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

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Abstract

Objective: Germline mutations of the aryl hydrocarbon receptor-interacting protein gene (AIP) have recently been described in three families with GH or prolactin-secreting tumors, as well as in a few patients with apparently sporadic somatotropinomas. The aim of the study was to determine the prevalence of AIP mutations in a large cohort of patients with apparently sporadic GH-secreting tumors.

Design: One hundred and fifty-four patients were included in a prospective cohort designed to study the genetic predisposition to GH-secreting tumors together with 270 controls.

Methods: In all these subjects, the entire coding sequence of the AIP gene was screened for germline mutations.

Results: AIP mutations were detected in 5 out of 154 patients (3%): nonsense mutations in exon 4 (p.Lys201X; n=2) and in exon 6 (p.Arg304X), one deletion in exon 3 (c.404delA; p.His135LeufsX21), and one mutation affecting the splice acceptor site of exon 4 (c.469-2 A>G). The five patients with an AIP mutation were significantly younger (mean age±s.d.: 25±10 vs 43±14 years, P=0.005) and three of them presented with gigantism. One missense mutation (p.Arg304Gln) was found in a single patient that was absent in all controls.

Conclusions: Germline mutations of the AIP gene were found in a small proportion of patients with sporadic pituitary somatotropinomas. This study shows that age and gigantism are simple clinical features which can help to select patients for mutation screening. It also supports the role of AIP in pituitary tumorigenesis.

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Introduction

Somatotrope tumors are rare pituitary neoplasms, responsible for growth hormone (GH) hypersecretion causing acromegaly in adult patients and gigantism when GH oversecretion occurs before the closure of ephysyeal growth plates. Although it may cause severe symptoms, including cardiac manifestations, and increase mortality from cancer, this condition is difficult to diagnose early whereas treatment of advanced or aggressive tumors may be difficult (1). The large majority of these tumors are sporadic, arising from a somatic mutation in a somatotrope cell allowing clonal expansion. The most frequent somatic molecular alterations identified to date are gain-of-function mutations in the guanine nucleotide-binding α-subunit 1 gene, encountered in about one-third of the tumors. They lead to constitutive activation of the adenyl cyclase, cellular proliferation, and GH hypersecretion. However, in many tumors, no Gsα mutations are found, even if cAMP-dependent pathways are activated, indicating that other unknown mechanism(s) of cAMP activation must exist in sporadic somatotrope tumors. Other molecular defects have been reported such as pituitary tumor-transforming gene overexpression (2, 3).

A small proportion of GH-secreting adenomas occur with a familial aggregation, as a component of either
the multiple endocrine neoplasia type 1 (MEN1) or the Carney complex, both syndromes resulting in a genetic predisposition to multiple endocrine tumors (4, 5). Isolated familial pituitary tumors were also described in the absence of MEN1 and PRKAR1A mutations (6). These syndromes require further genetic characterization. Recently, Vierimaa et al. (7) identified germline-inactivating mutations of the aryl hydrocarbon receptor-interacting protein gene (AIP, also called XAP2 or ARA9) in three kindreds with pituitary prolactin- and/or GH-secreting adenomas. Heterozygous mutations of the same gene were also found in ~10% of Finnish patients diagnosed with apparently sporadic somatotropinomas. It is noteworthy that, even in families, only a few members were diagnosed with prolactin or GH hypersecretion, suggesting that AIP is a low-penetration tumor susceptibility gene. Because of this low penetrance, inherited predisposition to AIP may be difficult to detect by patient interviews.

We had previously collected germline DNA and biochemical data from a prospective cohort of 154 consecutive patients with GH-producing pituitary tumor with no family history of pituitary tumor, MEN1 or Carney complex. This cohort is part of a program designed to study the potential genetic factors involved in the genesis of apparently sporadic somatotropinomas. In the present study, this cohort was studied to determine the prevalence of germline mutations of the AIP gene and the clinical characteristics associated with AIP mutations. We found five heterozygous inactivating mutations of the AIP gene and one missense mutation absent from 270 matched controls. This supports the role of AIP in the development of a subset of apparently sporadic pituitary tumors.

**Patients and methods**

**Patients and controls**

One hundred and fifty-four consecutive patients investigated for a GH-secreting pituitary adenoma in the Endocrine Departments of Cochin and Bicêtre Hospitals were prospectively included in a population cohort between December 2002 and September 2006. Patients with a family history of pituitary tumor or clinical features suggesting McCune Albright syndrome, Carney complex or MEN1 were excluded after examination by a senior endocrinologist. Patients were specifically asked for pituitary tumors symptoms among their family: we thus considered them as apparently sporadic acromegaly. However, none of the relatives have been directly interviewed or clinically examined. Clinical data (height, weight, thyroid palpation, age at time of diagnosis, and birth place), biochemical data before any treatment (basal and nadir plasma GH levels during oral glucose test, insulin-like growth factor-I (IGF-I)), as well as morphological data (adenoma size), and histological findings after transsphenoidal surgery were collected. Two hundred and seventy control subjects were also recruited by the same investigators. In controls, age, medical history, and birthplace were recorded. A clinical examination was performed in order to exclude subjects with personal or family history of MEN1 or Carney complex. All subjects gave informed consent to genetic studies and the protocol was approved by the Institutional Review Board of Cochin Hospital.

**DNA extraction and PCR amplification**

Genomic DNA was prepared from blood samples as previously described (9). For patients and controls, the six exons and the flanking intronic sequences of the AIP gene (GenBank accession number Hs. 412433) were amplified by PCR using the following specific primers:

- exon 1 sense 5'-CTA GGA GTT GCC GAA GCA AG-3',
- antisense 5'-AAA CCC AGA TAC CCG AGG AC-3';
- exon 2 sense 5'-GGA CTG GAC TTC TCC TTG GG-3',
- antisense 5'-GTG TAG CAG AGG GTG GAG GG-3';
- exon 3 sense 5'-AGG GGG TTA GGT GCT CTG TT-3',
- antisense 5'-GTT TCC TGG AGG AGG TGA CA-3';
- exons 4–5 sense 5'-CTC TGCT TGCTGGTTGGAT-3',
- antisense 5'-ATT CAT GCT TCA TTG GCA CA-3'; and
- exon 6 sense 5'-CCA ATG AAG CAT GAA TGG TG-3',
- antisense 5'-AAC AGC CAC CCA AGT ACC AG-3'.

The PCR conditions were: denaturation at 95 °C for 5 min, followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C, and a final extension step of 8 min at 72 °C. Direct sequencing of the purified fragments was performed using the Genetic Sequencer ABI3100 AppliedBiosystems apparatus (Foster City, CA, USA). All genetic alterations were confirmed by a second PCR and direct sequencing. The amplification and sequencing of genomic DNA for all of the coding sequences of Menin and PRKAR1A genes were realized in the same manner. Primers are available upon request.

**Statistical analysis**

Clinical, biochemical, and radiological data were recorded in a Microsoft Access 98 computerized database. Analyses included Student’s t-test, χ²-test and ANOVA, which were all carried out with the SAS package (SAS Institute Inc., Cary, NC, USA).

**Results**

**Clinical, hormonal, and radiological data in acromegalic patients**

The 154 patients (84 females and 70 males) were aged 15–79 years at the time of diagnosis, with a mean age of 42.6 ± 14.3 years (mean ± s.d.). Eighty percent of the patients were French Caucasians whereas the remaining...
had diverse ethnic origins. Anthropometric measures were as indicated: height = 171 ± 10.5 cm, weight = 83.4 ± 20.6 kg, body mass index (BMI) = 28 ± 5.4. Basal plasma GH levels = 66.3 ± 91.3 ng/ml, nadir GH during oral glucose load = 29.1 ng/ml, with a very large dispersion, ratio between patient plasma IGF-I level and the upper limit of normal for age = 2.97 ± 1.45. Twenty-three patients had microadenomas whereas 92 tumors were 10 mm or larger. The mean adenoma size was 18.8 ± 11.3 mm (Table 1).

**AIP mutations**

Four different mutations leading to premature stop codons or affecting a splice acceptor site were identified in 5 out of 154 acromegalic patients (Table 1): a one nucleotide deletion in exon 3 (c.404delA; p.His135Leufs21X) leading to a frameshift and a premature stop codon after 19 amino acids (patient 1); a nonsense mutation in exon 4 (p.Lys201X) found in two unrelated patients (patients 3 and 4); a nonsense mutation in exon 6 (p.Arg304X, patient 5) which has already been described in two apparently unrelated Italian families (7, 8; patient 5 and his parents were born in France). The last mutation is located in the splice acceptor site of exon 4 (c.469-2 A>G, patient 2). None of these mutations were observed in the 270 controls. Thus, the overall frequency is 3%, 95% confidence interval (CI) 1.5–4.8%. Twenty-three patients had microadenomas whereas 92 tumors were 10 mm or larger. The mean adenoma size was 18.8 ± 11.3 mm (Table 1).

**Clinical data in patients with AIP mutations**

**Patient 1** Male, presented at 14 years of age, complaining of excessive growth: 188 cm, whereas his mother and father were 163 and 168 cm tall respectively. Diagnosis of gigantism because of GH excess was established and surgical ablation of a pituitary macroadenoma was performed, followed by radiotherapy because of persistent GH hypersecretion. After 30 years, GH hypersecretion is still controlled but panhypopituitarism has developed. He has one son without known endocrine abnormality (Table 1).

**Patient 2** Male, GH hypersecretion was diagnosed at the age of 40 years because of headaches, morphological changes, sweating, and bilateral carpal tunnel syndrome; all symptoms had been noticed for at least 3 years. Hormonal and morphological data are shown in Table 1. After surgical treatment, somatostatin analog treatment was indicated because of persistent GH hypersecretion. Immunostaining was positive for prolactin and GH. After 15 years, GH secretion is still under control with octreotide acetate LAR 10 mg/2 months. There is no hypopituitarism.

**Patient 3** Female, presented at the age of 25 with secondary amenorrhoea. At the age of 27, acromegaly was diagnosed because of morphological changes and headaches. Biochemical features were consistent with acromegaly. An enlarged sella was observed on skull X-ray. She was treated by transsphenoidal surgery followed by radiotherapy. GH secretion was controlled but she developed panhypopituitarism. At the age of 41, a 19 mm incidental nonhypersecreting adrenocortical adenoma was surgically removed. After 10 years, an ulcerative colitis was diagnosed. At the age of 58, thyroidectomy was performed because of a 4 cm nodule. Histology concluded to a microfollicular and trabecular adenoma. She is now aged 61 and GH secretion is still low.

### Table 1 Clinical data of patients with aryl hydrocarbon receptor-interacting protein (AIP) germline mutation. The clinical, biological, and AIP sequence for the five patients with a germline AIP mutation are shown. The two last lines represent the mean clinical data of the patients without AIP gene mutation and of the whole cohort.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age at diagnosis (years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI</th>
<th>GH (ng/ml)</th>
<th>IGF-I (P/N ratio)</th>
<th>Tumor size (mm)</th>
<th>Mutation</th>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>15</td>
<td>197</td>
<td>102</td>
<td>26.3</td>
<td>NA</td>
<td>NA</td>
<td>NA (macro)</td>
<td>c.404delA; p.His135LeufsX21</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>40</td>
<td>173</td>
<td>80</td>
<td>26.7</td>
<td>33</td>
<td>2.5</td>
<td>15</td>
<td>c.469-2 A&gt;G</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>27</td>
<td>157</td>
<td>63</td>
<td>25.6</td>
<td>100</td>
<td>NA</td>
<td>NA (macro)</td>
<td>p.Lys201X</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>24</td>
<td>189</td>
<td>130</td>
<td>36.4</td>
<td>NA</td>
<td>NA</td>
<td>NA (macro)</td>
<td>p.Arg304X</td>
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<td>5</td>
<td>M</td>
<td>17</td>
<td>195</td>
<td>95</td>
<td>36.8</td>
<td>12</td>
<td>NA</td>
<td>NA (macro)</td>
<td></td>
</tr>
<tr>
<td>AIP non-</td>
<td>F</td>
<td>83 F 42.6 ± 14</td>
<td>164.5 (F)</td>
<td>74.6 (F)</td>
<td>27.5 (F)</td>
<td>51.8 (14 NA)</td>
<td>3 (42 NA)</td>
<td>18 (24 NA)</td>
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<tr>
<td>mutated n=149</td>
<td>M</td>
<td>66 M 43.2 ± 14.1</td>
<td>164.4 (M)</td>
<td>74.5 (F)</td>
<td>27.5 (F)</td>
<td>51.7 (16 NA)</td>
<td>2.99 (46 NA)</td>
<td>18 (28 NA)</td>
<td></td>
</tr>
<tr>
<td>Whole cohort</td>
<td></td>
<td>84 F 43.2 ± 14.1</td>
<td>164.4 (F)</td>
<td>74.5 (F)</td>
<td>27.5 (F)</td>
<td>51.7 (16 NA)</td>
<td>2.99 (46 NA)</td>
<td>18 (28 NA)</td>
<td></td>
</tr>
</tbody>
</table>

Sex, age at diagnosis, height, weight, body mass index (BMI), basal GH value, ratio between patient plasma IGF-I level and the upper limit of normal for age (P/N), tumor size and mutation are reported. NA, not available.
Patient 4 Male, presented at the age of 24 with severe headaches and visual impairment revealing a compressive pituitary tumor. No preoperative hormonal data are available. A somatotrope macroadenoma was partially removed by emergency transcranial surgery. Postoperatively, GH hypersecretion was diagnosed. His height suggested gigantism. The treatment was complemented by transsphenoidal resection of remaining tissue followed by radiotherapy. At the age of 38, he was operated for massive pulmonary embolism. At the age of 41, he developed a hemorrhagic stroke. Thereafter, GH secretion was controlled but the patient developed panhypopituitarism and severe obesity.

Patient 5 Male, presented at the age of 17 with headaches, visual field defect, and a height suggesting gigantism. A pituitary magnetic resonance imaging (MRI) scan showed a 15 mm pituitary macroadenoma. Acromegaly was confirmed by hormonal data without hyperprolactinemia. Transsphenoidal surgery was performed allowing complete cure of GH hypersecretion. Immunostaining was positive for GH. Twenty years after surgery, GH is normalized without any pituitary deficiency but he suffers from severe obesity (BMI: 37.2) complicated by diabetes mellitus and hypertension.

Patients with AIP mutations are significantly younger at the time of diagnosis (24.6 ± 9.9; P = 0.005); men are also taller (188.5 cm), although the difference did not reach significance (P = 0.09). We noted three cases of gigantism out of five patients with germline AIP mutations, whereas only 17 out of 149 unmutated patients presented with gigantism (P = 0.016). Tumor size and hormonal data, especially plasma IGF-I levels are not available in four patients diagnosed before 1980. Four mutated patients were male and only one was female. However, the difference in sex ratio did not reach significance (P = 0.12).

Missense mutations, polymorphism, and genetic variants

A missense mutation in exon 6 was observed in one acromegalic patient (p.Arg304Gln) and was absent from the 270 control subjects; this mutation has also been described in one young Polish patient diagnosed with Cushing’s disease, and an Italian acromegalic patient (10). This patient (female) presented at the age of 37 with severe headache, diplopia, and visual impairment revealing a compressive pituitary tumor. No preoperative hormonal data are available. Cerebral MRI found pituitary apoplexy. A somatotrope macroadenoma was partially removed by emergency surgery. Eighteen years after surgery, GH secretion is still under control using a somatostatin analog but she developed thyrotrope, corticotrope, and gonadotrope deficiencies and suffers from morbid obesity (BMI: 65.5) complicated by diabetes mellitus, sleep apnea, and hypertension. Four patients and two controls displayed nucleotide variations in the 3’ untranslated region: c.993 + 34 C > G (n = 1), c.993 + 56 C > G (n = 4), and c.993 + 60 G > A (n = 1).
A nonsynonymous mutation exon 1 (p.Arg16His, c.47 G>A) was present in two patients and two controls; this nucleotide change has been found in a family with isolated familial somatotropinoma (8), an Italian acromegalic patient without somatic LOH, unselected pituitary adenomas from US and Poland, and in a German control (10). Two other new polymorphisms were detected in patients: p.Gly12Gly (c.36 G>A) in exon 1 (n = 1) and p.Gln142Gln (c.429 G>A) in exon 3 (n = 1). A previously described single nucleotide polymorphism has been found in patients and controls with a similar frequency: c.468 C>T (rs 4084113) with an allele frequency of 36% in patients and 34% in controls, c.516 C>T (p.Asp172Asp, rs 2276020) in 0.7 and 1.6% in patients and controls respectively (Table 2).

No abnormality of PRKAR1A and Menin coding sequences has been found in the 28 patients under 30 years old without AIP mutations.

Discussion

Recently, mutations in the AIP gene were identified in familial pituitary tumors (mostly GH-secreting adenomas) as well as in 5 out of 51 patients from Northern Finland with sporadic acromegaly (7, 8). As most patients lacked a strong family history of pituitary tumor, AIP could be a low-penetrance susceptibility gene to GH-secreting pituitary adenomas (7). As these results were observed in a particular population, we studied the prevalence of AIP germline mutations or specific polymorphisms in a cohort of 154 consecutive acromegalic patients with a different ethnic origin (mostly French Caucasians). Patients with a family history of pituitary tumors were excluded, and Menin and PRKAR1A gene mutations were excluded in all patients under 30 years old. We found heterozygous inactivating mutations in five of them. Although we cannot exclude undiagnosed familial cases, according to statistical analysis, a germline mutation of AIP could therefore be expected in 1–7% of all apparently sporadic acromegalic patients. Considering the significant number of patients and the fact that it is a prospective recruitment of consecutive patients from two major academic centers devoted to pituitary diseases, these results are likely to be very representative of acromegaly in France. The prevalence may be different in different populations. However, our results are in agreement with two recently published series (10, 11).

One other patient displayed a missense mutation in exon 6, changing a basic residue (Arg) to Gln, in a region involved in the aryl hydrocarbon receptor (AHR) binding (12). This arginine is rather conserved among species, although it is sometimes replaced by another basic amino acid, lysine. The same amino acid changes have been found in a young Polish patient with Cushing’s disease, and an Italian acromegalic patient. The functional consequences should be evaluated. Unfortunately, tumoral DNA is not available to look for LOH. Two patients and two controls displayed a nonsynonymous mutation in exon 1, changing a lysine for another basic amino acid, histidine (p.Arg16His, c.47 G>A). This nucleotide change has been found in a family with isolated familial somatotropinoma (8), an Italian acromegalic patient without somatic LOH, unselected pituitary adenomas from US and Poland, as well as in one healthy German control (10). We considered this change as a rare polymorphism, although we cannot exclude its pathogenic value (13).

This study is discordant with data from Yu et al. (14) who very recently reported no mutations of the AIP gene in 66 patients with pituitary tumors. However, a much smaller number of GH-secreting adenomas were tested (n = 35), and only the three previously published mutations were looked for (14). In fact, there is no
L Cazabat and others

The mechanism of tumorigenesis induced by AIP mutations remains hypothetical. Heterozygous inactivating mutations are likely to result in a lower expression of the AIP protein. Moreover, loss of the wild-type allele was detected in somatotropinomas from mutated Finnish patients (7), and in one French patient studied separately (13), suggesting a tumor suppressor gene mechanism.

AIP is a 330 amino acid protein analog to steroid receptors associated immunophilins (15). AIP was also shown to bind the phosphodiesterase (PDE) 4A5 and attenuate its effect (22). PDEs form a superfamily of enzymes that hydrolyze cyclic nucleotides (23). PDE4A5 is a cAMP-specific isoform (24). Recently, mutations of PDE11A4 have been found in patients with Cushing’s syndrome due to micronodular adrenal hyperplasia (25). Consequences of AIP inactivation on cAMP signaling deserves further studies.

Clinical data analysis reveals that mutated patients are younger, and several had gigantism. This confirms data from Vierimaa et al. (7) and Georgitsi et al. (10). Younger age is also a clinical characteristic of patients with familial isolated pituitary adenoma (6). However, in the present cohort, acromegaly was diagnosed at the age of 40 in one case. This questions the age cut-off for genetic screening for AIP mutation in sporadic acromegaly. It is noteworthy that four out of five patients with AIP mutations of this cohort are male. This differs from the previous study by Vierimaa et al. (7) and Georgitsi et al. (10), which found a large male predominance in AIP mutations (six out of seven). Since AIP is autosomal, this might suggest that

eye of high spot region for the AIP gene and all mutated patients from our cohort except one had a new mutation. This result points out the need for sequencing the whole gene when genetic testing is proposed.

The mechanism of tumorigenesis induced by AIP mutations remains hypothetical. Heterozygous inactivating mutations are likely to result in a lower expression of the AIP protein. Moreover, loss of the wild-type allele was detected in somatotropinomas from mutated Finnish patients (7), and in one French patient studied separately (13), suggesting a tumor suppressor gene mechanism. AIP is a 330 amino acid protein analog to steroid receptors associated immunophilins (15). AIP was also shown to bind the phosphodiesterase (PDE) 4A5 and attenuate its effect (22). PDEs form a superfamily of enzymes that hydrolyze cyclic nucleotides (23). PDE4A5 is a cAMP-specific isoform (24). Recently, mutations of PDE11A4 have been found in patients with Cushing’s syndrome due to micronodular adrenal hyperplasia (25). Consequences of AIP inactivation on cAMP signaling deserves further studies.

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Table 2 Aryl hydrocarbon receptor-interacting protein mutation and polymorphisms in acromegalic patients. Each line corresponds to one genetic variant in acromegalic patients and in controls. For each genetic variant, coding sequence mutation and amino acid changes are reported. Nonsense and missense mutations in acromegalic patients only are in bold type. Allelic frequency and GenBank reference sequence are also given. New AIP mutations reported in this paper have been deposited in GenBank (nos EF553637-EF553639).

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<th>Exons</th>
<th>Change in DNA sequence</th>
<th>Change in protein sequence</th>
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<th>Allelic frequency in controls</th>
<th>Database reference</th>
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<tr>
<td></td>
<td>c.47 G&gt;A</td>
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ND, never reported in GenBank or Ensembl database.
AIP inactivation would be more oncogenic in males than females.

Genetic testing may be useful in young patients with acromegaly, even in the absence of family history, and could allow early diagnosis in relatives bearing the mutation, which is a potentially important advantage to improve outcome (26).

Germline mutations of the AIP gene are present in a small but significant proportion of patients with sporadic acromegaly, supporting a role – still to be determined – in somatotrope tumorigenesis. In the setting of genetic counseling, genetic testing could be proposed to the youngest patients in order to evaluate, among relatives, a population at high risk of developing pituitary tumors.

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