AIP: p.D262N VUS	AIP: p.R323W VUS	AIP: p.G326R VUS	AIP: p.Q315X	AIP: p.L251P	MEN1: p.P71PfsX46	MEN1: p.P263PfsX14

Regions of allelic loss are shown in gray.   NA, not analyzed; NI, not informative; NO: no LOH; VUS, variant of unknown significance.

Family 1, J Clin Endocrinol Metab 82:239-242 (1997); Families 2 and 3, J Clin Endocrinol Metab 85:707-714 (2000); Family 4, J Clin Endocrinol Metab 90:6580-6587 (2005); Family 5: J Endocrinol Invest 33:800-805 (2010); Family 6, Pituitary 15 Suppl 1:S61-67 (2012); Sporadic cases 7-10, Eur J Endocrinol 2013 Sep 19. [Epub ahead of print]; Family 11, present index patient; Family 12, unpublished case; Sporadic case 13, unpublished case; Family 14, J Clin Endocrinol Metab 83: 960-965 (1998); Sporadic case 15, J Clin Endocrinol Metab 8:2631-2634 (1998).
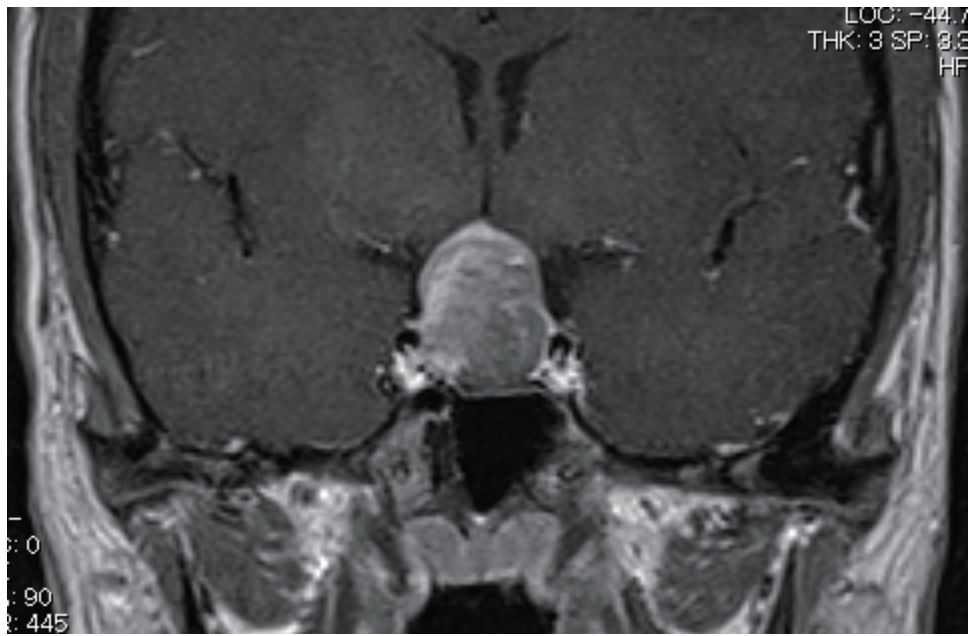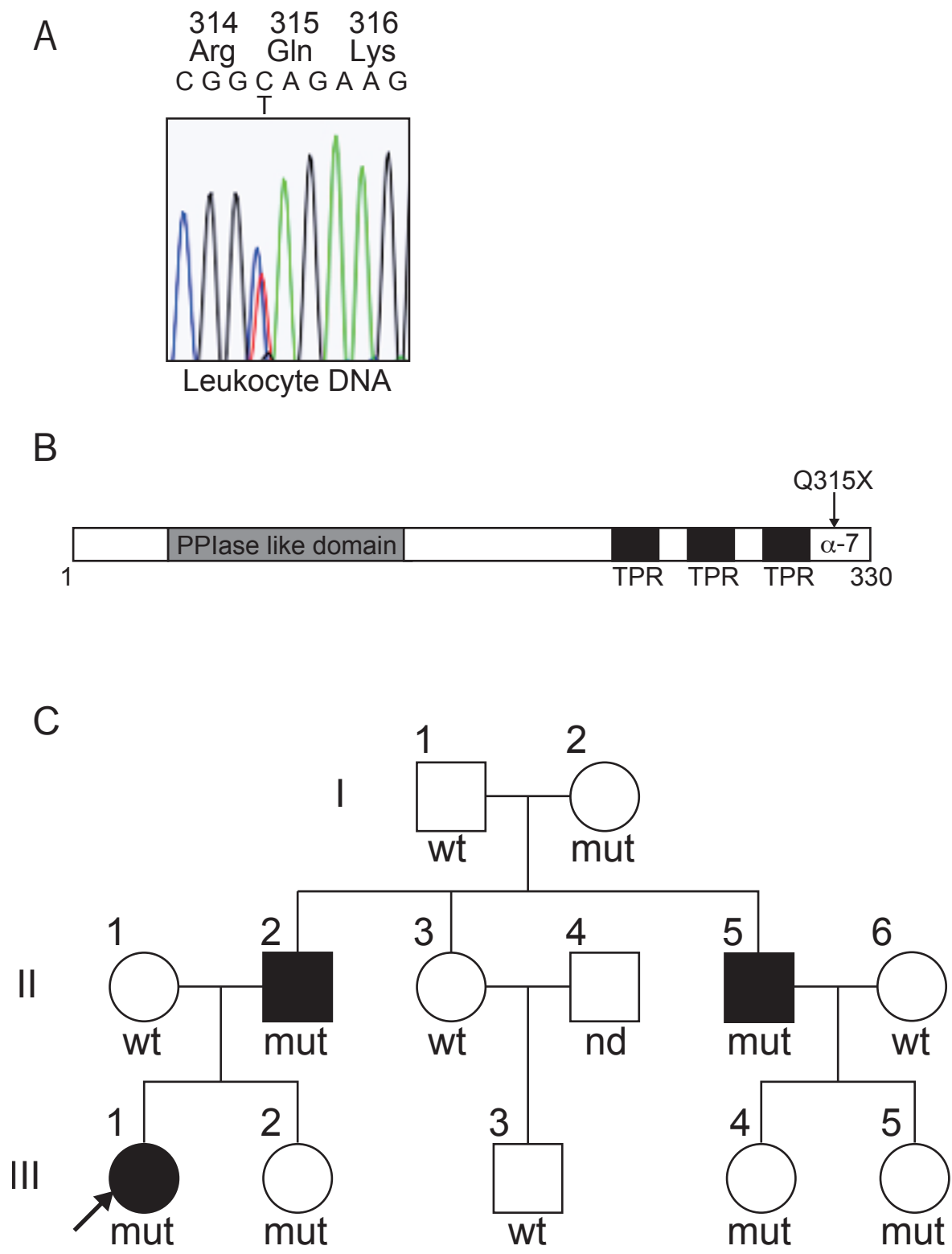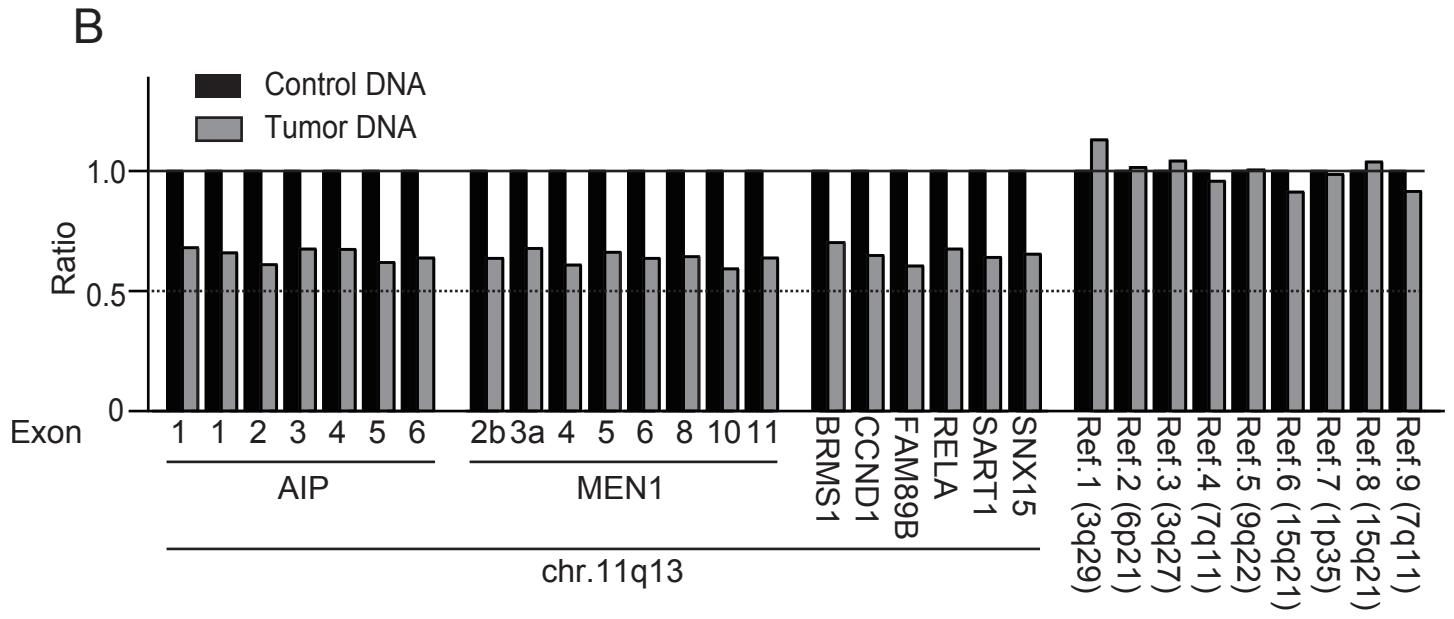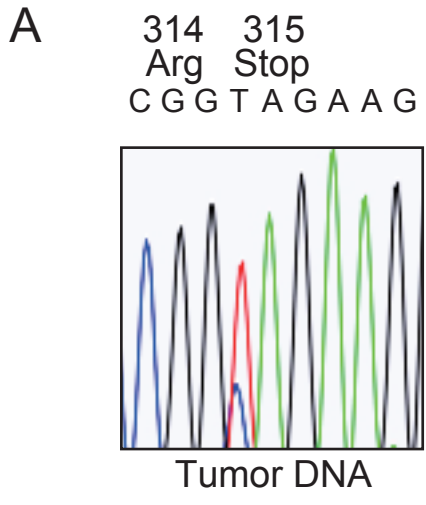
Figure 1

Figure 2

Figure 3

Figure 4