CLINICAL STUDY

Screening for *AIP* gene mutations in a Han Chinese pituitary adenoma cohort followed by LOH analysis

Feng Cai, Yi-Dan Zhang, Xiuli Zhao¹, Ya-Kun Yang, Si-Hai Ma², Cong-Xin Dai, Xiao-Hai Liu, Yong Yao, Ming Feng, Jun-Ji Wei, Bing Xing, Yong-Hui Jiao, Zhen-Qing Wei, Zhen-Ming Yin, Bo Zhang³, Feng Gu⁴ and Ren-Zhi Wang

Department of Neurosurgery, Peking Union Medical College, Chinese Academy of Medical Sciences, Peking Union Medical College Hospital, Beijing 100730, People's Republic of China, ¹State Key Laboratory of Medical Molecular Biology, Peking Union Medical College, Institute of Basic Medical Science, Chinese Academy of Medical Sciences, Beijing, People's Republic of China, ²Department of Neurosurgery, The Affiliated Yuquan Hospital of Tsinghua University, Beijing, People's Republic of China, ³Department of Neurosurgery, The First Affiliated Hospital of Dalian Medical University, Dalian, People's Republic of China and ⁴Department of Endocrinology, Peking Union Medical College, Chinese Academy of Medical Sciences, Peking Union Medical College Hospital, Beijing 100730, People's Republic of China

(Correspondence should be addressed to R-Z Wang; Email: renzhi.wang1957@yahoo.com.cn; F Gu; Email: gfpunch2010@126.com)

Abstract

Objective: The aryl hydrocarbon receptor interacting protein gene (*AIP*) is associated with pituitary adenoma (PA). *AIP* has not been sequenced in East Asian PA populations, so we performed this study in a Han Chinese cohort.

Design: Our study included six familial PA pedigrees comprising 16 patients and 27 unaffected relatives, as well as 216 sporadic PA (SPA) patients and 100 unrelated healthy controls.

Methods: *AIP* sequencing was carried out on genomic DNA isolated from blood samples. Multiplex ligation-dependent probe amplification and microsatellite marker analyses on DNA from the paired tumor tissues were performed for loss of heterozygosity analysis.

Results: We identified three common and four rare single nucleotide polymorphisms (SNPs), one intron insertion, one novel synonymous variant, four novel missense variants, and a reported nonsense mutation in three familial isolated PA (FIPA) cases from the same family. Large genetic deletions were not observed in the germline but were seen in the sporadic tumor DNA from three missense variant carriers. The prevalence of *AIP* pathogenic variants in PA patients here was low (3.88%), but was higher in somatotropinoma patients (9.30%), especially in young adults (≤ 30 years) and pediatric (≥ 18 years) patients (17.24% and 25.00% respectively). All *AIP* variant patients suffered from macroadenomas. However, the *AIP* mutation rate in FIPA families was low in this cohort (16.67%, 1/6 families).

Conclusion: *AIP* gene mutation may not be frequent in FIPA or SPA from the Han Chinese population. *AIP* sequencing and long-term follow-up investigations should be performed for young patients with large PAs and their families with PA predisposition.

European Journal of Endocrinology 169 867–884

Introduction

The pituitary gland plays a primary and crucial role in the regulation of metabolic processes. Pituitary adenomas (PAs) are identified by autopsy and radiological imaging and were shown to have an incidence of 8.4–22.5% (1). They account for 15% of primary intracranial neoplasms and are the second most common type of intracranial tumor in the 20- to 34-year age range (2). Clinically relevant PAs nearly occur in 1 in 1000 of the population in some similar cross-sectional studies (3, 4, 5). Tumor mass effects and hormone overproduction are the two main effects of PA on the human body.

The vast majority of PAs are sporadic. However, a small proportion is characterized by a familial aggregation and has been well described in the setting of

an endocrine tumor syndrome, such as multiple endocrine neoplasia type 1 (MEN1) and Carney complex (CNC), as well as familial isolated PA (FIPA), in which pituitary tumors occur in multiple members of a single family in the absence of MEN1 or CNC. FIPA was discovered to account for about 2% of all adenomas from two reference centers in one study (6), and family members can have either the same type of pituitary tumor or different types. PAs in FIPA family members generally appear at a younger age and/or a larger size than sporadic ones.

In 2006, Vierimaa *et al.* (7) described a probable association between familial acromegaly kindreds and mutations in the aryl hydrocarbon receptor interacting protein gene (*AIP*), which maps at 11q13, close to the *MEN1* gene. Loss of heterozygosity (LOH) at the *AIP* locus was identified through analysis of patient

tumor samples, suggesting that *AIP* acts as a tumor suppressor gene. Although more than 100 FIPA kindreds have been analyzed clinically and genetically, no such studies have been carried out in the Chinese population. To this end, we collected six FIPA kindreds comprising 16 affected patients and 27 healthy members from Peking Union Medical College Hospital (PUMCH) and attempted to identify possible *AIP* mutations among the Han Chinese population for the first time. As *AIP* mutations are also expected in some patients with sporadic pituitary tumors, especially those with somatotrinomas, another 216 sporadic PA (SPA) patients and 100 unrelated healthy control volunteers were also included in our study to evaluate their *AIP* gene mutation rate.

Subjects and methods

Subjects

Sixteen FIPA patients (six growth hormone (GH) secreting, five prolactin (PRL) secreting, three with nonfunctioning PA (NFPA), and two adrenocorticotrophic hormone (ACTH) secreting) and 27 relatives from six Han Chinese families (Fig. 1 and Table 1) with PA were included in the study. All affected members were clinically excluded from MEN1 and CNC and did not carry *MEN1* mutations as determined by conventional sequencing. In addition, 216 SPA patients were randomly selected for analyses of *AIP* gene mutations from Neurosurgery Departments of PUMCH between October 2010 and August 2012, during which time a total of 1263 PA patients were hospitalized, of which 1172 underwent surgery. All sporadic patients were confirmed to have no family members with PA symptoms and were clinically excluded from any

MEN1 syndrome and CNC symptoms. One hundred healthy, unrelated Han Chinese individuals were used as controls for the mutation validation experiments.

Standard protocol approvals and patient consents

Study procedures were in strict accordance with the ethics standards of the responsible committee on human experimentation and the Helsinki Declaration of 1975, as revised in 1983. Protocols were approved by the institutional ethics committee of PUMCH of the Chinese Academy of Medical Sciences functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research issued by the Royal College of Physicians of London. Blood samples were collected after the individuals had signed an informed consent document.

Hormone testing

Peripheral blood was collected from each subject on an empty stomach for hormone testing. GH concentrations were measured by an ELISA, while insulin-like growth factor 1 (IGF1) and PRL levels were measured by immunoradiometry.

Tumor DNA isolation

Tumor tissues from patients with *AIP* missense variants were microdissected from a series of 30 µm-thick paraffin-embedded tissue sections under direct visualization using laser capture microdissection. Microdissected tissue was digested with proteinase K, and genomic DNA was isolated using the TIANquick FFPE DNA Kit (DP330, Beijing, China), following the manufacturer's instructions.

Figure 1 Pedigrees of the six families (A, B, C, D, E and F). The proband of each family is indicated by an arrow. Male family members are represented by squares, females by circles, deceased members by diagonal lines, and affected members by black-filled symbols. The gray-filled symbol in Family F represents a with suspected somatotrinoma according to the statement of the proband.

Table 1 Clinical summary of FIPA patients with the data from follow-up and family history.

Family	Gender	Tumor subtype	AIP mutation/variant	Age (years) ^a	Max. tumor diameter (mm)	Hormone level ^b	Therapeutic responses (Y/N)	Immunohistochemistry	Follow-up/family study
A									
III1	M	GH-secreting	WT	11	15	GH: 13.9	Surgery (I)	NA	AI11: Octreotide GH IGF1 (H), residual tumor AI12: Octreotide GH (H) AI1 and AI3: hyperthyroidism AI13: multiple nodi goiter, suspected pituitary tumor
B									
III2	M	GH-secreting	WT	8	6	GH: 8.2	Octreotide (I)	NA	
I1	F	PRL-secreting	c.993+60G>C; c.468+111C>T	40 ^c	10	IGF1: 530 PRL: 86.3	N/T	NA	BI1: silent tumor BI17: PRL (N) tumor lesion NG
II7	F	PRL-secreting	c.993+60G>C; c.468+111C>T	38	12	PRL: 407.4	Bromocriptine (I) Surgery (E)	PRL(++) , GH(+), LH(+), Ki67: 2%	BI16: bromocriptine PRL (N) tumor size: unchanged
III6	F	PRL-secreting	c.993+60G>C; c.468+111C>T	21	8	PRL: 2089	Bromocriptine (E)	NA	
C									
CI1	M	GH-secreting	p.Q217*	43	26	GH: 13.9 IGH1: 685	N/T	NA	CI11: suspected residual mass; poor vision GH (N) CI12: residual pituitary tumor; amenorrhea, poor vision GH PRL (H)
CI1	F	GH-secreting	p.Q217*	19	45	GH: 40.2 IGF1: 897	Surgery (I)	GH(+) ^d	
CI2	M	GH-secreting	p.Q217*	17	38	GH: 73.2 IGF1: 851.8	Surgery (E)	GH(+), ACTH(±), PRL(±), Ki67: 2%	
D									
I1	F	NFPA	c.516C>T (p.(=))	62	31	Normal	Surgery (E)	FSH(+)	DI1: cured
II3	F	NFPA	c.516C>T (p.(=))	24	46	Hypopituitarism	Surgery (I)	ACTH(±), Ki67: 1%	DI3: aciesis; low level of hormones; blind
E									
I2	M	NFPA	c.516C>T (p.(=))	64	23	Normal	Surgery (E)	FSH(+), LH(+)	EI2: cured
II7	M	ACTH-secreting	c.516C>T (p.(=))	13	13	ACTH: 57	Surgery (I)	ACTH(+), Ki67: 5%; ACTH(+), Ki67: 2% ^e	EI17: ACTH(H) GH (L) FSH (L) LH(L) Urolithiasis osteoporosis
III10	M	ACTH-secreting	c.516C>T (p.(=))	11	4	ACTH: 49	N/T	N/A	
F									
III4	F	PRL-secreting	c.516C>T (p.(=)); c.468+111C>T; c.993+60G>C	42	15	PRL: 89.8	Bromocriptine (E)	PRL(±), p53(±), Ki67: 2%	FII13: cured
							Surgery (E)		FIV1: GH (H) IGF1 (H) FIV3: bromocriptine silent tumor

Table 1 Continued

Family	Gender	Tumor subtype	AIP mutation/ variant	Age (years) ^a	Max. tumor diameter (mm)	Hormone level ^b	Therapeutic responses (Y/N)	Immu- histochemistry	Follow-up/ family study
IV1	M	GH-secreting	c.516C>T (p.(=)); c.468+111C>T	40	12	GH: 9.5 IGF1: 617	Octreotide (I) Surgery (E)	GH(+), p53(+), Ki67: 3%	FI4: breast cancer FI11: hepatocellular carcinoma FI12: lung cancer FI14: esophageal and cardiac carcinoma FI11: suspected acro- megaly described by FI14
IV3	F	PRL-secreting	c.516C>T (p.(=)); c.468+111C>T	30	9	PRL: 86.8	Bromocriptine (E)	NA	

NA, not available; N/G, negative; N/T, no therapy; (H), higher than normal level; (Ho), homozygous; (N), normal; (L), lower than normal level; (E), effective; (I), ineffective.

^aAge at diagnosis.

^bGH and PRL (ng/ml); ACTH (pg/ml).

^cAge at first symptom is 40 years old.

^dPathological result provided by the local hospital.

^eTwo successive pathological results.

AIP genetic analysis

Genomic DNA was isolated from EDTA blood or tumor samples of patients and unaffected members of each FIPA family and 217 SPA subjects. The *AIP* sequence was based on Ensembl sequences: ENST00000279146, ENSG00000110711, and ENSP00000279146. PCR of *AIP* exonic and flanking intronic sequences was performed with five specific primer pairs (Supplementary Table 1, see section on supplementary data given at the end of this article), using AmpliTaq Gold DNA Polymerase (Qiagen) according to the manufacturer's instructions. PCR products were purified using the Ampure system (Agencourt Bioscience Corporation, Beverly, MA, USA) and were sequenced using ABI3100 and BigDye Terminator v3.1 Technology (Applied Biosystems). The complete *AIP* coding region was sequenced from both ends, including the exon/intron junctions, in both patients and control subjects. Identified mutations were re-amplified and re-sequenced from both ends. A total of 100 blood samples from non-PA subjects in China were analyzed for *AIP* polymorphisms.

Patients with negative *AIP* direct sequencing results were subjected to multiplex ligation-dependent probe amplification (MLPA) analysis using the MLPA kit (code #P244-B1 *AIP*-MEN1, MRC Holland, Amsterdam, The Netherlands), in accordance with the manufacturer's instructions. Data were further analyzed by Coffalyser MLPA DAT v8, which generated a relative ratio from a comparison of patients and controls. A ratio is considered 'normal' within the range 0.7–1.3, while a ratio <0.7 indicates a possible fragment deletion.

LOH analysis

LOH was investigated using MLPA as well as micro-satellite markers for the genetic locus flanking region of *AIP*. MLPA analysis was conducted as described earlier. Data from one patient were further analyzed by the Coffalyser MLPA DAT v8, which generated a relative ratio from a comparison of tumor and normal tissue (blood) from the same individual, both of which were normalized using unrelated healthy blood. A ratio between 0.65 and 0.85 indicates a suspected fragment deletion, while a ratio of <0.65 suggests a possible heterozygous deletion (LOH).

Four microsatellite markers for the genetic locus flanking region of *AIP* were selected based on their LOD scores from Ensembl. Selected markers, the primers used, and their *T_m* are listed in Supplementary Table 2, see section on supplementary data given at the end of this article. All PCRs were performed on tumor and normal tissues under the same conditions. PCRs were performed in a final volume of 25 µl and contained 2 µl purified DNA template, 2.5 mM MgCl₂, 2.5 µl 1× working solution buffer (Tris Borate EDTA), 0.15 µl Taq polymerase, 0.75 µl of each of forward and reverse primers at a concentration of 0.3 pM/µl, and 200 µM

dNTPs in 0.5 µl. Tubes were denatured for 5 min at 95 °C, followed by 38 cycles of denaturation at 94 °C for 30 s, relevant T_m for 30 s as indicated in [Supplementary Table 2](#), and extension at 72 °C for 30 s. Amplified products were detected via denaturing gel electrophoresis and analyzed by ImageJ Software (rsb.info.nih.gov/ij/).

LOH was calculated as the ratio between the short-allele normal (SN) and long-allele normal (LN) divided by the ratio of the short allele tumor (ST) and long allele tumor (LT), i.e. (SN:LN)/(ST:LT). LOH was indicated if one allele decreased by more than 50% in the tumor sample when compared with the same allele in normal tissue DNA, i.e. a score is <0.67 or >1.5 (8). When DNA from normal tissue (blood) was homozygous for the markers, the case was said to be non-informative.

Bioinformatic analysis

The effects of the newly detected AIP missense variants on protein structure or function were analyzed *in silico* with the prediction program: PonP (Pathogenic-or-Not-Pipeline; <http://bioinf.uta.fi/PON-P/index.shtml>), which integrates the predictions of different online software to give an overall pathogenic score. The effect of the variants on splicing and transcription processes was analyzed with Alamut, the software for studying the effect of synonymous variants on splicing (<http://www.interactive-biosoftware.com/software/alamut/features>). UCSC Genome Bioinformatics (genome.ucsc.edu/cgi-bin/hgGateway), human single nucleotide polymorphism databases (dbSNP) (<http://www.ncbi.nlm.nih.gov/SNP/snp.summary.cgi>), and Exome Variant Server (<http://evs.gs.washington.edu/EVS/>) were also consulted. Moreover, to underline the possible structural outcome of the missense substitutions, the three-dimensional (3D) structure of the AIP was predicted using the Phyre2 (Protein Homology Fold Recognition Engine; <http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>, last access: 04 May 2013) created by the Structural Bioinformatics Group, Imperial College, London (9). The AIP protein structure for prediction was based on the work by Linnert *et al.* (10) and Morgan *et al.* (11). The *.pdb model generated from Phyre2 was loaded and the 3D structure visualized using PyMOL Software (<http://www.pymol.org/>).

Results

Subject characteristics

Four of the six families (A–D) were homogeneous for PA subtype, including two somatotropinomas, one prolactinomas, and one NFPA family ([Table 1](#)). The grandfather of Family E had NFPA, while his two grandsons suffered from Cushing's disease. The proband of Family F and her niece had prolactinoma, while her son had somatotropinoma ([Table 1](#)). Of the FIPA patients, six

(37.5%) had somatotropinomas (only one female; mean $\pm$ s.d. age at diagnosis: 23.7 ± 14.7 years, mean $\pm$ s.d. tumor diameter: 23.7 ± 15.4 mm); five (31.25%) had prolactinomas (no males; mean $\pm$ s.d. age at diagnosis: 34.2 ± 8.7 years, mean $\pm$ s.d. tumor diameter: 10.8 ± 2.8 mm); and three (18.75%) had NFPA; two boys (12.5%) from Family E had Cushing's disease. Of the SPA patients, 80 had somatotropinomas (42 females; mean $\pm$ s.d. age at diagnosis: 36.75 ± 11.66 years, mean $\pm$ s.d. tumor diameter: 21.13 ± 10.27 mm); 39 had prolactinomas (23 females; mean $\pm$ s.d. age at diagnosis: 33.49 ± 9.77 years, mean $\pm$ s.d. tumor diameter: 16.18 ± 8.09 mm); 39 had Cushing's disease (31 females; mean $\pm$ s.d. age at diagnosis: 32.26 ± 11.68 years, mean $\pm$ s.d. tumor diameter: 7.82 ± 5.34 mm); and 58 had NFPA (26 females; mean $\pm$ s.d. age at diagnosis: 47.05 ± 13.11 years, mean $\pm$ s.d. tumor diameter: 26.22 ± 9.13 mm).

AIP sequencing

One novel synonymous (c.687G>A (p.(=)); [Fig. 2H](#)) and four missense novel heterozygote substitutions (c.90T>G (p.D30E), c.784G>A (p.D262N), c.955G>A (p.E319K), and c.976G>A (p.G326R)) were identified in SPA patients of this study ([Fig. 2A, J, K and M](#)). Another heterozygote substitution c.649C>T (p.Q217*) in exon 5 leading to a premature stop codon was discovered in all three GH-secreting PA members of Family C ([Fig. 2G](#)). This mutation was also detected in a GH-secreting PA family in a previous study (12). Moreover, variant c.144C>T (p.(=)) ([Fig. 2B](#)), found in an NFPA patient, was reported in an investigation on AIP variants in healthy people from six different ethnic populations (13), as well as in other previous reports (14, 15). Three missense variants, c.355C>T (p.R119W) from a GH patient ([Fig. 2C](#)), c.733G>A (p.E245K) ([Fig. 2I](#)) from a PRL patient, and the c.967C>T (p.R323W) ([Fig. 2L](#)) from a NFPA patient, are known SNP sites. None of these substitutions were detected in the controls. Another three common SNPs were also identified in this study: rs2276020 c.516C>T (p.(=)) ([Fig. 2E](#)) was found in the members of Family D, E, and F; 19 sporadic GH-secreting, nine PRL-secreting, 11 ACTH-secreting, 18 NFPA patients, as well as 40 in the controls; variant c.468+111C>T (rs4084113) in the intronic region ([Fig. 2D](#)) was also discovered in the members of Family F, 12 sporadic GH-secreting, eight PRL secreting, nine ACTH secreting, ten NFPA patients, as well as nine controls; and variant c.993+60G>C (rs146014363) in the 3' UTR ([Fig. 2N](#)) was detected in the members of Family B and F, ten sporadic GH-secreting PA, four PRL-secreting, six ACTH-secreting, eight NFPA patients, as well as 12 controls. Notably, the duplication variant c.645+41 dupG was observed in an NFPA patient, and we found no AIP substitutions in the members of Family A ([Tables 1 and 2](#)). Otherwise, PCR amplification for AIP

Figure 2 Sequencing chromatograms showing *AIP* mutations/variants. Red arrows show the positions of the nucleotide transitions. The amino acids on the right side of the leak lines replace the ones on the left following the nucleotide transitions.

sequencing failed in genomic DNA from the paraffin-embedded tumor samples, and no deletion was detected by MLPA in the blood samples of any patient.

Phenotype of the FIPA patients and sporadic cases with *AIP* variants

Three patients in Family C with p.Q217* mutation were diagnosed with gigantism (CII2) and acromegaly (CI1

and CII1). Notably, CI1 had not been diagnosed with GH-secreting PA until the *AIP* mutation was detected when magnetic resonance imaging (MRI) revealed a mass at the sellar region and an endocrinological examination indicated abnormal GH serum levels. Both CII1 and CII2 had high GH levels and aggressive macroadenomas invading into the suprasellar region and cavernous sinus bilaterally leading to poor eyesight and hemianopsia. CII1 had undergone two operations

Table 2 Genetic variants in AIP found in patients with PA and/or controls.

Position	Gender	Age diagnosis ^a	Phenotype (tumor size); hormone test	Immu- histochemistry	Nucleotide change	Amino acid substitution	Variant type	Patients with PA (GH=86, PRL=44, ACTH=41, NFPA=61)	Controls (n=127)	Status
Exon 1	M	23	(33); GH↑, IGF1↑	GH(+), PRL(±), TSH(±)	c.90T>G	p.D30E	Missense	One GH	0	Novel
Exon 2	M	58	(24); –	N/G	c.144C>T	p.(=)	Synonymous	One NFPA	0	Reported in an investigation from six ethnic population
Exon 3	F	32	(37); GH↑, IGF1↑	NA	c.355C>T	p.R119W	Missense	One GH	0	A rare variant rs4084113
Intron 3	–	–	–	–	c.468+111C>T	–	–	13 GH, 13 PRL, nine ACTH, ten NFPA	9	
Exon 4	–	–	–	–	c.516C>T	p.(=)	Synonymous	20 GH, 11 PRL, 13 ACTH, 21 NFPA	40	rs2276020
Intron 4	F	47	(60); –	N/G, Ki67: 5%, p53(+)	c.645+41 dupG	–	Duplication	One NFPA	0	Novel
Exon 5	–	–	–	–	c.649C>T	p.Q217*	Non-sense	One family with three GH patients	0	Reported in one family with two GH patients
Exon 5	M	24	(31); GH↑, IGF1↑	GH(+), p53(+), Ki67: 5%	c.687G>A	p.(=)	Synonymous	One GH	0	Novel
Exon 5	F	24	(33); PRL↑	PRL(+), p53(+), Ki67: 3%	c.733G>A	p.E245K	Missense	One PRL	0	rs150645662
Exon 5	M	35	(25); GH↑, IGF1↑	GH(+), Ki67: 2%	c.784G>A	p.D262N	Missense	One GH	0	Novel
Exon 6	M	11	(38); GH↑, IGF1↑	GH(+), p53(+), Ki67: 2%	c.955G>A	p.E319K	Missense	One GH	0	Novel
Exon 6	M	41	(25); –	FSH(+), p53(±), Ki67: 2%	c.967C>T	p.R323W	Missense	One NFPA	0	rs188965257
Exon 6	M	30	(47); GH↑, IGF1↑	Ki67: 2% GH(+), PRL(+), Ki67: 4%, p53(+)	c.976G>A	p.G326R	Missense	One GH	0	Novel
3' UTR	–	–	–	–	c.993+60G>C	–	–	10 GH, eight PRL, six ACTH, eight NFPA	12	rs146014363

^aIndex age at diagnosis (years).

for recurrence of symptoms of amenorrhea and visual acuity decrease, while CII2 had undergone surgery for a suspected residual mass and visual impairment of the left eye (Table 1).

Of the SPA patients with *AIP* missense variants, there were five GH-secreting PA cases (70.1%, 5/7). There was also a novel synonymous variant c.687G>A (p.(=)) in one GH-secreting PA patient. The p.D30E mutation patient was presenting with alteration of facial features and enlarged hands and feet. An MRI revealed that the tumor had invaded into the suprasellar cisterna. The patient's condition was not good with poor vision and high levels of GH and IGF1, despite receiving

octreotide therapy and undergoing two operations. Both of the c.687G>A (p.(=)) and p.G326R patients had atypical PAs (Ki67 > 3%, p53(+)). The former one suffered from hyperglycemia and was sensitive to octreotide therapy, while the latter had characteristics of acromegaly as well as suffering from diabetes and sleep apnea. In spite of receiving octreotide therapy, the rapid growth of the tumor invading into the suprasellar cisterna and bilateral cavernous sinus made him decide to have an operation. Six months later, he underwent the second surgery for the recurrence of the aggressive tumor. The p.R119W patient also had an aggressive GH-secreting adenoma that did not respond to

Figure 3 (legend continued)

Figure 3 Bar chart of the MLPA analysis performed on nine pairs of DNA from patients' blood and the matched tumor tissue samples.

octreotide therapy, and no further therapy was given at our hospital. The p.E319K patient was an 11-year-old boy with an invasive macroadenoma characterized by gigantism. His pathological report showed that in his PA tissue, Ki67 labeling index was 2% and p53 was positive (+). The p.D262N patient with a non-invasive adenoma underwent surgery and the follow-up endocrinological examination and imaging study were found to be normal. The NFPA case carrying the insertion variant c.645+41~c.645+42insG suffered from hypopituitarism and ablesia caused by the aggressive and refractory tumor (Table 2).

LOH analyses

Allelic loss of the *AIP* gene in tumor tissues was assessed by MLPA and microsatellite analysis of tumor and normal tissue (blood) of each patient. Additionally, we extracted nine eligible tumor DNA samples from PA patients with identified *AIP* variants in germline cells.

We first performed MLPA analyses on the nine pairs of DNA samples. The MLPA probe mix contains probes for several genes located at chromosome 11q13: (from centromere to telomere), *MEN1* (eight of the 11 exons), *SNX15*, *FAM89B*, *RELA*, *SART1*, *BRMS1*, *AIP* (all of six exons), and *CCND1*, covering a region of nearly 5 Mb. As shown in Fig. 3, the dosage quotients (DQs) of some probes were <0.85 in tumor DNA from the c.733G>A (p.E245K) patient and the c.955G>A (p.E319K) patient. All DQs (except for the *CCND1* probe) were <0.65 in tumor DNA from the patients carrying c.784G>A (p.D262N) and c.967C>T (p.R323W), while all DQs in tumor with c.976G>A (p.G326R) were also <0.65, indicating the presence of a large deletion in the almost 5 Mb region of ch11q13 in these tumor tissues; probes for *AIP* exons 3, 4, 5, and 6 had DQs <0.85 in the patient with the missense variant c.90T>G (p.D30E), suggesting that a possible deletion of *AIP* exons 3–6 was present in the patient's tumor DNA (Fig. 3).

Allelic loss of *AIP* in tumor tissues was also assessed by microsatellite analysis. The STR markers RH41895 and RH41858 are located around 4.7 and 1.9 kb 5' of *AIP* respectively, while WI-22393 and SHGC-132167 are around 660 bp and 4.2 kb 3' of *AIP* respectively. Table 3 illustrates that RH41895 is a noninformative locus for each case. No LOH was observed in the tumor samples of either the c.733G>A (p.E245K)- or c.955G>A (p.E319K)-carrying patient. Tumors that were from the patients with the *AIP* germline variants c.784G>A (p.D262N), c.967C>T (p.R323W), and c.976G>A (p.G326R) each showed LOH in the three markers, which was consistent with the above MLPA results (Fig. 4 and Table 3). MLPA results together with LOH for WI-22393 and SHGC-132167 markers in the tumor sample from the c.90T>G (p.D30E) patient indicate a deletion of at least some *AIP* exons.

Analyses of *AIP* variants in silico

Multi-alignment analysis confirmed that the R119, E319, R323, and G326 residues in *AIP* are phylogenetically conserved. The D30 and D262 residues are conserved to a lesser degree, while the E245 residue is not conserved at all. Prediction Software PON-P suggested that the two substitutions, E245K and D262N, could be neutral for the *AIP* protein with a score of 0. However, I-mutant3 indicated that the D262N substitution could decrease the *AIP* structure stability and found it to have the highest score of these variants. Each of the three substitutions E319K, R323W, and G326R was integrally predicted to have a neutral effect on *AIP*, although various tools provided different results with a low reliability index and I-mutant3 indicated that they could destroy the *AIP* structure stability. Substitutions D30E and R119W were suggested to be neutral and unclassified with a score of 0.01 and 0.38 respectively. As to the effects of the nucleotide substitutions on splicing variants, the Alamut predicted that neither c.645+111C>T nor c.967C>T would have an impact on splicing. Except the c.468+111C>T that might decrease the binding of splicing factors, other variants were predicted to affect the binding of various splicing factors at various sites, increasingly and/or decreasingly (Supplementary Tables 3 and 4, see section on supplementary data given at the end of this article).

3D modeling approach prediction of missense variants effect

Of the missense variants detected here, c.733G>A (p.E245K), c.784G>A (p.D262N), c.955G>A (p.E319K), c.967C>T (p.R323W), and c.976G>A (p.G326R) are located in the tetratricopeptide repeat (TPR) domain of the *AIP* protein, which has been recently proved to be sufficient for PA predisposition by interaction with some client proteins (11). And c.90T>G (p.D30E) and c.355C>T (p.R119W) lie in the FK506-binding protein (FKBP) homology domain (Fig. 5). This domain was also proved to have potential interaction with HSP90 (10). Molecular modeling indicated that these missense variants interfere with the secondary structure of the *AIP* peptidyl-prolyl *cis/trans*-isomerase (PPIase)-like and TPRs domains, including the interconversion of loops and α -helices/ β -sheets. Some key sites for binding surface and TPR motifs were also affected by the interconversion. For example, all seven variants would translate a loop into an α -helix structure at binding site AA 278 and, with the exception of p.R323W and p.D262N, the other five variants could also convert a loop part into an α -helix one at a TPR motif site AA 209. Moreover, the p.R119W variant might remove an α -helix structure part at the AA 188–192 binding site, while p.R323W would remove an α -helix structure part at the TPR motif site AA 284–289,

Table 3 Summary of LOH analyses of nine AIP variants.

Nucleotide change	Amino acid substitution	RH41895	RH41858	WI-22393	SHGC-132167
c.90T>G	p.D30E	□	○	●	●
c.144C>T	p.(=)	□	○	○	○
c.645+41 dupG	–	□	○	○	○
c.687G>A	p.(=)	□	○	□	○
c.733G>A	p.E245K	□	○	○	○
c.784G>A	p.D262N	□	●	●	●
c.955G>A	p.E319K	□	○	○	○
c.967C>T	p.R323W	□	●	●	●
c.976G>A	p.G326R	□	●	●	●

□, homozygous; ○, heterozygous; ●, loss of heterozygosity.

which is essential for inter-helix packing. These sites are strongly phylogenetically conserved in flies and worms (Fig. 6, Table 4).

Discussion

This is the first study to investigate the prevalence of *AIP* sequence variations among four subtypes of PA patients from a Han Chinese population of FIPA and SPA cases. Here, only a previously reported nonsense mutation (c.649C>T, p.Q217*) (12) was present in one FIPA family (16.67% (1/6)), from which all the three patients suffered from somatotropinoma. Sequencing analyses for the past few years demonstrated that germline mutations of *AIP* are almost always found in families with either homogeneous somatotroph adenomas or ones with mixed somatotroph and lactotroph adenomas, with an overall frequency of *AIP* mutations of 15% among FIPAs and of 50% among study cohorts with isolated familial somatotropinoma (IFS) (12). *AIP* mutations are rare in unselected SPA patients, with reported somatotropinoma frequencies of 0–4% in some cohorts (16, 17, 18).

Moreover, there was PA heterogeneity within two FIPA families (Family E and Family F), one with somatotroph and lactotroph adenoma, which is the most common, and the other involving NFPA and corticotroph adenomas, as described previously in some studies (7, 19, 20). However, there is no explanation as yet for this phenomenon.

To date, the c.649C>T (p.Q217*) mutation has only been observed in two IFS siblings from Belgium who suffered from invasive macroadenomas and high serum levels of GH and IGF1 (12). In our study, not only the two siblings but also their father carried the c.649C>T, p.Q217* variant and suffered from somatotropinoma. The tumors were both invasive and refractory. *AIP* is similar in nature to a tumor suppressor gene (19), and the C-terminal TPRs domain of *AIP* binds HSP90, PDE4A5, and aryl hydrocarbon receptor (AHR), which mediates signaling processes in carcinogenesis, immunosuppression, and teratogenesis (21, 22, 23, 24). The premature stop codon p. Q217* might contribute to FIPA tumorigenesis via a loss of this *AIP* binding function.

Figure 4 Display of denaturing gel electrophoresis for LOH analysis by four microsatellite markers (RH41895, RH41858, WI-22393, and SHGC-132167). N, normal tissue (blood); T, tumor tissue; A, c.90T>G; B, c.144C>T; C, c.645+41dupG; D, c.687G>A; E, c.733G>A; F, c.784G>A; G, c.955G>A; H, c.967C>T; and I, c.976G>A. DNA from normal tissue (blood) was homozygous for the marker RH41895, so it was non-informative; for RH41858, the value of (SN:LN)/(ST:LT) was <0.67 or >1.5 in lanes F, H, and I. SN, short allele normal; LN, long allele normal; ST, short allele tumor; and LT, long allele tumor.

Figure 5 Analyses of the 12 *AIP* variants. (A) Functional domains of AIP and locations of the nine missense mutations and three synonymous mutations are shown. TPR, tetratricopeptide repeat; AHR, aryl hydrocarbon receptor; FKBP, FK506-binding protein; HSP90, 90-kDa heat-shock protein; PDE, phosphodiesterases; ER α , the α subtype of estrogen receptor. The C-terminus of the protein binds AHR, HSP90, PDE, and ER- α . (B) Sequence alignment of AIP protein in different species via Alamut Software. (Rouen, France) Numbers along the top represent related sites in 14 different species at location of missense mutations in this study. The darker the blue is, the more conserved the residue of AIP protein.

With the exception of some common SNPs, we found no variants or previously reported mutations in the other FIPA families. This agrees with the work by Daly *et al.* (12) who found that *AIP* mutations only occurred in about 15% of FIPA families and that the majority had normal germline *AIP* sequences. Similarly, Beckers *et al.* (25) recently reported that only 20.4% of FIPA kindreds could be explained by *AIP* mutations. There are a few large studies that reported numerous AIP-negative FIPA families with various rates (19, 20, 22). Those indicate that the susceptible loci may vary among different populations and that other gene alterations could be involved in FIPA tumorigenesis. So far, besides *ch11q13* where *AIP* is located, several regions have been suggested to be involved in somatotropinoma susceptibility: 2p16–12 (26), 8q12.1 (7, 27), 13q14 (28), and 19q13.41 (27). Additional investigations of linkage analysis should be performed to identify the potential loci risk related to FIPA, specifically in the Han Chinese population. Furthermore, although we have not completely excluded the fact that some gene variants

related to CNC symptoms may be present in the present subjects, this has been ruled out clinically.

There are three common SNPs ((c.516C>T (p.(=)), c.468+111C>T, and c.993+60G>C) detected in both PA patients and healthy controls (Table 2). With the prediction from *in silico* analysis, all of them are considered to be non-pathogenic.

The current study identified the synonymous variant c.144C>T for the first time in an isolated PA patient; it has previously been identified in an Italian acromegalic patient with clinical signs of multiple tumors and was verified to not affect splicing *in vitro* (14, 15). This variant, together with the synonymous c.634C>T (p.(=)) variant, which was not found in the current study, was exclusively detected in two of 18 Chinese individuals in an evaluation of *AIP* SNPs in six different ethnic populations by Rowlands *et al.* (13). The allelic frequency discrepancies between the studies may be explained in several ways. First, the earlier research comprised only 18 Chinese subjects, so the relatively high SNP allelic frequency might be coincidental. Enlargement of samples

Figure 6 3D modeling structure prediction of the impact of seven missense variants on the secondary structure alteration of AIP (interconversion between loops and β -sheets, or loops and α -helices). Red, β -sheets; pink, loops; light blue, α -helices; blue, transformation from β -sheets or α -helices into loops; green, transformation from loops into β -sheets or α -helices; yellow, binding surface or TPR motif sites affected by interconversion.

from the Chinese population in Rowlands’ study would draw a different conclusion. Secondly, the genomic DNA samples for the earlier research were from immortalized lymphoblast cell lines stored in the Coriell Cell Repositories, and it is conceivable that the immortalization process might lead to the alteration. The methods for that study did not clearly describe the genetic relationship between the 18 subjects, or between the two synonymous variant carriers. In the current study, the c.144C>T variant carrier was a 58-year-old male patient with a nonfunctioning macroadenoma. Another novel synonymous alteration (c.687G>A) was detected in a 24-year-old male patient with acromegaly and a giant adenoma. This atypical PA had a high Ki67 index (5%) and p53-positive staining. To date, 31 synonymous alterations have been identified in all six exons of *AIP* (Ensembl data). Of these, only the c.249G>T and c.807C>T variants have been confirmed *in vitro* to be pathogenic through their effects on splicing regulation (22). However, MLPA and microsatellite analyses in this study revealed no *AIP* LOH in the tumor of the patient carrying c.687G>A. Moreover, the variant is not located near the intron–exon–intron splice junctions. Evaluation *in silico* suggested that the synonymous alteration may lead to diminished exonic enhancer binding at some sites and have little impact on the generation of potential new

splicing sites. Given this, the c.687G>A variant is unlikely to be pathogenic, although *in vitro* mRNA analysis should be performed to validate this prediction. Another four novel missense variants were identified in four somatotropinoma patients: c.90T>G (p.D30E), c.784G>A (p.D262N), c.955G>A (p.E319K), and c.976G>A (p.G326R). As none were found in unrelated healthy controls, they were considered to be variants of unknown significance. The first one, c.90T>G (p.D30E), was detected in a male patient with an aggressive adenoma diagnosed at the age of 23 years. Although *in silico* prediction indicated that the variant might be benign or neutral and have little effect on splicing regulation, it is nevertheless likely to be pathogenic for a number of reasons. First, LOH analysis indicated the presence of a large deletion (2.15 Mb) from *AIP* exon 3 of *CCND1* in the tumor tissue; this includes the region that encodes the AIP C-terminal structure, which plays a crucial role in the protein function. Secondly, the first part of the protein is essential for AIP regulation of the intracellular localization of AHR and the interaction with HSP90 (29), although it does not seem to be directly involved in protein–protein interaction. Igreja *et al.* (22) demonstrated that p.R16H, located in the same domain as p.D30E, shows a 61% loss of PDE4A5–AIP binding compared with WT,

Table 4 The prediction of the missense variants’ impact on the secondary structure (binding surface and motif in TPRs domain) of AIP protein.

	p.D30E	p.R119W	p.E245K	p.D262N	p.E319K	p.R323W	p.G326R
Binding surface ^a	194–195 277–278	190–191 277–278	194–195 277–278	277–278	277–278	194–195 277–278	277–278
Motif stability ^a	209	209	209	–	209	284, 288	209

^aThe site of amino acid sequence; it is referred to the database derived from UniProtKB/Swiss-Prot: O00170.2 (www.ncbi.nlm.nih.gov/protein/O00170.2). The number with line below represents the site that alteration of secondary structure takes place predicted by 3D modeling.

Table 5 A list of pathogenic and non-pathogenic AIP variants identified in this study.

Pathogenic AIP variants	Non-pathogenic AIP variants
c.90T>G (p.D30E)	c.144C>T (p.(=))
c.355C>T (p.R119W)	c.468+111C>T
c.649C>T (p.Q217*)	c.516C>T (p.(=))
c.784G>A (p.D262N)	c.645+41dupG
c.955G>A (p.E319K)	c.687G>A (p.(=))
c.967C>T (p.R323W)	c.733G>A (p.E245K)
c.976G>A (p.G326R)	c.993+60G>C

while p.R304Q shows only a 24% loss, although PDE4A5 binds the C-terminal part of AIP. Moreover, our 3D modeling prediction indicated that the missense variant may influence some AIP secondary structures essential for binding and TPR motif stability in the distant TPR domain (Fig. 6A). Thirdly, amino acid 30 of AIP is highly conserved in vertebrates and worms, implying that the site is essential for protein function.

Two of the novel missense variants c.784G>A (p.D262N) and c.955G>A (p.E319K) are located in the TPR domain of the protein, which is crucial for protein–protein interactions and other AIP functions (19, 21, 30). c.784G>A is likely to be pathogenic, as it was predicted to negatively impact on protein structure stability. It is also located at an exon–intron junction, so the alteration is more likely to affect splicing, and Alamut showed its location to be highly conserved between species. Moreover, MLPA and microsatellite analysis revealed LOH across the AIP region in the patient's tumor tissue. The novel variant c.955G>A was detected in the germline of an 11-year-old child, who carried an aggressive GH-secreting macroadenoma. It was predicted *in silico* that this variant may be deleterious. The negative LOH microsatellite analysis for the c.955G>A variant was unexpected and inconsistent with MLPA analysis. It could be explained as contamination by normal cells, or tumor heterogeneity, or a different LOD of referred microsatellite markers between populations. So we think that the c.955G>A variant may be pathogenic.

The last novel missense variant c.976G>A (p.G326R) is located in the C-terminal part of AIP, which is essential for the protein binding to AHR. Alamut displays that the p.G326 residue is highly conserved in vertebrates. LOH analysis indicated that there was a large deletion from *MEN1* to *CCND1* gene, including *AIP*. *In silico* analysis also suggested that it might be deleterious and affected the splicing process. So this alteration may also be pathogenic.

Another three missense SNPs were detected in the current study: c.355C>T (p.R119W), c.733G>A (p.E245K), and c.967C>T (p.R323W). Each of the three young patients carrying these variants suffered from a giant atypical PA, particularly those with

c.355C>T and c.967C>T. LOH analysis of the tumor from the c.355C>T variant patient could not be performed because of a lack of available tumor tissue, but the variant was predicted to be deleterious *in silico*. The c.733G>A (p.E245K) variant, a rare SNP Minor Allele Frequency (MAF)=0.0005), was from a young woman with a prolactinoma in this study. Alamut displayed that the residue E245 was not conserved in orthologs. It appeared to be benign from *in silico* prediction and LOH analysis. So we considered the c.733G>A variant unlikely to be pathogenic. The presence of LOH in the tumor from the c.967C>T variant patient indicated possible pathogenicity, although the variant is considered a rare SNP (MAF=0.0018, from NCBI (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=9049)). This could be explained by the incomplete penetrance of PAs in carriers of AIP mutations (7, 31). For example, variant p.R16H has been detected in both patients and healthy subjects (12, 18, 32) but was confirmed to have an impact on the protein–protein interaction of AIP (22). Hence, it is possible that some SNPs could predispose carriers to developing PAs.

None of the previously reported pathological hotspot mutations such as p.R304* (7, 12, 23, 32, 33), p.R304Q (19, 22, 32, 34), p.R271W (12, 22, 34, 35), p.K241* (31), p.K241E (12), p.R81* (19, 22, 36), and p.Q14* (7, 37) were identified in this Chinese cohort. Two nonsense mutations p.Q14* and p.R304* are considered founder mutations based on haplotype and genealogical data analyses. However, most identified PA patients harboring p.Q14* (18/19) come from a limited geographical region in Northern Finland, while only one patient comes from Estonia, a neighboring country east of Finland (7, 25). Haplotype analyses by Occhi *et al.* (38) and Chahal *et al.* (33) provided evidence of a founder effect for p.R304* in central Italy and in Northern Ireland respectively. Cazabat *et al.* (39) also detected the mutation in a child with SPA from France, and in the reply to their letter, Stals reported their further haplotype analysis of patients with R304* mutations from several countries, indicating that the mutation can occur independently in various areas of the world and also have a founder effect in some cases. As the sample size of our study was relatively small, it is

Table 6 The statistical calculations of pathogenic AIP variants in young (≤ 30 years) or pediatric (≤ 18 years) patients.

	Pathogenic AIP variants	Non-pathogenic/no AIP variants	Total
All PA patients	9 (3.88%)	223 (96.12%)	232
Young (≤ 30 years)	5 (6.49%)	72 (93.51%)	77
Pediatric (≤ 18 years)	2 (12.5%)	14 (87.5%)	16
GH PA patients	8 (9.30%)	78 (90.70%)	86
Young (≤ 30 years)	5 (17.24%)	24 (82.76%)	29
Pediatric (≤ 18 years)	2 (25.00%)	6 (75.00%)	8

conceivable that the hotspot mutations above or novel ones would be detected in the Han Chinese population in further studies. We also propose that a larger number of FIPA kindreds should be collected for the identification of founder mutations among specific geographical or cultural populations in China, as was done for p.Q14* in Northern Finland.

MLPA assays are usually performed if genomic sequencing results are negative during genetic testing. Some large genomic deletions have previously been reported in endocrine-related tumor-associated genes, such as *MEN1* (40), *PRKAR1A* (41), and *AIP* (20, 42), although most studies found no large *AIP* locus deletions in the germline (34, 43, 44). Here, large deletions spanning from *MEN1* to *AIP* were observed in tumors of some *AIP* missense variant-carrying patients but not in the germline. Barlier *et al.* (42) previously demonstrated a deletion of the *AIP* locus in a somatotropinoma specimen carrying a nonsense mutation (p.R22*) in the retained allele in both tumor and germline; they also detected another large deletion encompassing both *MEN1* and *AIP* in a different somatotropinoma sample but did not find any *MEN1* or *AIP* mutation in the retained allele. In the current study, it is uncertain whether the variants identified in the germline were the retained alleles in the tumors, or whether there were other pathogenic *AIP* variants in other alleles of the tumors, because PCR amplification failed in *AIP* sequencing of the paraffin-embedded tumors. Based on the aggressive and refractory phenotype of the tumors with large deletions and the absence of a *MEN1* mutation in the germline, this raises the possibility that either one or more mutations in genes within chr11q13 other than *AIP* and *MEN1* could be involved in pituitary tumorigenesis in these patients or possibly that the variants identified in one allele of the germline could be pathogenic. There would also be another pathogenic mutation or a deletion in the *AIP* locus in another allele of the tumor at the beginning of tumorigenesis than that detected in the tumor from the c.90T>G variant, and then chromosome instability in the tumor genome could lead to larger deletions during the progressive stage than those in tumors from the c.784G>A, c.967C>T, and c.976G>A variants. If this is the case, MLPA results combined with other tests across the *MEN1* to *AIP* region could be used to determine prognosis, especially in patients carrying *AIP* or *MEN1* germline mutations, as *MEN1* mutations are also identified in some sporadic isolated pituitary macroadenoma patients (44).

In this study, there are seven *AIP* variants considered to be pathogenic, which were detected in nine PA patients (Table 5). It is noticeable that 77.78% (7/9) of pathogenic *AIP* variants are identified in male patients, which is in line with previous results (22, 45). Moreover, the frequency of *AIP* pathogenic variants in patients with SPAs was very low (3.88%, 9/232); however, the prevalence in young (≤ 30 years) or

pediatric patients (≤ 18 years) increased to 6.49% (5/77) and 12.5% (2/16) respectively. Notably, the prevalence soars to 17.24% (5/29) and 25% (2/8) respectively in our somatotropinoma population (Table 6). Other researchers also previously observed that *AIP* mutations were low in SPAs (42, 46, 47). Current data indicate that up to 20.5% of pediatric patients may carry an *AIP* mutation (34) and the likelihood of an *AIP* mutation in pediatric somatotroph and lactotroph adenomas is even higher (31, 34, 39), whereas the rate of young adults with a macroadenoma is around 11% (32, 34, 37, 39). *AIP* mutation-positive subjects are also common among FIPA cohorts (7, 12, 19, 20, 22, 23), with integrated data from multicentric collaborations, suggesting a prevalence of around 20% (37), especially for those with gigantism or acromegaly (19, 22, 37, 48). Therefore, it is advised that *AIP* mutations should be tested for i) patients meeting the criteria of FIPA (49), or ii) pediatric patients with any PA (34, 43, 50) or with somatotropinomas (31, 39), or iii) young patients with large/aggressive adenomas (age ≤ 30 years (12, 32, 34, 35, 37, 51, 52) or even age ≤ 40 years (43)). However, no guidance for testing is available as yet, although a recommendation for screening has first been published in a Clinical Practice suggestion paper recently (49). Penetrance rates in recent larger and more completely *AIP* mutation-positive family studies are variable but are generally in the low range (around 20% on the average) (7, 12, 19, 22, 33, 53, 54). *De novo* mutations are rarely identified (7, 33, 43, 53), with a notable exception (31), indicating a familial origin of *AIP* mutations in PA patients. The Han nationality, with a large population, is known to be relatively genetically homogenous. The policy of birth control limits during the early 1980s has brought about ~ 100 million only children in China so far. It is possible that some latent FIPA kindreds in which the probands are the young only children would be missed out because they have no siblings, and generally, their parents or grandparents would be diagnosed with PAs at a significantly older age (6). Given all that, besides the above suggestions, we propose that in China, young (age ≤ 30 years) *AIP* mutation-positive patients' consanguinity, if available, should also be tested for *AIP* mutations, in order to get enough genetic information for mapping the exclusive *AIP* mutation spectrum and founder mutations in Han Chinese, identifying those at high risk of developing PAs and providing genetic counseling to affected families.

In conclusion, genetic screening for *AIP* mutations in a Han Chinese population for the first time confirmed the low prevalence of *AIP* germline variants in sporadic or FLPA patients, as previously reported in other ethnic populations. Besides *AIP*, other genes may be crucial for FIPA tumorigenesis in Chinese patients. Some novel variants were identified in the current study and further investigations should be performed to verify their pathogenicity, although some have been indicated via

LOH analysis. Most variants were detected in young patients with macroadenomas, suggesting that AIP mutation tests for such patients and their relatives should be carried out, if possible. MLPA and micro-satellite analysis may also be useful to identify some deletions from MEN1 to AIP locus and to promote a better understanding of the tumorigenesis and progression of PAs.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-13-0442>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was funded by the National Natural Science Foundation of China (2011AA020112). The funding organization had no role in: the design and conduct of the study; in the collection, management, analyses, and interpretation of the data; or in the preparation, review, and approval of the manuscript.

Author contribution statement

F Cai and Y-D Zhang contributed in the conception and design of the research; F Cai, Y-D Zhang, and X-L Zhao performed the experiments and contributed to the interpretation of the experiments' results and the preparation of the figures and tables; X-L Zhao and F Gu also revised the manuscript; F Cai, Y-D Zhang, and X-L Zhao analysed the data; F Cai contributed in the interpretation of the experiments' results; F Cai, Y-D Zhang, Y-K Yang, F Gu, C-X Dai, X-H Liu, Y Yao, B Xing, Y-H Jiao, Z-Q Wei, and Z-M Yin participated in the collection of blood samples and clinical data; F Cai participated in the preparation of the figures and tables; F Cai participated in drafting the manuscript; and F Cai, S-H Ma, M Feng, J-J Wei, B Zhang, and R-Z Wang edited and revised the manuscript. The final version of the manuscript was approved by F Cai, Y-D Zhang, and R-Z Wang. R-Z Wang and F Gu contributed equally as first authors to this work.

Acknowledgements

The authors thank the patients and their families, as well as the healthy controls, for their cooperation in the study. We are also grateful to Prof. Xue Zhang, as well as Yun-Zhou Gao, Zhang-Yu Zou, Wei Wang, Guo-Xing Yang, and Zhang Wen for their rationalization proposal and data analyses assistance.

References

- Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML & McCutcheon IE. The prevalence of pituitary adenomas: a systematic review. *Cancer* 2004 **101** 613–619. (doi:10.1002/cncr.20412)
- Gejman R, Swearingen B & Hedley-Whyte ET. Role of Ki-67 proliferation index and p53 expression in predicting progression of pituitary adenomas. *Human Pathology* 2008 **39** 758–766. (doi:10.1016/j.humpath.2007.10.004)
- Daly AF, Rixhon M, Adam C, Dempegioti A, Tichomirowa MA & Beckers A. High prevalence of pituitary adenomas: a cross-sectional study in the province of Liege, Belgium. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 4769–4775. (doi:10.1210/jc.2006-1668)
- Fernandez A, Karavitaki N & Wass JA. Prevalence of pituitary adenomas: a community-based, cross-sectional study in Banbury (Oxfordshire, UK). *Clinical Endocrinology* 2010 **72** 377–382. (doi:10.1111/j.1365-2265.2009.03667.x)
- Fontana E & Gaillard R. Epidemiology of pituitary adenoma: results of the first Swiss study. *Revue Médicale Suisse* 2009 **5** 2172–2174.
- Daly AF, Jaffrain-Rea ML, Ciccirelli A, Valdes-Socin H, Rohmer V, Tamburrano G, Borson-Chazot C, Estour B, Ciccirelli E, Brue T *et al.* Clinical characterization of familial isolated pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 3316–3323. (doi:10.1210/jc.2005-2671)
- Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, Raitila A, Tuppurainen K, Ebeling TM, Salmela PI, Paschke R *et al.* Pituitary adenoma predisposition caused by germline mutations in the AIP gene. *Science* 2006 **312** 1228–1230. (doi:10.1126/science.1126100)
- Rzepecka IK, Szafron L, Stys A, Bujko M, Plisiecka-Halasa J, Madry R, Osuch B, Markowska J, Bidzinski M & Kupryjanczyk J. High frequency of allelic loss at the BRCA1 locus in ovarian cancers: clinicopathologic and molecular associations. *Cancer Genetics* 2012 **205** 94–100. (doi:10.1016/j.cancergen.2011.12.005)
- Kelley LA & Sternberg MJ. Protein structure prediction on the Web: a case study using the Phyre server. *Nature Protocols* 2009 **4** 363–371. (doi:10.1038/nprot.2009.2)
- Linnert M, Lin YJ, Manns A, Haupt K, Paschke AK, Fischer G, Weiwad M & Lucke C. The FKBP-type domain of the human aryl hydrocarbon receptor-interacting protein reveals an unusual Hsp90 interaction. *Biochemistry* 2013 **5** 2097–2107. (doi:10.1021/bi301649m)
- Morgan RM, Hernandez-Ramirez LC, Trivellin G, Zhou L, Roe SM, Korbonits M & Prodromou C. Structure of the TPR domain of AIP: lack of client protein interaction with the C-terminal α -7 helix of the TPR domain of AIP is sufficient for pituitary adenoma predisposition. *PLoS ONE* 2012 **7** e53339. (doi:10.1371/journal.pone.0053339)
- Daly AF, Vanbellinghen JF, Khoo SK, Jaffrain-Rea ML, Naves LA, Guitelman MA, Murat A, Emy P, Gimenez-Roqueplo AP, Tamburrano G *et al.* Aryl hydrocarbon receptor-interacting protein gene mutations in familial isolated pituitary adenomas: analysis in 73 families. *Journal of Clinical Endocrinology and Metabolism* 2007 **92** 1891–1896. (doi:10.1210/jc.2006-2513)
- Rowlands JC, Urban JD, Wikoff DS & Budinsky RA. An evaluation of single nucleotide polymorphisms in the human aryl hydrocarbon receptor-interacting protein (AIP) gene. *Drug Metabolism and Pharmacokinetics* 2011 **26** 431–439. (doi:10.2133/dmpk.DMPK-11-SC-013)
- Ceccato F, Occhi G, Albiger NM, Rizzati S, Ferasin S, Trivellin G, Mantero F & Scaroni C. Adrenal lesions in acromegaly: do metabolic aspects and aryl hydrocarbon receptor interacting protein gene have a role? Evaluation at aseline and after long-term follow-up. *Journal of Endocrinological Investigation* 2011 **34** 353–360. (doi:10.3275/7145)
- Occhi G, Trivellin G, Ceccato F, De Lazzari P, Giorgi G, Dematte S, Grimaldi F, Castello R, Davi MV, Arnaldi G *et al.* Prevalence of AIP mutations in a large series of sporadic Italian acromegalic patients and evaluation of CDKN1B status in acromegalic patients with multiple endocrine neoplasia. *European Journal of Endocrinology* 2010 **163** 369–376. (doi:10.1530/EJE-10-0327)
- Raitila A, Georgitsi M, Karhu A, Tuppurainen K, Makinen MJ, Birkenkamp-Demtroder K, Salmenkivi K, Orntoft TF, Arola J, Launonen V *et al.* No evidence of somatic aryl hydrocarbon receptor interacting protein mutations in sporadic endocrine neoplasia. *Endocrine-Related Cancer* 2007 **14** 901–906. (doi:10.1677/ERC-07-0025)
- Iwata T, Yamada S, Mizusawa N, Golam HM, Sano T & Yoshimoto K. The aryl hydrocarbon receptor-interacting protein

- gene is rarely mutated in sporadic GH-secreting adenomas. *Clinical Endocrinology* 2007 **66** 499–502. (doi:10.1111/j.1365-2265.2007.02758.x)
- 18 Georgitsi M, Raitila A, Karhu A, Tuppurainen K, Makinen MJ, Vierimaa O, Paschke R, Saeger W, van der Luijt RB, Sane T *et al.* Molecular diagnosis of pituitary adenoma predisposition caused by aryl hydrocarbon receptor-interacting protein gene mutations. *PNAS* 2007 **104** 4101–4105. (doi:10.1073/pnas.0700004104)
 - 19 Leontiou CA, Gueorguiev M, van der Spuy J, Quinton R, Lolli F, Hassan S, Chahal HS, Igreja SC, Jordan S, Rowe J *et al.* The role of the aryl hydrocarbon receptor-interacting protein gene in familial and sporadic pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 2390–2401. (doi:10.1210/jc.2007-2611)
 - 20 Georgitsi M, Heliovaara E, Paschke R, Kumar AV, Tischkowitz M, Vierimaa O, Salmela P, Sane T, De Menis E, Cannavo S *et al.* Large genomic deletions in AIP in pituitary adenoma predisposition. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 4146–4151. (doi:10.1210/jc.2008-1003)
 - 21 Bell DR & Poland A. Binding of aryl hydrocarbon receptor (AhR) to AhR-interacting protein. The role of hsp90. *Journal of Biological Chemistry* 2000 **275** 36407–36414. (doi:10.1074/jbc.M004236200)
 - 22 Igreja S, Chahal HS, King P, Bolger GB, Srirangalingam U, Guasti L, Chapple JP, Trivellin G, Gueorguiev M, Guegan K *et al.* Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families. *Human Mutation* 2010 **31** 950–960. (doi:10.1002/humu.21292)
 - 23 Jaffrain-Rea ML, Angelini M, Gargano D, Tichomirowa MA, Daly AF, Vanbellinghen JF, D'Innocenzo E, Barlier A, Giangaspero F, Esposito V *et al.* Expression of aryl hydrocarbon receptor (AHR) and AHR-interacting protein in pituitary adenomas: pathological and clinical implications. *Endocrine-Related Cancer* 2009 **16** 1029–1043. (doi:10.1677/ERC-09-0094)
 - 24 Vargiolu M, Fusco D, Kurelac I, Dirnberger D, Baumeister R, Morra I, Melcarne A, Rimondini R, Romeo G & Bonora E. The tyrosine kinase receptor RET interacts *in vivo* with aryl hydrocarbon receptor-interacting protein to alter survivin availability. *Journal of Clinical Endocrinology and Metabolism* 2009 **94** 2571–2578. (doi:10.1210/jc.2008-1980)
 - 25 Beckers A, Aaltonen LA, Daly AF & Karhu A. Familial isolated pituitary adenomas (FIPA) and the pituitary adenoma predisposition due to mutations in the aryl hydrocarbon receptor interacting protein (AIP) gene. *Endocrine Reviews* 2013 **34** 239–277. (doi:10.1210/er.2012-1013)
 - 26 Gadelha MR, Une KN, Rohde K, Vaisman M, Kineman RD & Frohman LA. Isolated familial somatotropinomas: establishment of linkage to chromosome 11q13.1–11q13.3 and evidence for a potential second locus at chromosome 2p16–12. *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 707–714. (doi:10.1210/jc.85.2.707)
 - 27 Khoo SK, Pendek R, Nickolov R, Luccio-Camelo DC, Newton TL, Massie A, Petillo D, Menon J, Cameron D, Teh BT *et al.* Genome-wide scan identifies novel modifier loci of acromegalic phenotypes for isolated familial somatotropinoma. *Endocrine-Related Cancer* 2009 **16** 1057–1063. (doi:10.1677/ERC-08-0287)
 - 28 Donangelo I, Araujo PB, Antenuzi D, Farage M, Marcondes J, Filho PN & Gadelha MR. Tumor deletion mapping of chromosomal region 13q14 in 43 growth hormone secreting pituitary adenomas. *Endocrine* 2005 **28** 131–136. (doi:10.1385/ENDO:28:2:131)
 - 29 Kazlauskas A, Poellinger L & Pongratz I. Two distinct regions of the immunophilin-like protein XAP2 regulate dioxin receptor function and interaction with hsp90. *Journal of Biological Chemistry* 2002 **277** 11795–11801. (doi:10.1074/jbc.M200053200)
 - 30 Bolger GB, Peden AH, Steele MR, MacKenzie C, McEwan DG, Wallace DA, Huston E, Baillie GS & Houslay MD. Attenuation of the activity of the cAMP-specific phosphodiesterase PDE4A5 by interaction with the immunophilin XAP2. *Journal of Biological Chemistry* 2003 **278** 33351–33363. (doi:10.1074/jbc.M303269200)
 - 31 Stratakis CA, Tichomirowa MA, Boikos S, Azevedo MF, Lodish M, Martari M, Verma S, Daly AF, Raygada M, Keil MF *et al.* The role of germline AIP, MEN1, PRKAR1A, CDKN1B and CDKN2C mutations in causing pituitary adenomas in a large cohort of children, adolescents, and patients with genetic syndromes. *Clinical Genetics* 2010 **78** 457–463. (doi:10.1111/j.1399-0004.2010.01406.x)
 - 32 Cazabat L, Libe R, Perlemoine K, Rene-Corail F, Burnichon N, Gimenez-Roqueplo AP, Dupasquier-Fediaevsky L, Bertagna X, Clauser E, Chanson P *et al.* Germline inactivating mutations of the aryl hydrocarbon receptor-interacting protein gene in a large cohort of sporadic acromegaly: mutations are found in a subset of young patients with macroadenomas. *European Journal of Endocrinology* 2007 **157** 1–8. (doi:10.1530/EJE-07-0181)
 - 33 Chahal HS, Stals K, Unterlander M, Balding DJ, Thomas MG, Kumar AV, Besser GM, Atkinson AB, Morrison PJ, Howlett TA *et al.* AIP mutation in pituitary adenomas in the 18th century and today. *New England Journal of Medicine* 2011 **364** 43–50. (doi:10.1056/NEJMoa1008020)
 - 34 Tichomirowa MA, Barlier A, Daly AF, Jaffrain-Rea ML, Ronchi C, Yaneva M, Urban JD, Petrossians P, Elenkova A, Tabarin A *et al.* High prevalence of AIP gene mutations following focused screening in young patients with sporadic pituitary macroadenomas. *European Journal of Endocrinology* 2011 **165** 509–515. (doi:10.1530/EJE-11-0304)
 - 35 Jennings JE, Georgitsi M, Holdaway I, Daly AF, Tichomirowa M, Beckers A, Aaltonen LA, Karhu A & Cameron FJ. Aggressive pituitary adenomas occurring in young patients in a large Polynesian kindred with a germline R271W mutation in the AIP gene. *European Journal of Endocrinology* 2009 **161** 799–804. (doi:10.1530/EJE-09-0406)
 - 36 Guaraldi F, Corazzini V, Gallia GL, Grottoli S, Stals K, Dalantaeva N, Frohman LA, Korbonits M & Salvatori R. 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. (doi:10.1007/s11102-012-0391-y)
 - 37 Daly AF, Tichomirowa MA, Petrossians P, Heliovaara E, Jaffrain-Rea ML, Barlier A, Naves LA, Ebeling T, Karhu A, Raappana A *et al.* Clinical characteristics and therapeutic responses in patients with germ-line AIP mutations and pituitary adenomas: an international collaborative study. *Journal of Clinical Endocrinology and Metabolism* 2010 **95** E373–E383. (doi:10.1210/jc.2009-2556)
 - 38 Occhi G, Jaffrain-Rea ML, Trivellin G, Albiger N, Ceccato F, De Menis E, Angelini M, Ferasin S, Beckers A, Mantero F *et al.* The R304X mutation of the aryl hydrocarbon receptor interacting protein gene in familial isolated pituitary adenomas: mutational hot-spot or founder effect? *Journal of Endocrinological Investigation* 2010 **33** 800–805. (doi:10.3275/6956)
 - 39 Cazabat L, Bouligand J & Chanson P. AIP mutation in pituitary adenomas. *New England Journal of Medicine* 2011 **364** 1973–1974 (author reply 1974–1975). (doi:10.1056/NEJMc1101859)
 - 40 Kikuchi M, Ohkura N, Yamaguchi K, Obara T & Tsukada T. Gene dose mapping delineated boundaries of a large germline deletion responsible for multiple endocrine neoplasia type 1. *Cancer Letters* 2004 **208** 81–88. (doi:10.1016/j.canlet.2003.10.011)
 - 41 Horvath A, Bossis I, Giatzakis C, Levine E, Weinberg F, Meoli E, Robinson-White A, Siegel J, Soni P, Groussin L *et al.* Large deletions of the PRKAR1A gene in Carney complex. *Clinical Cancer Research* 2008 **14** 388–395. (doi:10.1158/1078-0432.CCR-07-1155)
 - 42 Barlier A, Vanbellinghen JF, Daly AF, Silvy M, Jaffrain-Rea ML, Trouillas J, Tamagno G, Cazabat L, Bours V, Brue T *et al.* Mutations in the aryl hydrocarbon receptor interacting protein gene are not highly prevalent among subjects with sporadic pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* 2007 **92** 1952–1955. (doi:10.1210/jc.2006-2702)

- 43 Cazabat L, Bouligand J, Salenave S, Bernier M, Gaillard S, Parker F, Young J, Guiochon-Mantel A & Chanson P. Germline AIP mutations in apparently sporadic pituitary adenomas: prevalence in a prospective single-center cohort of 443 patients. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** E663–E670. (doi:10.1210/jc.2011-2291)
- 44 Cuny T, Pertuit M, Sahnoun-Fathallah M, Daly A, Occhi G, Odou MF, Tabarin A, Nunes ML, Delemer B, Rohmer V *et al.* Genetic analysis in young patients with sporadic pituitary macroadenomas: besides AIP don't forget MEN1 genetic analysis. *European Journal of Endocrinology* 2013 **168** 533–541. (doi:10.1530/EJE-12-0763)
- 45 Cazabat L, Guillaud-Bataille M, Bertherat J & Raffin-Sanson ML. Mutations of the gene for the aryl hydrocarbon receptor-interacting protein in pituitary adenomas. *Hormone Research* 2009 **71** 132–141. (doi:10.1159/000197869)
- 46 DiGiovanni R, Serra S, Ezzat S & Asa SL. AIP mutations are not identified in patients with sporadic pituitary adenomas. *Endocrine Pathology* 2007 **18** 76–78. (doi:10.1007/s12022-007-0010-z)
- 47 Yu R, Bonert V, Saporta I, Raffel LJ & Melmed S. Aryl hydrocarbon receptor interacting protein variants in sporadic pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 5126–5129. (doi:10.1210/jc.2006-1731)
- 48 Chahal HS, Chapple JP, Frohman LA, Grossman AB & Korbonits M. Clinical, genetic and molecular characterization of patients with familial isolated pituitary adenomas (FIPA). *Trends in Endocrinology and Metabolism* 2010 **21** 419–427. (doi:10.1016/j.tem.2010.02.007)
- 49 Korbonits M, Storr H & Kumar AV. Familial pituitary adenomas – who should be tested for AIP mutations? *Clinical Endocrinology* 2012 **77** 351–356. (doi:10.1111/j.1365-2265.2012.04445.x)
- 50 Personnier C, Cazabat L, Bertherat J, Gaillard S, Souberbielle JC, Habrand JL, Dufour C, Clauser E, SainteRose C & Polak M. Clinical features and treatment of pediatric somatotropinoma: case study of an aggressive tumor due to a new AIP mutation and extensive literature review. *Hormone Research in Paediatrics* 2011 **75** 392–402. (doi:10.1159/000327831)
- 51 Georgitsi M, De Menis E, Cannavo S, Makinen MJ, Tuppurainen K, Pauletto P, Curto L, Weil RJ, Paschke R, Zielinski G *et al.* Aryl hydrocarbon receptor interacting protein (AIP) gene mutation analysis in children and adolescents with sporadic pituitary adenomas. *Clinical Endocrinology* 2008 **69** 621–627. (doi:10.1111/j.1365-2265.2008.03266.x)
- 52 Pinho LK, Vieira Neto L, Wildemberg LE, Moraes AB, Takiya CM, Frohman LA, Korbonits M & Gadelha MR. Familial isolated pituitary adenomas experience at a single center: clinical importance of AIP mutation screening. *Arquivos Brasileiros de Endocrinologia e Metabologia* 2010 **54** 698–704. (doi:10.1590/S0004-27302010000800006)
- 53 Naves LA, Daly AF, Vanbellinghen JF, Casulari LA, Spilioti C, Magalhaes AV, Azevedo MF, Giacomini LA, Nascimento PP, Nunes RO *et al.* Variable pathological and clinical features of a large Brazilian family harboring a mutation in the aryl hydrocarbon receptor-interacting protein gene. *European Journal of Endocrinology* 2007 **157** 383–391. (doi:10.1530/EJE-07-0533)
- 54 Beckers A & Daly AF. The clinical, pathological, and genetic features of familial isolated pituitary adenomas. *European Journal of Endocrinology* 2007 **157** 371–382. (doi:10.1530/EJE-07-0348)

Received 23 October 2012

Revised version received 17 September 2013

Accepted 19 September 2013