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

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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.

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 somatotropinomas, 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 adrenocorticotropic 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. Micro-dissected 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 somatotropinoma according to the statement of the proband.
<table>
<thead>
<tr>
<th>Family</th>
<th>Tumor subtype</th>
<th>Gender</th>
<th>AP mutation/variant</th>
<th>Age (years)</th>
<th>Max. tumor diameter (mm)</th>
<th>Hormone level</th>
<th>Therapeutic responses (Y/N)</th>
<th>Follow-up/ family study</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M</td>
<td>M</td>
<td>GH-secreting</td>
<td>11</td>
<td>15</td>
<td>GH 1.39</td>
<td>Surgery (I)</td>
<td>AIII1: Octreotide GH (H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>F</td>
<td>F</td>
<td>PRL-secreting</td>
<td>11</td>
<td>15</td>
<td>PRL 86.3</td>
<td>Surgery (I)</td>
<td>AIII1: Octreotide GH (H)</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>M</td>
<td>GH-secreting</td>
<td>21</td>
<td>26</td>
<td>GH 1.39</td>
<td>Surgery (I)</td>
<td>AIII2: Octreotide GH (H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>F</td>
<td>F</td>
<td>NFPA</td>
<td>62</td>
<td>31</td>
<td>Normal</td>
<td>Surgery (I)</td>
<td>AIII1: Octreotide GH (H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>M</td>
<td>ACTH-secreting</td>
<td>42</td>
<td>15</td>
<td>Normal</td>
<td>Surgery (I)</td>
<td>AIII1: Octreotide GH (H)</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>F</td>
<td>F</td>
<td>NFPA</td>
<td>42</td>
<td>15</td>
<td>Normal</td>
<td>Surgery (I)</td>
<td>AIII1: Octreotide GH (H)</td>
</tr>
</tbody>
</table>

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

**AIP sequencing in a Han Chinese population**
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 microsatellite 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 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, 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 Tm 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 1X 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 7 ms 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 ± s.d. age at diagnosis: 23.7 ± 14.7 years, mean ± s.d. tumor diameter: 23.7 ± 15.4 mm); five (31.25%) had prolactinomas (no males; mean ± s.d. age at diagnosis: 34.2 ± 8.7 years, mean ± 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 ± s.d. age at diagnosis: 36.75 ± 11.66 years, mean ± s.d. tumor diameter: 21.13 ± 10.27 mm); 39 had prolactinomas (23 females; mean ± s.d. age at diagnosis: 33.49 ± 9.77 years, mean ± s.d. tumor diameter: 16.18 ± 8.09 mm); 39 had Cushing’s disease (31 females; mean ± s.d. age at diagnosis: 32.26 ± 11.68 years, mean ± s.d. tumor diameter: 7.82 ± 5.34 mm); and 58 had NFPA (26 females; mean ± s.d. age at diagnosis: 47.05 ± 13.11 years, mean ± 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 NFP A patient, are known SNP sites. None of these substitutions in the members of Family A (Tables 1 and 2). Otherwise, PCR amplification for AIP

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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 CI1 and CII2 had high GH levels and aggressive macroadenomas invading into the suprasellar region and cavernous sinus bilaterally leading to poor eyesight and hemianopsia. CI1 had undergone two operations...

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**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.
<table>
<thead>
<tr>
<th>Position</th>
<th>Gender</th>
<th>Age diagnosis</th>
<th>Phentype (tumor size); hormone test</th>
<th>Immuno-histochemistry</th>
<th>Nucleotide change</th>
<th>Amino acid substitution</th>
<th>Variant type</th>
<th>Patients with PA</th>
<th>Controls (n = 127)</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>M</td>
<td>23</td>
<td>(33); GH↑, IGF1↑</td>
<td>GH(+), PRL(±), TSH(±)</td>
<td>c.90T&gt;G</td>
<td>p.D30E Missense</td>
<td>One GH</td>
<td>0</td>
<td>0</td>
<td>Novel</td>
</tr>
<tr>
<td>Exon 2</td>
<td>M</td>
<td>58</td>
<td>(24); –</td>
<td>N/G</td>
<td>c.144C&gt;T</td>
<td>p.(=) Synonymous</td>
<td>One NFPA</td>
<td>0</td>
<td>0</td>
<td>Reported in an investigation from six ethnic population</td>
</tr>
<tr>
<td>Exon 3</td>
<td>F</td>
<td>32</td>
<td>(37); GH↑, IGF1↑</td>
<td>NA</td>
<td>c.355C&gt;T</td>
<td>p.R119W Missense</td>
<td>One GH</td>
<td>0</td>
<td>9</td>
<td>A rare variant rs4084113</td>
</tr>
<tr>
<td>Intron 3</td>
<td>–</td>
<td>–</td>
<td>Course</td>
<td></td>
<td>c.468 + 111C&gt;T</td>
<td>– Synonymous</td>
<td>One GH</td>
<td>0</td>
<td>0</td>
<td>Novel</td>
</tr>
<tr>
<td>Intron 4</td>
<td>F</td>
<td>47</td>
<td>(60); –</td>
<td>N/G, Ki67: 5%, p53(+)</td>
<td>c.645 + 41 dupG</td>
<td>– Duplication</td>
<td>One NFPA</td>
<td>0</td>
<td>0</td>
<td>Novel</td>
</tr>
<tr>
<td>Exon 5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>c.649C&gt;T</td>
<td>p.Q217* Non-sense</td>
<td>One family with three GH patients</td>
<td>0</td>
<td>Reported in one family with two GH patients</td>
<td></td>
</tr>
<tr>
<td>Exon 1</td>
<td>M</td>
<td>24</td>
<td>(31); GH↑, IGF1↑</td>
<td>GH(+), p53(+), KI67: 5%</td>
<td>c.687G&gt;A</td>
<td>p.(=) Synonymous</td>
<td>One GH</td>
<td>0</td>
<td>0</td>
<td>Novel</td>
</tr>
<tr>
<td>Exon 5</td>
<td>F</td>
<td>24</td>
<td>(33); PRL↑</td>
<td>PRL(+), p53(+), KI67: 3%</td>
<td>c.733G&gt;A</td>
<td>p.E245K Missense</td>
<td>One PRL</td>
<td>0</td>
<td>0</td>
<td>rs150645662</td>
</tr>
<tr>
<td>Exon 5</td>
<td>M</td>
<td>35</td>
<td>(25); GH↑, IGF1↑</td>
<td>GH(+), KI67: 2%</td>
<td>c.784G&gt;A</td>
<td>p.D262N Missense</td>
<td>One GH</td>
<td>0</td>
<td>0</td>
<td>Novel</td>
</tr>
<tr>
<td>Exon 6</td>
<td>M</td>
<td>11</td>
<td>(38); GH↑, IGF1↑</td>
<td>GH(+), p53(+), KI67: 1%</td>
<td>c.955G&gt;A</td>
<td>p.E319K Missense</td>
<td>One GH</td>
<td>0</td>
<td>0</td>
<td>Novel</td>
</tr>
<tr>
<td>Exon 6</td>
<td>M</td>
<td>41</td>
<td>(25); –</td>
<td>FSH(+), p53(±), KI67: 2%</td>
<td>c.967C&gt;T</td>
<td>p.R323W Missense</td>
<td>One NFPA</td>
<td>0</td>
<td>0</td>
<td>rs188965257</td>
</tr>
<tr>
<td>Exon 6</td>
<td>M</td>
<td>30</td>
<td>(47); GH↑, IGF1↑</td>
<td>GH(+), PRL(+), KI67: 4%, p53(+)</td>
<td>c.976G&gt;A</td>
<td>p.G326R Missense</td>
<td>One GH</td>
<td>0</td>
<td>0</td>
<td>Novel</td>
</tr>
<tr>
<td>3' UTR</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>c.993 + 60G&gt;C</td>
<td>– – 10 GH, eight PRL, six ACTH, eight NFPA</td>
<td>12</td>
<td>rs146014363</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Index 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 cisterns. 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 cisterns 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 NFP A case carrying the insertion variant c.645+41~c.645+42insG suffered from hypopituitarism and apoplexy 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, FAM59B, 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 tumors 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 peptide-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 motives 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.
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 homo- or 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.

**Table 3** Summary of LOH analyses of nine AIP variants.

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Amino acid substitution</th>
<th>RH41895</th>
<th>RH41858</th>
<th>WI-22393</th>
<th>SHGC-132167</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.90T&gt;G</td>
<td>p.D30E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.144C&gt;T</td>
<td>p.(=)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.645+41dupG</td>
<td>p.(=-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.687G&gt;A</td>
<td>p.(=-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.733G&gt;A</td>
<td>p.E245K</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.784G&gt;A</td>
<td>p.D262N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.955G&gt;A</td>
<td>p.E319K</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.967G&gt;T</td>
<td>p.R322W</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.976G&gt;A</td>
<td>p.3526R</td>
<td></td>
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</tr>
</tbody>
</table>

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

**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.967G>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; for WI-22393 and SHGC-132167, the satisfactory value was in lanes A, F, H, and I. SN, short allele normal; LN, long allele normal; ST, short allele tumor; and LT, long allele tumor.
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

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**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; ERα, 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.
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, MLP A 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.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Binding surface</th>
<th>Motif stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.R119W</td>
<td>190–191</td>
<td>209</td>
</tr>
<tr>
<td>p.D262N</td>
<td>277–278</td>
<td>209</td>
</tr>
<tr>
<td>p.E319K</td>
<td>277–278</td>
<td>209</td>
</tr>
<tr>
<td>p.R323W</td>
<td>277–278</td>
<td>209</td>
</tr>
<tr>
<td>p.G326R</td>
<td>277–278</td>
<td>209</td>
</tr>
</tbody>
</table>

*The 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.
A list of pathogenic and non-pathogenic AIP variants identified in this study.

<table>
<thead>
<tr>
<th>Pathogenic AIP variants</th>
<th>Non-pathogenic AIP variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.90T&gt;G (p.D30E)</td>
<td>c.144C&gt;T (p.(=))</td>
</tr>
<tr>
<td>c.355C&gt;T (p.R119W)</td>
<td>c.468+111C&gt;T</td>
</tr>
<tr>
<td>c.649C&gt;T (p.D217*)</td>
<td>c.516C&gt;T (p.(=))</td>
</tr>
<tr>
<td>c.784G&gt;A (p.D262N)</td>
<td>c.645+41dupG</td>
</tr>
<tr>
<td>c.955G&gt;A (p.E319K)</td>
<td>c.687G&gt;A (p.(=))</td>
</tr>
<tr>
<td>c.967C&gt;T (p.R323W)</td>
<td>c.733G&gt;A (p.E245K)</td>
</tr>
<tr>
<td>c.976G&gt;A (p.G326R)</td>
<td>c.993+60G&gt;C</td>
</tr>
</tbody>
</table>

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 macroadrenocortical. 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).

Thus, 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>
<thead>
<tr>
<th>Pathogenic AIP variants</th>
<th>Non-pathogenic/ no AIP variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>All PA patients</td>
<td>9 (3.88%)</td>
</tr>
<tr>
<td>Young (≤30 years)</td>
<td>5 (6.49%)</td>
</tr>
<tr>
<td>Pediatric (≤18 years)</td>
<td>2 (12.5%)</td>
</tr>
<tr>
<td>GH PA patients</td>
<td>8 (9.30%)</td>
</tr>
<tr>
<td>Young (≤30 years)</td>
<td>5 (17.24%)</td>
</tr>
<tr>
<td>Pediatric (≤18 years)</td>
<td>2 (25.00%)</td>
</tr>
</tbody>
</table>

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

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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; 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.

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References
17. Iwata T, Yamada S, Mizusawa N, Golam HM, Sano T & Yoshimoto K. The aryl hydrocarbon receptor-interacting protein


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)


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