Do the Aryl Hydrocarbon Receptor Interacting Protein Variants (Q228K and Q307R) Play a Role in Patients with Familial and Sporadic Hormone-Secreting Pituitary Adenomas?

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Aim: The aim of this study was to examine mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene by sequence analysis of exons 1–6 using leukocyte genomic DNA obtained from a cohort of familial isolated pituitary adenoma (FIPA) and apparent sporadic functional pituitary adenoma Turkish patients. **Methods:** Fourteen FIPA and 90 sporadic pituitary adenoma (somatotrophinoma, prolactinoma, and corticotrophinoma) patients, 1 sporadic gigantism case, and 70 healthy controls were included in the study. **Results:** We did not detect *AIP* mutations in patients with FIPAs or sporadic pituitary adenomas, including the gigantism case. Only two exonic homozygous missense single-nucleotide polymorphisms (rs641081 [Q228K] and rs4930195 [Q307R]) were identified in the *AIP* locus. Minor allele frequencies of the Q307R and Q228K variants were significantly higher in FIPA patients compared to controls. In addition, the minor allele frequency of the Q228K variant was significantly increased in patients with sporadic somatotrophinomas compared to controls, whereas the minor allele frequency of the Q307R variant was significantly increased in corticotrophinoma patients compared to controls. Conversely, the minor allele frequencies of Q228R and Q307R variants were similar between patients with prolactinomas and controls. No *AIP* gene mutation or variant was observed in the sporadic gigantism patient. These results suggest that Q228K and Q307R variants in the *AIP* gene might be involved in the genetic susceptibility to familial and sporadic pituitary adenomas (somatotrophinoma and corticotrophinoma) in the Turkish population.

Introduction

THE MAJORITY OF PITUITARY ADENOMAS occur sporadically, but heterozygous germline inactivating mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene confer a predisposition to pituitary adenomas in different races of familial isolated pituitary adenoma (FIPA) patients, and particularly patients with somatotrophinoma and prolactinomas (Aaltonen, 2007; Barlier *et al.*, 2007; Beckers and Daly, 2007; Cazabat *et al.*, 2012; Beckers *et al.*, 2013). *AIP* gene mutations have been identified in 15–20% of all FIPA cases, with 50–80% of these cases being somatotrophinomas (Daly *et al.*, 2006, 2007; Vierimaa *et al.*, 2006; Yu *et al.*, 2006; Aaltonen, 2007; Beckers and Daly, 2007; Georgitsi *et al.*, 2007; Daly *et al.*, 2009; Fontana *et al.*, 2010; Igreja *et al.*, 2010; Cazabat *et al.*, 2012).

In addition to FIPA cases (typically early-onset disease and larger pituitary adenomas), *AIP* screening in young adult patients (age <40 years) with isolated sporadic macro-

adenomas has found that nearly 12% of patients possess germline *AIP* mutations (Tichomirowa *et al.*, 2006; Beckers and Daly, 2007; Daly *et al.*, 2007). Extensive genetic analyses have been conducted on FIPAs and sporadic pituitary adenomas, and many mutations identified in the *AIP* gene have been shown to be associated with familial or sporadic pituitary adenomas. However, the cause of tumorigenesis in FIPA remains unknown in a high number of cases. Therefore, the aim of the present study was to investigate the entire coding sequence of the *AIP* gene in a cohort of Turkish patients with FIPAs and apparent sporadic functional pituitary adenomas.

Materials and Methods

A total of 105 pituitary patients attending an outpatient pituitary clinic at the Medical Faculty of Istanbul University and 70 healthy control subjects were included in this study. The study was approved by the Ethics Committee of the

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Medical Faculty of Istanbul University, Istanbul. All subjects provided informed consent for participation in the study. Seven families with FIPAs (four homogeneous for GH-secreting adenomas and three heterogeneous with GH/PRL/ACTH/nonfunctional pituitary adenomas, GH/PRL-secreting adenomas, and GH/nonfunctional pituitary adenomas, respectively) were identified as having at least two family members with pituitary adenoma without MEN1- or Carney complex-associated lesions and laboratory features. The mean age at diagnosis of FIPA patients was 39.2 ± 13.6 years (range: 20–64 years). The pituitary adenoma types in our cohort of seven FIPAs patients ($n=16$) were somatotrophinomas ($n=11$), prolactinomas ($n=2$), nonfunctional adenomas ($n=2$), and corticotrophinomas ($n=1$). Two patients with isolated familial somatotrophinomas (IFSs) were excluded from the genomic analysis because one of them was deceased and the other was unreachable. In addition, patients with acromegaly ($n=47$), Cushing's disease ($n=23$), and prolactinomas ($n=21$) were negative for pituitary tumors within their family and therefore considered as apparently sporadic. Patients received regular follow-up at the clinic and 70 healthy controls were eligible for this study. Clinical and radiological features of the patients with FIPA and sporadic pituitary adenomas (somatotrophinoma, corticotrophinoma, and prolactinoma) are shown in Table 1.

In addition to this cohort, a 54-year-old female was also included who had first presented at the age of 17 years with a tall stature and accelerated growth velocity that was first noticed when she was 5 years old. The diagnosis was gigantism, and she received surgery, conventional radiotherapy, and medical therapy for treatment. The patient has been monitored regularly under medical therapy. This represented a childhood-onset case that was not included in the sporadic acromegaly group, but was included for AIP gene analysis.

AIP mutation analysis

DNA extraction. Whole blood from patients ($n=105$) and healthy controls ($n=70$) was collected in tubes containing K₂EDTA. Genomic DNA was extracted using a commercial kit (QIAamp DNA Blood Mini Kit; Qiagen, Milano, Italy). The concentration was measured using a common spectrophotometric method and stored at -20°C for further use.

Polymerase chain reaction and DNA sequencing. Polymerase chain reaction (PCR) was carried out on a Perkin Elmer 9700 (ABI, Vernon, CA) using the following conditions: an initial denaturation at 95°C for 10 min, followed by 35 cycles at 94°C for 45 s, 60°C (0.5°C decrease per cycle) for 60 s, 72°C for 1 min, and a final extension step at 72°C for

10 min. This basic protocol was modified depending on the DNA sample, the primer annealing temperatures, and the amplification outcome of the template (Vierimaa *et al.*, 2006). PCR products were run on 1–2% agarose gels at 120 V for 30 min stained with ethidium bromide and visualized under UV light. The PCR products were purified by ExoSAP-IT (USB Corporation, Cleveland, OH) according to the manufacturer's instructions. DNA sequencing was performed using the BigDye 3.1 Termination Chemistry (Applied Biosystems, Foster City, CA) on an ABI 3130 DNA sequencer (Applied Biosystems). Each PCR product was sequenced in separate reactions with the forward (F) and reverse (R) primers as follows: Exon 1-F: CCGAGACATTCTAGGCTCC, Exon 1-R: CTCTCGCTAAGGCCTCC, Exon 2-F: GGACTG GACTTCTCCTTGGG, Exon 2-R: GTCTAGCAGAGGGT GGAGGG, Exon 3-F: GATGGTGGTGGGGAAGG, Exon 3-R: ACCCCTGGGTGGACAGG, Exon 4–5-F: ATGTGGGT CAGGTCTGCTG, Exon 4–5-R: AAAGGCTAGGTCTTGA CCCC, Exon 6-F: AGGAGACATGAGGGCAGGC, and Exon 6-R: AACAGCCACCCAAGTACCAG (Metabion International AG, Planegg, Germany). Sequencing chromatograms were visualized using the Sequencing Analysis 5.3.1 version (Applied Biosystems) and aligned to the reference sequence(s) using the SeqScape v.2.6 software (Applied Biosystems). All mutations and variants have been annotated according to the nomenclature for human genomic sequence variations (The Human Genome Variation Society 2008, at www.hgvs.org). Results were compared with the human AIP single-nucleotide polymorphisms (SNPs) documented in the National Center for Biotechnology Information (NCBI) dbSNP database (www.ncbi.nlm.nih.gov/snp).

Data analysis

Calculations were performed using the SPSS v21.0 software system. An evaluation of continuous variables, such as age, gender, and maximum tumor diameter working groups, was conducted and presented as the mean and standard deviation. Nominal variables were expressed as a percentage. The chi-square test, odds ratio, and confidence interval were used to detect possible differences in minor allele frequency of variants between pituitary adenoma patients and healthy controls. Statistical significance was set at $p < 0.05$ for the results that were two tailed.

Results

The allelic frequency of AIP variants in patients with FIPAs, sporadic functional pituitary adenomas, and healthy controls is summarized in Table 2. We did not detect AIP

TABLE 1. CLINICAL AND RADIOLOGICAL FEATURES OF THE PATIENTS WITH FAMILIAL ISOLATED PITUITARY ADENOMAS AND SPORADIC FUNCTIONAL PITUITARY ADENOMAS

Patients	Age at diagnosis mean $\pm$ SD (range, years)	Sex (F/M)	Maximum tumor diameter on MRI scan mean $\pm$ SD (range, mm)
FIPAs ($n=14$)	39.2 ± 13.6 (20–64)	10/4	17 ± 8.7 (5–40)
Somatotrophinoma ($n=47$)	35.6 ± 9.1 (18–58)	25/22	16.9 ± 7.8 (10–45)
Corticotrophinoma ($n=23$)	33.0 ± 9.8 (17–64)	19/4	10.4 ± 6.0 (5–25)
Prolactinoma ($n=21$)	27.2 ± 9.2 (15–49)	11/10	22.8 ± 11.3 (8–42)

FIPA, familial isolated pituitary adenoma; SD, standard deviation.

TABLE 2. DETECTED ARYL HYDROCARBON RECEPTOR-INTERACTING PROTEIN VARIANTS, AND COMPARISON OF THE VARIANTS OF CASES AND HEALTHY CONTROLS

Variants	FIPA patients (n=14)	Sporadic acromegaly (n=47)	Sporadic Cushing's disease (n=23)	Sporadic prolactinoma (n=21)	Healthy controls (n=70)
Q228K					
Allelic frequency (%)	78.6	38.3	21.7	0.0	10.0
<i>p</i> -Value ^a	0.0001	0.0001	0.137	0.148	
OR (95% CI)	33 (7.4–147.4)	5.6 (2.1–14.8)			
Q307R					
Allelic frequency (%)	78.6	21.3	34.9	0.0	8.6
<i>p</i> -Value ^a	0.0001	0.057	0.005	0.197	
OR (95% CI)	39.1 (8.5–180.1)		5.7 (1.7–18.8)		

^aChi-square test, $p < 0.05$ significant.

CI, confidence Interval; OR, odds ratio.

mutations in Turkish patients with FIPAs, sporadic acromegaly, prolactinomas, and Cushing's disease, and the sporadic gigantism case based on DNA sequencing analysis. However, two homozygous missense variants (rs641081 [Q228K] and rs4930195 [Q307R]) in exon 5 and exon 6 were found in the coding region of the *AIP* gene in patients with FIPA, sporadic acromegaly, and Cushing's disease (Fig. 1a, b). Furthermore, a heterozygous silent variant (rs2276020 [D172D]) in exon 4 was found in only one sporadic acromegaly patient in addition to the Q228K variant (Fig. 1c). The minor allele frequencies of *AIP* variants Q228K and Q307R were significantly different between FIPA patients (78.6% vs. 10% and 78.6% vs. 8.57%; $p = 0.0001$ and < 0.0001 , respectively). Interestingly, the minor allele frequencies of both *AIP* variants were more than sevenfold higher than controls. In addition, the minor allele frequency of the combination of both *AIP* variants was 57% in FIPA patients, which was significantly higher than controls ($p = 0.0001$). Furthermore, the minor allele frequency of the Q228K variant in patients with sporadic acromegaly was significantly higher than the controls (38.3% vs. 10%; $p = 0.0001$, respectively), and the minor allele frequency of the Q307R variant in patients with Cushing's disease was significantly higher when compared to the control group (34.9% vs. 8.57%; $p = 0.005$, respectively). However, the minor allele frequencies of the Q228K and Q307R variants did not differ significantly between patients with prolactinomas and controls ($p = 0.148$ and 0.196 , respectively). Interestingly, in addition to the Q228K variant, a heterozygote silent D172D variant in exon 4 was detected in only one young sporadic acromegalic patient, and his mother had died of liver cancer at a young age. There was a strong family history for Hepatitis B and hepatocellular carcinoma for this patient on the mother's side. Moreover, we only had one sporadic gigantism patient, and she had neither a family history of pituitary adenoma nor any germline *AIP* mutation or variant. Nevertheless, no significant difference was observed in age, gender, or maximum adenoma diameter between IFS ($n = 9$) and sporadic somatotrophinoma ($n = 47$) patients who were positive for *AIP* variants ($p > 0.05$). Furthermore, there were no significant differences in patient age (younger and older than the age of 40), gender, or maximum adenoma diameter between sporadic somatotrophinoma or

FIG. 1. (a) Sequence chromatogram showing a homozygous Q228K variant in exon 5. (b) Sequence chromatogram showing a homozygous Q307R variant in exon 6. (c) Sequence chromatogram showing a heterozygous D172D variant in exon 4.

Cushing's disease patients who were positive or negative for an *AIP* variant ($p > 0.05$).

Discussion

To the best of our knowledge, this is the first combined study involving the analysis of both FIPAs (four IFSs and three heterogeneous FIPAs) and sporadic functional pituitary adenomas (somatotrophinoma, corticotrophinoma, and prolactinoma) for *AIP* mutations in a Turkish patient cohort. The gene encoding *AIP* is located on chromosome 11q13.3 and several inactivating mutations have been identified to date, which cause the development of familial or sporadic pituitary adenomas (Daly *et al.*, 2006, 2007; Barlier *et al.*, 2007; Cazabat *et al.*, 2007; Igreja *et al.*, 2010; Occhi *et al.*, 2010; Chahal *et al.*, 2011; Beckers *et al.*, 2013). The first *AIP* germline mutations identified were from a Finnish cohort of FIPA patients (nonsense mutation Q14X and splice site mutation IVS3-1G>A) and an Italian cohort of pituitary adenoma patients (nonsense mutation R304X). However, no *AIP* mutation was identified in a Turkish family with isolated somatotrophinoma when using both *AIP* sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA) assays (Vierimaa *et al.*, 2006; Georgitsi *et al.*, 2008). Daly *et al.* (2007) reported that 50% of familial pure acromegalic cases assessed had no mutation in the coding region of the *AIP* gene. In the present study, no *AIP* mutations were identified in IFS patients by sequence analysis of exons 1–6. Istanbul is a cosmopolitan city, and therefore, the patients and healthy controls who participated in this study were from different geographical regions of Turkey. Nevertheless, a nonsense R304X mutation in the *AIP* gene was recently identified in a large Turkish family with isolated somatotrophinomas by exonic sequencing (Niyazoglu *et al.*, 2014). The frequency of *AIP* mutations in young patients (younger than the age of 30) with sporadic acromegaly was reported to be 13.3% in one study, but another study did not identify germline or somatic *AIP* mutations in leukocyte gDNA or tumor tissue cDNA in any sporadic acromegaly patients assessed, including those with sporadic gigantism (Barlier *et al.*, 2007; Tichomirowa *et al.*, 2011). In a separate study, *AIP* gene mutations were identified with very low prevalence in apparent sporadic acromegaly (4.1%), prolactinomas (4.5%), corticotrophinomas (6.8%), and nonfunctioning adenomas (0.9%) (Cazabat *et al.*, 2012). In addition, Preda *et al.* (2014) suggested a relatively low prevalence of *AIP* gene mutations in young patients (younger than the age of 40) with apparent somatotrophin macroadenomas (3%) by sequence analysis of exons 1–6 from leukocyte genomic DNA as well as by using the MLPA method. In the present study, *AIP* gene mutations were not detected in patients with apparent sporadic pituitary adenomas (younger and older than the age of 40) by sequencing. Interestingly, the present study showed that two exonic homozygous missense SNPs (rs641081 [Q228K] and rs4930195 [Q307R]) in the coding region of *AIP* were significantly increased in FIPA patients. Furthermore, significant associations were detected between the Q307R variant and Cushing's disease, as well as the Q228K variant and sporadic acromegaly. These variants are present in the dbSNP database within the NCBI. To date, a total of 14 exonic SNPs have been identified within the *AIP* locus in DNA samples from six ethnic populations (Rowlands *et al.*,

2010, 2011). The minor allele frequency of the Q228K variant was 0.13, whereas the minor allele frequency of the Q307R variant has not been identified in the NCBI dbSNP database to date. In the present study, the minor allele frequency of the Q228K variant in healthy controls was 0.10, which is similar to the rate in Caucasians. Moreover, we found that the minor allele frequency of the Q307R variant in our population was 0.086, but it has not been defined yet in the literature. The *AIP* gene appears to function as a tumor suppressor and consists of six exons that encode a 330 amino acid protein. The C-terminal region of the molecule contains three tetratricopeptide repeat (TPR) domains that are 34 amino acid long conserved structures consisting of an α -7 helix that is crucial for protein–protein interactions (Das *et al.*, 1998; Leontiou *et al.*, 2008; Chahal *et al.*, 2010; Trivellin and Korbonits, 2011). The majority of the mutations alter the C-terminal structure of the *AIP* gene, and most of the *AIP* mutations identified to date change the amino acid sequence due to nonsense, deletion, or insertion mutations, which results in a truncation of the protein and loss of the C-terminus (Leontiou *et al.*, 2008). All of these *AIP* variants exhibit some disruption of AIP-phosphodiesterase-4A5 binding important for the TPR structure, but it is uncertain which of these play a role in pituitary tumorigenesis (Trivellin and Korbonits, 2011). In the current study, the two exonic homozygous missense mutations were located in the coding region of the *AIP* gene; however, we do not know the potential functional consequences of these variations. No studies to date have listed these variants in the dbSNP database or provided functional analyses. The Q228K and Q307R variants may be involved in genetic susceptibility to familial and sporadic pituitary adenomas in the cohort of patients in this study or may lead to decreased *AIP* expression or disruption of protein function.

Finally, no *AIP* gene mutations were identified in FIPA or sporadic functioning adenoma patients in this study. We also did not identify any significant differences in age, gender, or tumor size between patients with and without *AIP* mutations. These results suggest that other genes may play a role in the pathogenesis of *AIP*-negative FIPAs and sporadic pituitary adenomas in the Turkish population. Since the sample size of the study was limited, further studies are needed using a larger sampling of patients with familial and sporadic pituitary adenomas to confirm that specific mutations/variants have a functional impact on the gene.

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Author Disclosure Statement

No competing financial interests exist.

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