



HAL
open science

Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families

Susana C Igreja, Harvey S Chahal, Peter J King, Graeme B Bolger, Umasuthan Srirangalingam, Leonardo Guasti, Paul J Chapple, Giampaolo Trivellin, Maria Gueorguiev, Katie Guegan, et al.

► To cite this version:

Susana C Igreja, Harvey S Chahal, Peter J King, Graeme B Bolger, Umasuthan Srirangalingam, et al.. Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families. *Human Mutation*, 2010, 31 (8), pp.950. 10.1002/humu.21292 . hal-00552399

HAL Id: hal-00552399

<https://hal.science/hal-00552399>

Submitted on 6 Jan 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families

Journal:	<i>Human Mutation</i>
Manuscript ID:	humu-2010-0068.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	08-Apr-2010
Complete List of Authors:	<p>Igreja, Susana; Barts and the London Medical School, Queen Mary University of London, Endocrinology Chahal, Harvey; Barts and the London Medical School, Queen Mary University of London, Endocrinology King, Peter; Barts and the London Medical School, Queen Mary University of London, Endocrinology Bolger, Graeme; Comprehensive Cancer Center, University of Alabama Srirangalingam, Umasuthan; Barts and the London Medical School, Queen Mary University of London, Endocrinology Guasti, Leonardo; Barts and the London Medical School, Queen Mary University of London, Endocrinology Chapple, Paul; Barts and the London Medical School, Queen Mary University of London, Endocrinology Trivellin, Giampaolo; Barts and the London Medical School, Queen Mary University of London, Endocrinology Gueorguiev, Maria; Barts and the London Medical School, Queen Mary University of London, Endocrinology Guegan, Katie; Department of Molecular Genetics, Royal Devon and Exeter Foundation Trust Stals, Karen; Department of Molecular Genetics, Royal Devon and Exeter Foundation Trust Khoo, Bernard; UCL Medical School, Department of Endocrinology Kumar, Ajith; Barts and the London Medical School, Queen Mary University of London, Endocrinology Ellard, Sian; Department of Molecular Genetics, Royal Devon and Exeter Foundation Trust Grossman, Ashley; Barts and the London Medical School, Queen Mary University of London, Endocrinology Korbonits, Marta; Barts and the London Medical School, Queen</p>

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Key Words:	pituitary, familial isolated pituitary adenoma, AIP, mutation, penetrance
------------	---



For Peer Review

Humu-2010-0068

Research Article

Supporting Information for this preprint is available from the
Human Mutation editorial office upon request (humu@wiley.com)

Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families

Susana Igreja^{1*}, Harvinder S. Chahal^{1*}, Peter King¹, Graeme B. Bolger², Umasuthan Srirangalingam¹,
Leonardo Guasti¹, J. Paul Chapple¹, Giampaolo Trivellin¹, Maria Gueorguiev¹, Katie Guegan³, Karen
Stals³, Bernard Khoo⁴, Ajith V. Kumar⁵, Sian Ellard³, Ashley B. Grossman¹, Márta Korbonits¹ and the
International FIPA consortium**

¹*Department of Endocrinology, Barts and the London School of Medicine, Queen Mary University of
London, London, UK, EC1M 6BQ*

²*Comprehensive Cancer Center, University of Alabama, Birmingham AL, USA, 35294*

³*Department of Molecular Genetics, Royal Devon and Exeter Foundation Trust, Exeter, UK, EX2 5DW*

⁴*Department of Endocrinology, UCL Medical School, Royal Free Campus, London, UK, NW3 2PF*

⁵*North East Thames Regional Genetics Service, Great Ormond Street Hospital, London, WC1N 3JH, UK*

**These authors contributed equally to this work*

*** See list of consortium members in Appendix*

Correspondence to: Professor Márta Korbonits

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Queen Mary University of London, Barts & The
London School of Medicine, Department of Endocrinology
Charterhouse Square, London, UK, EC1A 6BQ
Tel +44 20 7882 6238, Fax +44 20 7882 6197
E-mail: m.korbonits@qmul.ac.uk

For Peer Review

ABSTRACT

Familial isolated pituitary adenoma (FIPA) is an autosomal dominant condition with variable genetic background and incomplete penetrance. Germline mutations of the aryl hydrocarbon-interacting protein (*AIP*) gene have been reported in 15-40% of FIPA patients. Limited data are available on the functional consequences of the mutations or regarding the regulation of the *AIP* gene. We describe a large cohort of FIPA families and characterize missense and silent mutations using minigene constructs, luciferase and β -galactosidase assays as well as in silico predictions. Patients with *AIP* mutations had a lower mean age at diagnosis (23.6 ± 11.2 yr) than *AIP* mutation-negative patients (40.4 ± 14.5 yr). A promoter mutation showed reduced in vitro activity corresponding to lower mRNA expression in patient samples. Stimulation of the protein kinase A-pathway positively regulates the *AIP* promoter. Silent mutations led to abnormal splicing resulting in truncated protein or reduced *AIP* expression. A two-hybrid assay of protein-protein interaction of all missense variants showed variable disruption of *AIP*-phosphodiesterase-4A5 binding. In summary, exonic, promoter, splice-site and large deletion mutations in *AIP* are implicated in 31% of families in our FIPA cohort. Functional characterization of *AIP* changes is important to identify the functional impact of gene sequence variants.

Keywords: pituitary adenoma, FIPA, acromegaly, *AIP*, tumor suppressor

Introduction

The majority of pituitary adenomas are sporadic, but occasionally they may also occur in a familial setting (Melmed, 2006). Familial isolated pituitary adenoma (FIPA; MIM# 102200) is an increasingly recognized autosomal dominant disease with low or variable penetrance (Beckers and Daly, 2007; Chahal et al., 2010). Heterozygous germline mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*; MIM# 605555) have been identified in 15-40% of FIPA families, while in the majority of the families the disease causing gene or genes are not known. *AIP* mutation positive families most commonly present with somatotroph, or with both somatotroph and lactotroph adenomas, but rarely other types of pituitary tumors can be observed (Vierimaa et al., 2006; Daly et al., 2007; Iwata et al., 2007; Toledo et al., 2007; Leontiou et al., 2008). Young onset, seemingly sporadic somatotroph adenoma patients can also harbor germline *AIP* mutations (Georgitsi et al., 2008a). Patients have loss of heterozygosity (LOH) in the tumor tissue at the locus of the *AIP* gene at the 11q13 area (Yamada et al., 1997; Gadelha et al., 2000; Soares et al., 2005; Vierimaa et al., 2006). However, LOH at 11q13 has been observed frequently in sporadic pituitary adenomas (Farrell and Clayton, 2000) and there are a number of FIPA families with 11q13 LOH, but no detectable *AIP* mutations (Soares et al., 2005; Leontiou et al., 2008). Interestingly, the 11q13 locus also contains the Multiple Endocrine Neoplasia type 1 gene (*MEN1*; MIM# 131100), 2.4 megabases upstream from the *AIP* gene, which can cause familial pituitary adenomas; however, the phenotype of *MEN1* and FIPA is different.

The *AIP* gene consists of six exons encoding a 330 amino-acid protein with three typical tetratricopeptide repeat (TPR) domains and a final extended α -helix (α -7). *AIP* appears to function as a tumor suppressor gene: we have previously reported data showing that mutant *AIP* proteins lose the ability of wild-type (WT) *AIP* to decrease cell proliferation and are unable to bind protein partners (Leontiou et al., 2008). Most of the described *AIP* mutations identified via standard sequencing techniques change the amino-acid sequence due to nonsense, deletion or insertion mutations, and result in a loss of the C-terminal end of the protein. Large gene deletions also severely disrupt the protein

1
2
3 structure. Exon/intron junction mutations may affect splicing or RNA stability and promoter mutations
4 affect RNA expression, but these have not been previously characterized.
5
6

7
8 We have systematically examined our large cohort of patients with FIPA specifically looking for
9 large deletions, promoter and splice-site mutations in addition to exonic mutations; we describe 11 new
10 *AIP* mutation-positive families with six novel mutations. We present novel functional data on an *AIP*
11 promoter mutation, a splice-mutation and a synonymous change which leads to reduced *AIP* mRNA
12 expression, as well as quantitative data on the effects of all the published missense mutations on the
13 interaction of AIP with one of its binding proteins, phosphodiesterase-4A5 (PDE4A5). These data allow
14 us to characterize the clinical features of FIPA and to provide the most accurate data for the functional
15 impact of *AIP* variants, which has an important role in genetic counseling of patients and their families
16 and inform the need for predictive testing and biochemical and imaging screening.
17
18
19
20
21
22
23
24
25
26
27

28 **Patients and Methods**

29 **FIPA Patients**

30
31
32 We studied 38 novel families with FIPA (Supp. Table S1) who were identified as having at least two
33 family members with pituitary adenoma and no features of MEN1 or Carney complex (MIM# 160980).
34 All patients provided written informed consent and Institutional Review Board approval was obtained.
35 For the clinical analysis we combined these novel families with our previously described cohort of 26
36 families (Supp. Table S2) (Leontiou et al., 2008). The clinical features of some of these families have
37 previously been described, as detailed in Supp. Table S1 and S2 (Pestell et al., 1989; McCarthy et al.,
38 1990; Matsuno et al., 1994; Gadelha et al., 1999; Gadelha et al., 2000; Soares et al., 2005; Georgitsi et al.,
39 2007; Leontiou et al., 2008; Georgitsi et al., 2008b). Direct sequencing of *AIP* (NM_003977.2) included
40 the entire coding sequence, conserved splice sites (from the conserved A of the upstream branch site to
41 +10 downstream of each exon) and 1200 base-pairs of the promoter region. Nucleotide numbering
42 throughout the manuscript reflects cDNA numbering with +1 corresponding to the A of the ATG
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 translation initiation codon in the reference sequence, according to the guidelines of the Human Genome
4 Variation Society (www.hgvs.org/mutnomen). The initiation codon is codon 1. *AIP* sequencing data were
5 compared with Caucasian (n=96) and Japanese (n=78) subjects from the general population, as previously
6 described (Leontiou et al., 2008). Multiplex-ligation dependent probe amplification (MLPA, P244-kit
7 MRC-Holland, Amsterdam, Netherlands) dosage analysis was carried out to look for partial or whole
8 gene deletions in all the families that tested negative by direct sequencing for germline *AIP* mutations and
9 for whom a suitable quality of DNA sample was available.
10
11
12
13
14
15
16
17
18
19

20 21 **RNA extraction, RT-PCR, cell culture**

22 RNA from whole blood was extracted using PaxGene tube and extraction kit (Qiagen, Crawley, UK).
23 RNA from rat pituitary cell line GH3 cells was extracted using the RNeasy Mini Kit (Qiagen). Both
24 protocols include a DNase step. RT-PCR on 1µg RNA was performed as described previously (Leontiou
25 et al., 2008). Real-time PCR was performed with the TaqMan system using ready-made or custom-made
26 (surrounding the c.807C>T mutation) *AIP* probe-primer kits (ABI, Warrington, UK). Reactions were
27 performed in triplicate using β -actin or GAPDH as a housekeeping gene. Data were analyzed using the
28 standard curve method. Rat somatotroph GH3 cell line was cultured and transfected as previously
29 described (Leontiou et al., 2008).
30
31
32
33
34
35
36
37
38
39
40
41
42

43 **Promoter mutation**

44 The c.[-270_-269CG>AA;-220G>A] changes in the promoter area were identified in a Japanese
45 somatotroph adenoma family (Family VI Supp. Table S2). These changes were not detected in Japanese
46 (n=78) and Caucasian (n=96) individuals from the general population and in any of the studied family
47 members (affected or unaffected *AIP* mutation carriers or their non-carrier family members or *AIP*
48 mutation negative patients, n=150) of our familial cohort, or in any of our sporadic pituitary adenoma
49 cases studied (n=98), altogether 844 chromosomes. The transcription factor binding-site searching
50
51
52
53
54
55
56
57
58
59
60

1
2
3 programs TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) and Alibaba (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html>) were used to identify possible binding site disruption
4
5
6
7
8 caused by the c.[-270_-269CG>AA;-220G>A] sequence changes in the *AIP* promoter area (Figure 1A
9
10 and B). A 1.2 kb (upstream from the start codon) PCR fragment was amplified from gDNA (forward
11
12 5'TACAACCTCCATCTCCTGGG3', reverse 5'GAGTCCGGAAGTTGCCGAAA3'). The WT fragment
13
14 was cloned into the *SacI* and *SpeI* sites of the promoterless firefly luciferase vector pGL3-basic (Promega,
15
16 Southampton, UK). Three mutated constructs were prepared from the WT template using the QuikChange
17
18 site-directed mutagenesis kit (Stratagene, LaJolla, CA): a dibasic -270_-269AA mutant, a single -220A
19
20 mutant and a construct containing both -270_-269AA&-220A mutations (Figure 1C). To study basal and
21
22 stimulated activity, GH3 cells were transfected with the four different constructs and 48h later were
23
24 treated with db-cAMP (2mM), forskolin (10μM), phorbol-12-myristate 13-acetate (PMA, 20nM) and H89
25
26 (30μM) for five hours. A plasmid encoding renilla luciferase, pRL-CMV, was included to control for
27
28 transfection efficiency and promoter activity was analyzed using the dual luciferase assay (Promega).
29
30 Peripheral blood-derived cDNA samples were studied from the two affected patients and three ethnically
31
32 matched controls.
33
34
35
36
37

38 **Splicing mutation c.249G>T**

39
40 The ALAMUT program (<http://www.interactive-biosoftware.com/alamut/doc/1.5/splicing.html>) was used
41
42 to predict whether the novel synonymous sequence variant c.249G>T, p.= (affecting the third nucleotide
43
44 of codon 83 originally coding glycine) changes the splicing characteristics of the *AIP* gene (Family 28,
45
46 Figure 2A). The primers (forward 5'GCGGATATCATCGCAAGACT3', reverse
47
48 5'CCTCATCTTCCACATGGAGA3') were designed on exons 1&3 to detect aberrant splicing events.
49
50
51
52
53
54
55
56
57
58
59
60

Synonymous mutation c.807C>T

The ALAMUT, the ESEfinder v.3.0 (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>) and the RESCUE-ESE (<http://genes.mit.edu/burgelab/rescue-ese/>) programs were used to predict whether the synonymous c.807C>T, p.= (affecting the third member of codon 269 originally coding phenylalanine) sequence variant of exon 6 changes the splicing characteristics of the *AIP* gene (Figure 3A) (Leontiou et al., 2008). Conventional and real-time RT-PCRs were performed on patient blood-derived cDNA using primers spanning the exon 5-6 junction to compare *AIP* expression between patients carrying the mutation and control individuals. To further characterise the splicing events around this sequence abnormality we performed a minigene splicing study. To construct the minigene, gDNA from a patient harboring the c.807C>T mutation was PCR amplified with primers covering part of the exon 5, intron-exon region and part of exon 6 including the mutation (forward 5'AAGCTGGTGGTCGAGGAGTA3', reverse 5'CAAAGTGCTGGAGCTGGAC3'). WT and mutant minigene constructs were prepared and fragments were cloned into the EcoRI site of pcDNA3.1+ (Invitrogen, Paisley, UK). GH3 cells were transiently transfected for 24-48h with WT and mutant minigenes separately or together in equal amounts. Transfection efficiency was followed by co-transfection with an expression plasmid coding for green fluorescent protein (GFP, pEGFP-N1) at a pcDNA3.1+ with insert:pEGFP-N1 ratio of 1:10. Expression of the minigene transcripts was detected using vector-specific primers.

PDE4A5 interaction

Interactions between *AIP* and *PDE4A5* were studied to clarify the role of missense sequence changes in protein-protein binding. *PDE4A5* was cloned into the *NotI*-site of pLEXAN to generate a LexA DNA-binding-domain fusion (Bolger et al., 2003; Leontiou et al., 2008). WT *AIP* and nine missense *AIP* sequence variants (c.47G>A, p.R16H (Daly et al., 2007; Georgitsi et al., 2007; Cazabat et al., 2007;

1
2
3 Buchbinder et al., 2008; Yaneva et al., 2008); c.145G>A, p.V49M (Iwata et al., 2007); c.308A>G,
4
5 p.K103R (Beckers et al., 2008), c.713G>A, p.C238Y (Leontiou et al., 2008), c.721A>G, p.K241E (Daly
6
7 et al., 2007), c.769A>G, p.I257V (Montanana et al., 2009), c.811C>T, p.R271W (Daly et al., 2007;
8
9 Jennings et al., 2009; Igreja et al., 2010), c.896C>T, p.A299V (Georgitsi et al., 2007), the hotspot
10
11 mutation c.911G>A, p.R304Q and a novel stop mutation (c.490C>T, p.Q164X) were cloned into the
12
13 *NotI*-site of pGADN to generate GAL4 activation-domain fusions. Quantitative β -galactosidase assays
14
15 were performed in the *S. cerevisiae* strain L40 by the method of Guarente et al. using *O*-nitrophenyl- β -D-
16
17 galactopyranoside as a substrate (Guarente, 1983). Each mutation was tested in at least two different yeast
18
19 clones.
20
21

22 23 24 25 ***In silico* analysis**

26
27 Location of *AIP* missense mutations were compared to the available consensus sequences of TPR
28
29 proteins. A hypothetical model of AIP was constructed using the Phyre (Protein Homology/analogy
30
31 Recognition Engine) program (Kelley and Sternberg, 2009), based on the crystal structure of FKBP52
32
33 (MIM# 600611), the closest related protein with available crystal structure. The alignment is showing
34
35 28% identical amino-acids and 50% similar amino-acids in the N-terminal region (amino-acid 5-90 of
36
37 AIP) and the TPR domain area (amino-acid 165-328 of AIP) (Supp. Figure S1). Alignment was
38
39 performed with CLUSTAL 2.0.12 (<http://www.clustal.org/>) and BL2SEQ
40
41 (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) sequence alignment tools.
42
43
44
45

46 47 **Statistics**

48
49 Statistical analysis was performed with StatsDirect software (Addison-Wesley-Longman, Cambridge,
50
51 UK). Distribution of data was analyzed by the Shapiro-Wilk test. Comparisons were calculated with the
52
53 Student t-test and the Kruskal-Wallis-test followed by the Conover-Inman test, as appropriate. Data are
54
55 shown as mean \pm standard error, unless otherwise stated. Significance was taken at $P < 0.05$.
56
57
58
59
60

Results

Genetic studies

We report here 38 novel families with FIPA, including eleven families with an *AIP* mutation (Supp. Table S1). Six of these 11 families harbor a novel mutation including two complete gene deletions.

The novel mutations revealed via sequencing include a nonsense mutation (c.490C>T, p.Q164X), a single base-pair duplication (c.662dupC, p.E222X), a splice-site mutation (c.249G>T, p.G83AfsX15, see data below) and a complex deletion-insertion mutation (c.74_81delins7, p.L25PfsX130), all resulting in a frameshift and/or premature stop-codon. Three families have been found to have the previously well-described c.910C>T, p.R304X hotspot (Vierimaa et al., 2006; Daly et al., 2007; Leontiou et al., 2008; Jaffrain-Rea et al., 2009) mutation and one family the c.911G>A, p.R304Q hotspot (Daly et al., 2007; Leontiou et al., 2008) mutation. We also identified a previously described variant (c.896C>T, p.A299V) in one of our families which also harbors the p.R304X mutation.

We screened all affected patients in our cohort (Supp. Table S1 and S2) for large deletions using MLPA, where no *AIP* mutations were identified by sequencing and suitable DNA was available. Three large deletions were identified among the 36 families (8.3%, Family 9, Family IV and Family XXI, Supp. Table S1 and S2) all three originally reported to have WT *AIP* sequences by conventional sequencing (Georgitsi et al., 2007; Leontiou et al., 2008). Two of these resulted in the loss of the full length *AIP* gene and one was the previously described exon 2 deletion (Georgitsi et al., 2008b).

For clinical characteristics we analyzed the data from the currently reported 38 families together with the 26 families we have reported earlier (Leontiou et al., 2008). In our cohort of 64 FIPA families with 160 affected subjects (Supp. Table S1 and S2), the mean age (\pm SD) at diagnosis of pituitary adenoma was 33.7 ± 15.5 yr (23.6 ± 11.2 yr in the *AIP* mutant families and 40.4 ± 14.5 yr in the *AIP* negative families; $P<0.00001$). If we re-calculate the age of onset only for the successfully performed MLPA negative families this was 38.4 ± 13.8 yr (not significantly different from the full cohort of *AIP* negative families). Families with FIPA show variable penetrance. We are fully aware that penetrance calculations

1
2
3 are subject to bias due to symptomatic patient referral and due to incomplete genealogical data as well as
4
5 variable ages of the subjects studied, but these calculations could still provide some useful information.
6
7 Penetrance was calculated in our families with taking into account affected and obligate carrier subjects
8
9 as well as half of the subjects with 50% risk to inherit the mutation (Ogino et al., 2007). We have a
10
11 mean±SD penetrance of 42±21% for AIP positive families with a minimum of 8% and a maximum of
12
13 83%. The penetrance in the AIP negative families was not significantly lower at 38±16%, minimum 19%
14
15 and maximum 67%. However, we observed a difference in the reported number of affected patients
16
17 between AIP mutation-positive and negative families, suggesting a difference in penetrance: the mean
18
19 number of affected subjects in AIP mutation-positive families was 3.2±1.8 while in AIP mutation-
20
21 negative families it was 2.2±0.4 ($P<0.001$). Interestingly, 16 of our 20 families with an AIP mutation had
22
23 at least one member with gigantism and/or disease onset <18yr, while only three of our 44 AIP-negative
24
25 FIPA families had a member with gigantism and/or disease onset <18yr. In the 20 AIP-positive families
26
27 seven of the 63 affected subjects (11%) were diagnosed above the age of 35yr, three out of these (5%)
28
29 were above 50yr. In the 44 AIP-negative families 56 of 96 patients (58%) were diagnosed above the age
30
31 of 35yr, 24 of these (25%) above the age of 50yr. Five of 63 AIP mutation patients (8%) presented with
32
33 pituitary apoplexy, three of them were in childhood. We studied whether the type of AIP mutation
34
35 (presence or absence of C-terminal end of the protein) would influence penetrance. We have 16 families
36
37 with mutations resulting in no protein or truncated proteins having 3.43±1.9 patients per family and four
38
39 families with full length proteins having 2.5±0.58 patients per family (no significant difference),
40
41 suggesting the lack of genotype-phenotype correlation in these 20 AIP positive families in terms of
42
43 penetrance. Out of the 64 families, 59 had at least one member with a prolactinoma or acromegaly, while
44
45 five families had pure non-functioning adenomas. Out of the 63 AIP mutation patients only 6 had 'pure'
46
47 prolactinoma and all of these were macroadenomas. The AIP mutation-positive patients who clinically
48
49 presented with a non-functioning adenoma all had positive GH and/or PRL staining in the adenoma
50
51 tissue, suggesting that all subjects with AIP mutations have GH or PRL-synthesizing adenomas in our
52
53 cohort.
54
55
56
57
58
59
60

***AIP* promoter mutation causes decreased promoter activity**

Two mutations (c.[-270_-269CG>AA;-220G>A] *in cis*) had been identified in a Japanese FIPA family with two sisters suffering from gigantism (Family VII Supp. Table S2, Figure 1A and B) (Leontiou et al., 2008). These changes are located 160 and 111bp upstream of the 5'UTR. The transcription factor binding-site searching programs suggested that the -270_-269CG>AA promoter mutation causes disruption of several transcription factor binding sites. To investigate whether the observed sequence changes affected the transcriptional activity of the *AIP* promoter, we cloned a 1.2 kb upstream fragment of the *AIP* gene into the pGL3-basic luciferase reporter vector (WT construct). To identify which of the mutations were functional, promoter constructs were prepared with a dibasic change (-270_-269AA), a single base change (-220A), and a construct with both changes (-270_-269AA & -220A) (Figure 1C). These were transiently transfected into GH3 cells and the activity of the promoter was measured using a luciferase assay.

Transfection of the WT promoter and the single -220A promoter construct resulted in an 18-fold increase of promoter activity compared to empty vector pGL3-basic (Figure 1D). Compared to the WT promoter construct the dibasic -270_-269AA promoter construct and the double -270_-269AA & -220A promoter construct had significantly decreased promoter activities ($P<0.001$). These results suggest that the dibasic -270_-269AA promoter mutation, but not the single -220A variant, reduces promoter activity which is likely to have a functional impact in the patients carrying this dibasic mutation and suggests that this area is important for the regulation of the *AIP* promoter.

Additional support for the reduced function of this *AIP* promoter was generated with real-time PCR study of the two affected subjects' leukocyte cDNA (heterozygote for the promoter mutation) compared to three healthy controls from the same ethnic background. This showed reduced *AIP* expression (*AIP*/*GAPDH* mRNA ratio, mean \pm SEM, 0.86 \pm 0.2 vs. 0.58 \pm 0.1, $P=0.05$), supporting the reduced activity of the *AIP* promoter in these cells.

***AIP* promoter is regulated via the cAMP-PKA signaling pathway**

The cyclic AMP pathway is important for somatotroph pathogenesis as its upregulation via somatic *gsp* mutations (MIM# 139320) or via *PRKARI* mutations lead to somatotropinomas (Horvath and Stratakis, 2008). cAMP stimulates AIP binding partner AhR (Oesch-Bartlomowicz et al., 2005). In addition, the extracellular signal-regulated kinase (ERK) pathway has recently been shown to be deregulated in pituitary adenomas (Dworakowska et al., 2009). No previous data are available about the regulation of the *AIP* promoter. We therefore treated GH3 cells transfected with the 1.2kb *AIP* promoter constructs with db-cAMP (a cAMP analog which activates PKA), forskolin (an adenylate cyclase activator that activates PKA) and PMA (a protein kinase C activator leading to activation of the ERK/MAPK pathway) and measured luciferase activity. Treatment with db-cAMP and forskolin increased WT and -220A promoter activity compared to vehicle (Figure 1E). The effect of forskolin on the WT promoter was inhibited by the PKA inhibitor H89 (Figure 1F). However, db-cAMP and forskolin treatment did not have any effect on the dibasic -270_-269AA mutated promoter or the double -270_-269AA & -220A mutated promoter. Treatment with PMA did not alter *AIP* promoter activity in any of the four promoter constructs. These results implicate the involvement of the cAMP-PKA signaling pathway in regulating *AIP* expression, and suggest that the -270-269 region is required for cAMP-induced *AIP* promoter activity.

Splice mutation c.249G>T causes truncated AIP

A novel mutation (c.249G>T) was identified in a family (Family 28 Supp. Table S1) with two childhood-onset pituitary adenomas (a somatotrophinoma and a prolactinoma) and an adult-onset prolactinoma (Figure 2A). The mutation at the end of exon 2 did not change the amino-acid sequence, but *in silico* analysis using ALAMUT predicted that this mutation created a novel 5'splice-site 32 bases upstream from the normal splice-site resulting in a frameshift and a stop-codon after 35 novel amino-acids (Figure 2B). In order to determine whether this mutation indeed causes alternative splicing, we extracted RNA from

1
2
3 blood of one of the patients. RT-PCR with primers upstream and downstream of the mutation was
4 performed and compared to cDNA from a control individual. RT-PCR of cDNA from the patient
5 heterozygous for the mutation generated the expected WT product plus an additional band that
6 corresponded to the size of the predicted alternative product (425bp, Figure 2C). Sequencing of this
7 smaller product confirmed the identity of the alternatively-spliced transcript. These results confirmed the
8 *in silico* prediction that the nucleotide change c.249G>T causes alternative splicing leading to a
9 frameshift and a truncated AIP protein (p.G83AfsX15).
10
11
12
13
14
15
16
17
18
19
20

21 **The synonymous c.807C>T mutation results in reduced mRNA expression**

22
23 The mutation c.807C>T, p.= at the beginning of exon 6 had been identified in a family with two
24 somatotrophinomas (Family XVI Supp. Table S2) (Leontiou et al., 2008). *In silico* analysis (ESEfinder
25 and RESCUE-ESE) suggested the c.807C>T mutation might result in the loss of a binding site for splice
26 enhancers SRp40 and SRp55 (splicing regulatory proteins that recognize exonic splicing enhancer
27 sequences) in the final exon of AIP (Figure 3A). We explored whether this mutation affected the splicing
28 of the last AIP exon. cDNA obtained from affected or carrier individuals was amplified with different
29 primer sets covering the various sections of the exon 5 3'UTR region and compared to the WT gene. PCR
30 amplification with the different primer sets did not show any novel alternatively-spliced transcripts, but
31 instead patients with the mutation showed decreased AIP mRNA expression compared to control
32 individuals using both conventional (Figure 3B) and real-time PCR (Figure 3C). Thus, these data suggests
33 that the c.807C>T mutation may have a functional impact by reducing AIP transcript levels.
34
35
36
37
38
39
40
41
42
43
44
45
46

47 To further investigate whether the c.807C>T mutation leads to decreased mRNA expression, we
48 performed *in vitro* studies using a minigene construct. The minigene was constructed with an amplified
49 DNA fragment from a patient carrying the mutation. A WT (807C) and a mutated (807T) minigene
50 including the 3' end of exon 5, the intron between exon 5-6 and the 5'-end of exon 6 were cloned into the
51 pcDNA3.1(+) expression vector (Figure 3D). The WT minigene and the mutated minigene were then
52
53
54
55
56
57
58
59
60

1
2
3 transiently transfected into GH3 cells for 48h. To mimic the patient's heterozygous mutation, cells were
4
5 co-transfected with equal amounts of the WT and mutated minigenes. Using conventional (Figure 3E) and
6
7 real-time PCR (Figure 3F), we detected decreased transcriptional products of the minigene in the cells
8
9 transfected with the mutated minigene (807T) and an intermediate amount with the co-transfection of WT
10
11 and mutated minigenes compared to cells with the WT minigene construct. These data corroborate the *in*
12
13 *vivo* data showing that the c.807C>T variant is associated with reduced levels of *AIP* mRNA, supporting
14
15 the functional impact of the mutation.
16
17

20 **Does the c.896C>T, p.A299V variant have a functional impact?**

21
22 In Family 10, two *AIP* changes were identified in two asymptomatic male carriers (70yr and 65yr): the
23
24 c.910C>T, p.R304X (a known pathogenic mutation (Leontiou et al., 2008)) and the c.896C>T, p.A299V
25
26 (previously reported in a patient with sporadic acromegaly (Georgitsi et al., 2007), but in none of the
27
28 general population subjects studied by us or others) variants. Other family members showed only the
29
30 c.910C>T or the c.896C>T change. We cloned this region of the *AIP* gene from the gDNA of a subject
31
32 carrying both mutations and sequenced several colonies. Some of the colonies carried the c.910C>T
33
34 change while others the c.896C>T change. These data confirm that the two sequence changes reside on
35
36 different chromosomes (*in trans*), and therefore the asymptomatic subjects are compound heterozygotes
37
38 for these two *AIP* changes. Interestingly, one of the female carriers of the p.A299V variant, but not the
39
40 p.R304X mutation, was diagnosed with a microprolactinoma at the age 30yr. Out of our seven 'pure'
41
42 prolactinoma patients found in our *AIP* mutation positive families she is the only one with a
43
44 microprolactinoma and most of the others needed surgery and radiotherapy. We consider that her case
45
46 might be a phenocopy, similar to the phenocopies reported in the Finnish Q14X mutation family
47
48 (Vierimaa et al., 2006), however, as she carries the p.A299V *AIP* variant this is uncertain. We performed
49
50 a functional assay to study the interaction of *AIP* and PDE4A5. The p.A299V variant of *AIP* did not show
51
52 a profoundly reduced binding to PDE4A5 compared to WT controls (Figure 4A). In summary, the clinical
53
54
55
56
57
58
59
60

1
2
3 data on the compound heterozygote subjects support that the c.896C>T, p.A299V change might be a rare
4 polymorphism and the *in vitro* data is compatible with this.
5
6
7
8

9 10 **Analysis of missense AIP mutations**

11 We decided to study the protein-protein interaction properties of all missense mutations reported in the
12 literature. We have previously shown with a filter β -galactosidase assay that the R81X, R217X and
13 R304X nonsense mutations disrupt the binding between AIP and PDE4A5. Here we used a quantitative β -
14 galactosidase assay to study all nine published missense mutations. Figure 4A shows that the K103R,
15 C238Y, K241E and R271W mutations completely disrupt the binding with values 5-fold less than WT,
16 similar to that of the novel Q164X nonsense mutation ($P<0.01$ for each comparison). The V49M change
17 showed no effect (mean \pm SD, 97 \pm 1% of WT) while the R16H, I257V, A299V and R304Q variants
18 showed a small drop in binding compared to WT (39 \pm 8%, 35 \pm 25%, 58 \pm 13% and 76 \pm 17% of WT,
19 respectively).
20
21
22
23
24
25
26
27
28
29
30

31 We used a combination of clinical, experimental and *in silico* methods to analyse how AIP
32 mutations would change the AIP protein structure and function. Out of the known 49 AIP variants 35
33 result in a truncated or no protein (12 nonsense mutations, 12 frameshifts, six splice-site, four large
34 deletions and one promoter mutation) (Tahir et al., 2010). Previous studies convincingly showed that loss
35 of the C-terminal end of the protein results in loss of function (Petruilis and Perdew, 2002; Leontiou et al.,
36 2008). The in-frame insertion mutation of exon 6 has been shown to disrupt the anti-proliferative effect of
37 AIP (Leontiou et al., 2008). No functional data are available for the four in-frame deletions. The nine
38 missense changes result in full-length proteins (R16H, V49M, K103R, C238Y, K241E, I257V, R271W,
39 A299V and R304Q). The location of these missense mutations are shown on a model of AIP which was
40 based on the structure of FKBP52 (Figure 4B).
41
42
43
44
45
46
47
48
49
50
51
52

53 R16H has been identified in control subjects as well as sporadic and familial patients and no LOH
54 was shown in a tumor sample (Daly et al., 2007; Georgitsi et al., 2007; Cazabat et al., 2007; Buchbinder
55 et al., 2008; Yaneva et al., 2008). A reduction in PDE4A5 binding was observed, but this was less than 3-
56
57
58
59
60

1
2
3 fold difference from WT. It is important to note that the PDE4A5-AIP interaction is at the C-terminal end
4 of the molecule (Bolger et al., 2003). Although the R16 amino-acid is conserved in vertebrates, the R16H
5 change probably represents a rare polymorphism as previously suggested (Raitila et al., 2007).
6
7
8

9
10 The V49M change was found in a sporadic childhood-onset somatotroph adenoma case but no
11 LOH was found in the tumor sample (Iwata et al., 2007). The PDE4A5 binding data was similar to WT,
12 as expected, as PDE4A5 binds the C terminal of the AIP molecule while this variant affects the N
13 terminal of the protein. This change is conserved in most vertebrates, but not in lower species. While no
14 further data are available the clinical significance of this variant is uncertain.
15
16
17
18
19

20
21 The K103R variant was identified in a patient with a childhood-onset corticotroph adenoma
22 (Beckers et al., 2008). Although the K>R is conservative substitution, the K103 residue is a conserved
23 amino-acid in most species and the PDE binding assay suggests a profoundly reduced activity ($8\pm 5\%$ of
24 WT) supporting the possibility that this mutation has a functional impact.
25
26
27
28

29
30 The C238Y mutation was found in a Mexican family with three acromegalic brothers (Leontiou
31 et al., 2008). It disrupts a consensus TPR motif amino-acid. To understand the relevance of this amino-
32 acid change we need to examine the structure of the TPR domains. AIP has three TPR motifs (Figure 4B
33 and 4C). Each TPR motif is composed of a pair of antiparallel-helices, termed helices A and B. The
34 consecutive helices of TPR domains are packaged together so each helix shares two immediate helix
35 neighbors (Das et al., 1998). A typical TPR consensus sequence pattern of small and large hydrophobic
36 residues has been defined (Sikorski et al., 1990; D'Andrea and Regan, 2003). Consensus amino-acids
37 (TPR residue positions marked as numbered diamonds on Figure 4C) are located at position 4, 7, 8 and 11
38 in helix A and position 20, 24, 27 and 32 in helix B (Sikorski et al., 1990; D'Andrea and Regan, 2003).
39 Small hydrophobic residues are commonly observed at positions 8, 20 and 27 within the TPR motif.
40 Residues 8 and 20 are located at the position of closest contact between the A and B helices of a TPR,
41 whereas residue 27 on helix B is located at the interface of three helices (A, B and the A helix of the next
42 TPR motif) within a 3-helix bundle (Das et al., 1998). The C238Y mutation is at position 8 of the A helix
43 of the 2nd TPR domain of AIP and its mutation could destabilize the A and B helix packaging of the 2nd
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 TPR domain (Figure 4C) (D'Andrea and Regan, 2003; Hidalgo-de-Quintana et al., 2008). Both the cell
4 proliferation (Leontiou et al., 2008) and the PDE binding assays show that this mutant loses its activity in
5 these two different types of functional assays. Therefore all the data strongly suggest that this mutation
6 has a functional impact.
7
8
9

10
11 The K241E mutation was identified in a FIPA family (Daly et al., 2007). Interestingly the same
12 amino-acid can also be affected by a stop mutation (Beckers et al., 2008). This large conserved residue is
13 part of the TPR sequence consensus at position 11 (Figure 4C) and is suggested to be required for the
14 packing of adjacent TPR α -helices (D'Andrea and Regan, 2003; Hidalgo-de-Quintana et al., 2008). Our
15 experimental data on protein interaction show a reduced ability to interact with PDE4A5 supporting the
16 possible functional impact of the variant.
17
18
19
20
21
22
23

24
25 The I257V change affects a conserved small residue in most vertebrates in the B helix of the 2nd
26 TPR domain at position 27, which is thought to be important in the packaging of the adjacent helices (Das
27 et al., 1998), in this case A and B helices of TPR2 and A helix of TPR3 (Figure 4C). It was found in a
28 sporadic patient with unusual cell type (TSHoma) (Montanana et al., 2009). A reduction in PDE4A5
29 binding was observed, but this was less than 3-fold difference from WT. LOH data are not available
30 therefore the possibility that it has a functional impact cannot be excluded.
31
32
33
34
35
36
37

38 The R271W is the second most common mutational hotspot in the AIP gene. This mutation has
39 been identified in three independent families (Daly et al., 2007; Jennings et al., 2009) as well as in a
40 sporadic giant (Igreja et al., 2010). It disrupts a conserved amino-acid in the 3rd TPR, it corresponds to an
41 arginine in several other TPR proteins at the equivalent location such as in protein phosphatase 5 (PP5A),
42 AIP-L1, FKBP51 and FKBP52 proteins (Figure 4C). Interestingly this amino-acid was selected for
43 mutation to alanine experimentally even before human mutations were identified in this gene (Bolger et
44 al., 2003; Laenger et al., 2009). Both the human mutation R>W (Figure 4A) and the experimental
45 mutation of R>A (Bolger et al., 2003) completely disrupts the AIP-PDE binding. These experimental and
46 clinical data strongly support the pathogenic role of this mutation.
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 The A299V change was described in a sporadic acromegaly patient (Georgitsi et al., 2007) and in
4 the current manuscript (see above). This variant, which is conserved in most vertebrates, is located at the
5 beginning of the α -7 helix in the area which is relevant for PDE4A5 interaction but binding *in vitro* was
6 less than 3-fold difference from WT. We identified this change in four subjects of a family where the
7 R304X mutation was also detected. Two unaffected patients carried both changes, one unaffected patient
8 carried only the A299V change while one young female carried only the A299V change and was
9 diagnosed with a microprolactinoma at the age of 30yr. As AIP knockout mice are not viable (Lin et al.,
10 2007), and the subjects with the compound heterozygote genotype with the certainly pathogenic R304X
11 mutation are unaffected, this variant is unlikely to have a functional impact, although the patient with a
12 possible phenocopy (small prolactinoma in a young female is not typical of AIP mutation patients)
13 carrying this change makes the status of this variant uncertain.
14
15
16
17
18
19
20
21
22
23
24
25
26

27 The R304Q locus is part of a CpG island mutational hotspot. This amino-acid change is relatively
28 conservative changing a longer side-chain positively charged amino-acid to a slightly shorter, uncharged,
29 hydrophilic residue in the α -7 helix. The amino-acid is conserved in mammals, but not in lower species.
30 Our protein interaction data do not show a reduced ability to bind with PDE4A5. The pathogenic role of
31 R304Q, however, is overwhelmingly supported by the fact that it has been identified in several
32 independent FIPA families as well as in sporadic patients (Georgitsi et al., 2007; Cazabat et al., 2007;
33 Leontiou et al., 2008; Vargiolu et al., 2009) (and the current study).
34
35
36
37
38
39
40
41

42 In summary, our two-hybrid analysis of the missense mutations showed that they fell into two
43 groups: mutations that were generally considered to have a functional impact had β -galactosidase activity
44 values more than 5-fold different from WT, while the mutants not generally considered to have a
45 functional impact had β -galactosidase values less than 3-fold different from WT. Of note, none of the β -
46 galactosidase values for the missense mutants fell between these two values, providing a clear distinction
47 between functionally-significant and functionally non-significant mutants in this PDE4A5-related assay.
48
49
50
51
52
53
54
55
56
57
58
59
60

Discussion

We have analyzed clinical data and performed genetic and functional studies to investigate the possible functional impact of some of the reported *AIP* sequence changes. The main findings of this study are: **1)** all our *AIP*-positive families have at least one member with GH/PRL-staining adenomas, have significantly higher number of subjects affected suggesting higher penetrance than in *AIP*-negative FIPA families, and have at least one childhood-onset case in the majority of the families. **2)** Six novel *AIP* germline and five previously described mutations were identified using sequencing and MLPA and our data support the suggestion that in addition to exon and exon-intron junction sequencing, study of the promoter area and a technique suitable for the detection of large deletions should be part of the DNA analysis of patients with FIPA. **3)** Our data suggest a functional impact for the dibasic promoter mutation we have identified, as it causes decreased promoter activity compared to the WT promoter. In addition, we reveal that the cAMP-PKA signaling-pathway positively regulates the activity of the *AIP* promoter. **4)** The exonic synonymous mutations studied were shown to have a functional impact due to abnormal splicing or decreased *AIP* mRNA expression. **5)** We combined clinical, *in silico* prediction and protein-protein binding assay data to characterize all the missense *AIP* changes identified in pituitary adenoma patients to help define those that may represent rare single nucleotide polymorphisms, and which may have a role in the disease process.

Previously described mutations affecting exon-intron junctions were hypothesized to cause abnormal *AIP* protein based on *in silico* predictions. Here we show actual data from patient cDNA samples regarding the consequences of the c.249G>T mutation confirming the possible functional impact of this change. Studying another silent mutation (c.807C>T), we predicted the abnormal splicing of exon 6 due to the disrupted binding of the splicing regulatory proteins. As this abnormality affects the last exon, studying its role is not as straight forward as other splicing mutations at earlier exons. Although our search for alternative splice-products using various sets of oligonucleotides did not reveal an alternative

1
2
3 splice-product, a minigene approach confirmed the lack of a normal exon 5-6 product and patient cDNA
4
5 samples showed reduced AIP mRNA expression. We hypothesize that this mutation may either cause
6
7 destabilization of the mRNA leading to reduced expression levels, or alternative splicing to a new isoform
8
9 not detected by our RT-PCR assays.
10

11
12
13
14 *AIP* promoter regulation has not previously been studied. We suggest that the c.-270_-269 area is
15
16 part of the basic promoter as its disruption significantly reduces basal promoter activity *in vitro*. Based on
17
18 prediction programs, the mutation at -270_-269 position would disrupt several transcription factor binding
19
20 sites, including the zinc-finger protein *SPI*. *SPI* can be activated by the cAMP-PKA pathway (Rohlf et
21
22 al., 1997), which is important in proliferation and hormone release of somatotroph cells: gain-of-function
23
24 mutations in the α -subunit of the G-protein (*GNAS*) and loss-of-function mutations of the regulatory-
25
26 subunit of PKA (*PRKARIA*) lead to deregulation of the cAMP-PKA pathway and play a role in
27
28 somatotroph tumorigenesis. Here we show that the activity of the *AIP* promoter can be up-regulated by
29
30 the stimulation of this pathway and the effect is inhibited by a PKA inhibitor. This first appears to be a
31
32 paradox, as sporadic somatotroph adenomas show an *overactive* PKA-pathway (Bertherat et al., 1995).
33
34 However, our data are compatible with increased *AIP* mRNA and protein expression described previously
35
36 (Leontiou et al., 2008; Jaffrain-Rea et al., 2009) and corresponds to the fact that low AIP levels do not
37
38 play a role in sporadic somatotroph tumorigenesis when *AIP* mutations are not present (Leontiou et al.,
39
40 2008).
41
42
43
44
45

46
47 PDEs can also regulate the PKA-pathway and PDE4A5 and PDE2A are known partners of AIP
48
49 (Bolger et al., 2003; de Oliveira et al., 2007). The AIP-PDE4A5 interaction was shown to be lost in the
50
51 presence of *AIP* mutations (Leontiou et al., 2008). Here we further studied all the described missense *AIP*
52
53 changes as well as our novel nonsense mutation with a quantitative assay. We found that a stop mutation
54
55 and four missense mutations (K103R, C238Y, K241E and R271W) completely disrupted the PDE4A5
56
57 binding. Three of these affected amino-acids are known to be important for TPR structure (Das et al.,
58
59
60

1
2
3 1998), and two have been experimentally shown to disrupt the function of the AIP protein (Bolger et al.,
4
5 2003; Leontiou et al., 2008; Laenger et al., 2009). Clinical data suggest, although do not prove, that the
6
7 R16H (found in normals as well), V49M (lack of LOH in tumor sample) and the A229V (also identified
8
9 in unaffected subjects with compound heterozygosity for the pathogenic R304X mutation) changes might
10
11 not have a functional impact and this is supported by the weak or no effect on PDE4A5 binding. The
12
13 I257V change affects a conserved amino-acid at a location known to be important for the α -helix
14
15 packaging of the TPR domains and despite the weak effect on PDE4A5 binding a possible functional
16
17 impact is possible. The pathogenic role of the R304Q variant is beyond doubt. The lack of profound
18
19 reduction in PDE4A5 binding suggests that this part of the molecule may not be important in this
20
21 PDE4A5-AIP interaction. On the other hand, missense variants can often act by altering the stability or
22
23 folding the protein, rather than affecting amino-acids directly involved in a protein-protein interaction.
24
25 Clearly, the normal binding to PDE4A5 does not rule out other types of dysfunction of these variant AIP
26
27 molecules, and further studies are needed to clarify whether the reduced or lack of AIP-PDE4A5
28
29 interaction as measured in this *in vitro* assay is important for the pituitary pathogenic process.
30
31
32
33
34
35

36 We identified two asymptomatic carrier subjects with two changes in the *AIP* gene: p.R304X &
37
38 p.A299V and showed that these variants are located on opposite parental chromosomes. It has previously
39
40 been demonstrated that the p.R304X hotspot mutation disrupts the function of the AIP molecule. The
41
42 p.A299V change (first described in a sporadic patient with acromegaly (Georgitsi et al., 2007)) involves
43
44 an amino-acid substitution of alanine to valine. This residue is not part of the TPR-domain consensus.
45
46 The PDE4A5 binding data did not support a profound loss of function due to this missense mutation. Also
47
48 if this change has functional impact, our subjects (65&70yr, both asymptomatic) would be compound-
49
50 heterozygotes for *AIP* mutations, while *AIP* knockout mice are not viable (they die at embryonic age
51
52 e10.5-e14.5 due to cardiac malformations (Lin et al., 2007)) thus suggesting this change has no functional
53
54 impact. We, however, identified a p.A299V female carrier with a microprolactinoma. Combining all these
55
56 data with the fact that her phenotype is not typical of AIP mutation positive patients, her case may
57
58
59
60

1
2
3 represent a phenocopy. The A299V change is a variant with unknown significance, and further studies are
4
5 needed to confirm that it has no functional impact.
6
7
8

9
10 More than two-third of FIPA families do not have a detectable mutation in the *AIP* gene, and
11
12 show differences in age of onset and level of penetrance as well as heterogeneity of tumor types
13
14 compared to AIP positive patients, suggesting that there is a phenotypic difference between FIPA families
15
16 with AIP mutations and FIPA families where the disease is caused by another gene or genes. Currently,
17
18 work is being carried out to identify these new disease loci.
19
20

21
22 In summary, 31% of our FIPA families harbor *AIP* mutations. They show a predilection for
23
24 GH/PRL-synthesizing adenomas, younger age of onset and higher penetrance of disease compared to
25
26 *AIP*-negative FIPA families. The *AIP* promoter is positively regulated by the cAMP-PKA pathway and
27
28 functional characterization of *AIP* mutations reveal that our promoter mutation decreases promoter
29
30 activity, while some synonymous changes result in abnormal splicing or reduced AIP expression.
31
32 Searching for large deletions using MLPA and analysis of clinical data and functional characterization of
33
34 rare *AIP* changes helps to clarify the pathogenic role of AIP in patients with familial and sporadic
35
36 pituitary adenomas, and assists in the appropriate genetic counseling and clinical management of these
37
38 patients and their families.
39
40
41
42
43

44 **Acknowledgments**

45
46
47 We are very grateful for the patients and family members who provided samples. We thank Profs
48
49 David Barford and Carol Shoulders as well as Drs Chrysanthia Leontiou and Lou Metherell for the
50
51 helpful comments. HSC was supported by an MRC Training Fellowship. Projects in Prof Korbonits
52
53 laboratory are supported by the Cancer Research Committee of St. Bartholomew's Hospital, by the
54
55
56
57
58
59
60

1
2
3 Wellcome Trust and by the NIHR. Studies in Dr. Bolger's laboratory are supported by the Bolger Prostate
4
5 Cancer Research Fund.
6
7
8

9 10 **Appendix**

11 List of International FIPA Consortium members

12
13 Akker, Scott, London, UK; Atkinson, Brew, Belfast, Northern Ireland; Aylwin, Simon, London, UK;
14
15 Baldeweg, Stephanie, London, UK; Bevan, John, Aberdeen, UK; Cheetham, Tim, Newcastle, UK; Chew,
16
17 Shern, London, UK; Choudry, Kiran, Texas, USA; Clayton, Richard, Stoke-On-Trent, UK; Damjanovic,
18
19 Svetozar, Belgrade, Serbia; Darzy, Ken, Milton Keynes, UK; Dattani, Mehul, London, UK; Davis, Julian,
20
21 Manchester, UK; Drake, Will, London, UK; Dzeranova, Larisa, Moscow, Russia; Edén, Britt, Engström,
22
23 Sweden; Eguchi, Kuniki, Hiroshima, Japan; Fica, Simona, Bucharest, Romania; Flanagan, Daniel,
24
25 Plymouth, UK; Frohman, Lawrence, Chicago, USA; Gadelha, Monica, Rio de Janeiro, Brazil; Gallego,
26
27 Patricia, Brisbane, Australia; Gláz, Edit, Budapest, Hungary; Goldman, James, Boston, USA; Goldstone,
28
29 Tony, London, UK; Howlett, Trevor, Leicester, UK; Inder, Warrick, Melbourne, Australia; Iwata, Takeo,
30
31 Tokushima, Japan; Kaplan, Felicity, Stevenage, UK; Karavitaki, Niki, Oxford, UK; Laws, Ed, Boston,
32
33 USA; Lechan, Ron, Boston, USA; Levy, Miles, Leicester, UK; Matsuno, Akira, Chiba, Japan; Miljic,
34
35 Dragana, Belgrade, Serbia; Modenesi, Silvia, Belo Horizonte, Brazil; Molitch, Mark, Chicago, USA
36
37 Musat, Mádâlina, Bucharest, Romania; Orme, Steve, Leeds, UK; Patócs, Attila, Budapest, Hungary;
38
39 Popovic, Vera, Belgrade, Serbia; Powell, Michael, London, UK; Quinton, Richard, Newcastle, UK;
40
41 Randeve, Harpal, Warwick, UK; Ribeiro de Oliveira JR, Antônio, Belo Horizonte, Brazil; Schöfl,
42
43 Christof, Erlangen, Germany; Soares, Beatriz, Belo Horizonte, Brazil; Spada, Anna, Milan, Italy
44
45 Strasburger, Christian, Berlin, Germany; Swords, Francesca, Norwich, UK; Tsagarakis, Stylianos,
46
47 Athens, Greece; Vaks, Vladimir, Oxford, UK; Wass, John A.H., Oxford, UK; Widell, Hakan, Gotheborg,
48
49 Sweden; Yarman, Sema, Istanbul, Turkey; Yoshimoto, Katsuhiko, Tokushima, Japan.
50
51
52
53
54
55
56
57
58
59
60

References

- 1
2
3
4
5
6
7
8 Beckers A, Daly AF. 2007. The clinical, pathological, and genetic features of familial isolated
9
10 pituitary adenomas. *Eur J Endocrinol* 157:371-382.
11
12
13
14 Beckers A, Vanbellinhen JF, Boikos S, Martari M, Verma S, Daly AF, Raygada M, Keil M,
15
16 Papademetriou J, Drori-Herishanu L, Horvath A, Nesterova M, Tichomirowa MA, Bours
17
18 V, Marx S, Agarwal SK, Salvatori R, Stratakis CA. 2008. Germline *AIP*, *MEN1*,
19
20 *PRKARIA*, *CDKN1B* (*p27^{Kip1}*) and *CDKN2C* (*p18^{INK4c}*) gene mutations in a large cohort
21
22 of pediatric patients with pituitary adenomas occurring in isolation or with associated
23
24 syndromic features. *Proc of the 90th Annual Meet of the Endocrine Soc:OR38-1*.
25
26
27
28
29 Bertherat J, Chanson P, Montminy M. 1995. The cyclic adenosine 3',5'-monophosphate-
30
31 responsive factor CREB is constitutively activated in human somatotroph adenomas. *Mol*
32
33 *Endocrinol* 9:777-783.
34
35
36
37 Bolger GB, Peden AH, Steele MR, MacKenzie C, McEwan DG, Wallace DA, Huston E, Baillie
38
39 GS, Houslay MD. 2003. Attenuation of the activity of the cAMP-specific
40
41 phosphodiesterase PDE4A5 by interaction with the immunophilin XAP2. *J Biol Chem*
42
43 278:33351-33363.
44
45
46
47
48 Buchbinder S, Bierhaus A, Zorn M, Nawroth PP, Humpert P, Schilling T. 2008. Aryl
49
50 hydrocarbon receptor interacting protein gene (*AIP*) mutations are rare in patients with
51
52 hormone secreting or non-secreting pituitary adenomas. *Exp Clin Endocrinol Diabetes*
53
54 116:625-628.
55
56
57
58
59
60

1
2
3 Cazabat L, Libe R, Perlemoine K, Rene-Corail F, Burnichon N, Gimenez-Roqueplo AP,
4
5 Dupasquier-Fediaevsky L, Bertagna X, Clauser E, Chanson P, Bertherat J, Raffin-Sanson
6
7 ML. 2007. Germline inactivating mutations of the aryl hydrocarbon receptor-interacting
8
9 protein gene in a large cohort of sporadic acromegaly: mutations are found in a subset of
10
11 young patients with macroadenomas. *Eur J Endocrinol* 157:1-8.
12
13
14
15

16 Chahal HS, Chapple JP, Frohman LA, Grossman AB, Korbonits M. 2010. Clinical, genetic and
17
18 molecular characterisation of patients with familial isolated pituitary adenomas. *Trends*
19
20 *Endocrinol Metab*.
21
22
23

24 D'Andrea LD, Regan L. 2003. TPR proteins: the versatile helix. *Trends Biochem Sci* 28:655-
25
26 662.
27
28
29

30 Daly AF, Vanbellinhen JF, Khoo SK, Jaffrain-Rea ML, Naves LA, Guitelman MA, Murat A,
31
32 Emy P, Gimenez-Roqueplo AP, Tamburrano G, Raverot G, Barlier A, De Herder W,
33
34 Penfornis A, Ciccarelli E, Estour B, Lecomte P, Gatta B, Chabre O, Sabate MI, Bertagna
35
36 X, Garcia BN, Stalldecker G, Colao A, Ferolla P, Wemeau JL, Caron P, Sadoul JL,
37
38 Oneto A, Archambeaud F, Calender A, Sinilnikova O, Montanana CF, Cavagnini F, Hana
39
40 V, Solano A, Delettieres D, Luccio-Camelo DC, Basso A, Rohmer V, Brue T, Bours V,
41
42 Teh BT, Beckers A. 2007. Aryl hydrocarbon receptor-interacting protein gene mutations
43
44 in familial isolated pituitary adenomas: analysis in 73 families. *J Clin Endocrinol Metab*
45
46 92:1891-1896.
47
48
49
50

51
52 Das AK, Cohen PW, Barford D. 1998. The structure of the tetratricopeptide repeats of protein
53
54 phosphatase 5: implications for TPR-mediated protein-protein interactions. *EMBO J*
55
56 17:1192-1199.
57
58
59
60

- 1
2
3 de Oliveira SK, Hoffmeister M, Gambaryan S, Muller-Esterl W, Guimaraes JA, Smolenski AP.
4
5 2007. Phosphodiesterase 2A forms a complex with the co-chaperone XAP2 and regulates
6
7 nuclear translocation of the aryl hydrocarbon receptor. *J Biol Chem* 282:13656-13663.
8
9
10
11 Dworakowska D, Wlodek E, Leontiou C, Igreja S, Cakir M, Teng M, Prodromou N, Goth M,
12
13 Grozinsky-Glasberg S, Gueorguiev M, Kola B, Korbonits M, Grossman A. 2009.
14
15 Activation of Raf/MEK/ERK and PI3K/Akt/mTOR pathways in pituitary adenomas and
16
17 their effects on downstream effectors. *Endocr Relat Cancer* 16:1329-1338.
18
19
20
21 Farrell WE, Clayton RN. 2000. Molecular pathogenesis of pituitary tumors. *Front*
22
23 *Neuroendocrinol* 21:174-198.
24
25
26
27
28 Gadelha MR, Prezant TR, Une KN, Glick RP, Moskal SF, Vaisman M, Melmed S, Kineman RD,
29
30 Frohman LA. 1999. Loss of heterozygosity on chromosome 11q13 in two families with
31
32 acromegaly/gigantism is independent of mutations of the multiple endocrine neoplasia
33
34 type I gene. *J Clin Endocrinol Metab* 84:249-256.
35
36
37
38 Gadelha MR, Une KN, Rohde K, Vaisman M, Kineman RD, Frohman LA. 2000. Isolated
39
40 familial somatotropinomas: establishment of linkage to chromosome 11q13.1-11q13.3
41
42 and evidence for a potential second locus at chromosome 2p16-12. *J Clin Endocrinol*
43
44 *Metab* 85:707-714.
45
46
47
48
49 Georgitsi M, De ME, Cannavo S, Makinen MJ, Tuppurainen K, Pauletto P, Curto L, Weil RJ,
50
51 Paschke R, Zielinski G, Wasik A, Lubinski J, Vahteristo P, Karhu A, Aaltonen LA.
52
53 2008a. Aryl hydrocarbon receptor interacting protein (AIP) gene mutation analysis in
54
55
56
57
58
59
60

1
2
3 children and adolescents with sporadic pituitary adenomas. *Clin Endocrinol (Oxf)*
4
5 69:621-627.
6
7

8
9 Georgitsi M, Heliövaara E, Paschke R, Kumar AV, Tischkowitz M, Vierimaa O, Salmela P,
10
11 Sane T, De ME, Cannavo S, Gundogdu S, Lucassen A, Izatt L, Aylwin S, Bano G,
12
13 Hodgson S, Koch CA, Karhu A, Aaltonen LA. 2008b. Large genomic deletions of aryl
14
15 hydrocarbon receptor interacting protein (AIP) gene in pituitary adenoma predisposition.
16
17 *J Clin Endocrinol Metab* 93:4146-4151.
18
19

20
21
22 Georgitsi M, Raitila A, Karhu A, Tuppurainen K, Makinen MJ, Vierimaa O, Paschke R, Saeger
23
24 W, van der Luijt RB, Sane T, Robledo M, De ME, Weil RJ, Wasik A, Zielinski G,
25
26 Lucewicz O, Lubinski J, Launonen V, Vahteristo P, Aaltonen LA. 2007. Molecular
27
28 diagnosis of pituitary adenoma predisposition caused by aryl hydrocarbon receptor-
29
30 interacting protein gene mutations. *Proc Natl Acad Sci USA* 104:4101-4105.
31
32

33
34
35 Guarente L. 1983. Yeast promoters and lacZ fusions designed to study expression of cloned
36
37 genes in yeast. *Methods Enzymol* 101:181-191.
38
39

40
41 Hidalgo-de-Quintana J, Evans RJ, Cheetham ME, van der Spuy J. 2008. The Leber congenital
42
43 amaurosis protein AIPL1 functions as part of a chaperone heterocomplex. *Invest*
44
45 *Ophthalmol Vis Sci* 49:2878-2887.
46
47

48
49 Horvath A, Stratakis CA. 2008. Clinical and molecular genetics of acromegaly: MEN1, Carney
50
51 complex, McCune-Albright syndrome, familial acromegaly and genetic defects in
52
53 sporadic tumors. *Rev Endocr Metab Disord* 9:1-11.
54
55

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Igreja SC, Chahal HS, Leontiou CA, Bolger GB, Chapple JP, Grossman AB, Korbonits M. 2010. Functional characterisation of aryl hydrocarbon receptor interacting protein (AIP) promoter and silent mutations. *Endocr Abst* 21:P210.
- Iwata T, Yamada S, Mizusawa N, Golam HM, Sano T, Yoshimoto K. 2007. The aryl hydrocarbon receptor-interacting protein gene is rarely mutated in sporadic GH-secreting adenomas. *Clin Endocrinol (Oxf)* 66:499-502.
- Jaffrain-Rea ML, Angelini M, Gargano D, Tichomirowa MA, Daly AF, Vanbellinghen JF, D'Innocenzo E, Barlier A, Giangaspero F, Esposito V, Ventura L, Arcella A, Theodoropoulou M, Naves LA, Fajardo C, Zacharieva S, Rohmer V, Brue T, Gulino A, Cantore G, Alesse E, Beckers A. 2009. Expression of aryl hydrocarbon receptor (AHR) and AHR-interacting protein in pituitary adenomas: pathological and clinical implications. *Endocr Relat Cancer* 16:1029-1043.
- Jennings J, Georgitsi M, Holdaway I, Daly A, Tichomirowa M, Beckers A, Aaltonen L, Karhu A, Cameron F. 2009. Aggressive pituitary adenomas occurring in young patients in a large Polynesian kindred with a germline R271W mutation in the AIP gene. *Eur J Endocrinol* 161:799-804.
- Kelley LA, Sternberg MJ. 2009. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* 4:363-371.
- Laenger A, Lang-Rollin I, Kozany C, Zschocke J, Zimmermann N, Ruegg J, Holsboer F, Hausch F, Rein T. 2009. XAP2 inhibits glucocorticoid receptor activity in mammalian cells. *FEBS Lett* 583:1493-1498.

1
2
3 Leontiou CA, Gueorguiev M, van der SJ, Quinton R, Lolli F, Hassan S, Chahal HS, Igreja SC,
4
5 Jordan S, Rowe J, Stolbrink M, Christian HC, Wray J, Bishop-Bailey D, Berney DM,
6
7 Wass JA, Popovic V, Ribeiro-Oliveira A, Jr., Gadelha MR, Monson JP, Akker SA, Davis
8
9 JR, Clayton RN, Yoshimoto K, Iwata T, Matsuno A, Eguchi K, Musat M, Flanagan D,
10
11 Peters G, Bolger GB, Chapple JP, Frohman LA, Grossman AB, Korbonits M. 2008. The
12
13 role of the AIP gene in familial and sporadic pituitary adenomas. *J Clin Endocrinol*
14
15 *Metab* 93:2390-2401.
16
17

18
19
20
21 Lin BC, Sullivan R, Lee Y, Moran S, Glover E, Bradfield CA. 2007. Deletion of the aryl
22
23 hydrocarbon receptor-associated protein 9 leads to cardiac malformation and embryonic
24
25 lethality. *J Biol Chem* 282:35924-35932.
26
27

28
29 Matsuno A, Teramoto A, Yamada S, Kitanaka S, Tanaka T, Sanno N, Osamura RY, Kirino T.
30
31 1994. Gigantism in sibling unrelated to multiple endocrine neoplasia: case report.
32
33 *Neurosurgery* 35:952-955.
34
35

36
37 McCarthy MI, Noonan K, Wass JA, Monson JP. 1990. Familial acromegaly: studies in three
38
39 families. *Clin Endocrinol (Oxf)* 32:719-728.
40
41

42
43 Melmed S. 2006. Medical progress: Acromegaly. *N Eng J Med* 355:2558-2573.
44
45

46
47 Montanana CF, Daly AF, Tichomirowa MA, Vanbellinthen JF, Jaffrain-Rea ML, Trescoli
48
49 Serrano C, Riesgo Suares P, Gomez Vela J, Tenes S, Bours V, Beckers A. 2009. TSH-
50
51 secreting pituitary adenoma in a male patient with a novel missense AIP mutation. *Proc*
52
53 *of the 91st Annual Meet of the Endocrine Soc* P1-668.
54
55
56
57
58
59
60

- 1
2
3 Oesch-Bartlomowicz B, Huelster A, Wiss O, ntoniou-Lipfert P, Dietrich C, Arand M, Weiss C,
4
5 Bockamp E, Oesch F. 2005. Aryl hydrocarbon receptor activation by cAMP vs. dioxin:
6
7 divergent signaling pathways. *Proc Natl Acad Sci USA* 102:9218-9223.
8
9
10
11 Ogino S, Wilson RB, Gold B, Flodman P. 2007. Bayesian risk assessment in genetic testing for
12
13 autosomal dominant disorders with age-dependent penetrance. *J Genet Couns* 16:29-39.
14
15
16
17 Pestell RG, Alford FP, Best JD. 1989. Familial acromegaly. *Acta Endocrinol (Copenh)* 121:286-
18
19 289.
20
21
22
23 Petrulis JR, Perdew GH. 2002. The role of chaperone proteins in the aryl hydrocarbon receptor
24
25 core complex. *Chem Biol Interact* 141:25-40.
26
27
28
29 Raitila A, Georgitsi M, Karhu A, Tuppurainen K, Makinen MJ, Birkenkamp-Demtroder K,
30
31 Salmenkivi K, Orntoft TF, Arola J, Launonen V, Vahteristo P, Aaltonen LA. 2007. No
32
33 evidence of somatic aryl hydrocarbon receptor interacting protein mutations in sporadic
34
35 endocrine neoplasia. *Endocr Relat Cancer* 14:901-906.
36
37
38
39 Rohlff C, Ahmad S, Borellini F, Lei J, Glazer RI. 1997. Modulation of transcription factor Sp1
40
41 by cAMP-dependent protein kinase. *J Biol Chem* 272:21137-21141.
42
43
44
45 Sikorski RS, Boguski MS, Goebel M, Hieter P. 1990. A repeating amino acid motif in CDC23
46
47 defines a family of proteins and a new relationship among genes required for mitosis and
48
49 RNA synthesis. *Cell* 60:307-317.
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Soares BS, Eguchi K, Frohman LA. 2005. Tumor deletion mapping on chromosome 11q13 in
4
5 eight families with isolated familial somatotropinoma and in 15 sporadic
6
7 somatotropinomas. *J Clin Endocrinol Metab* 90:6580-6587.
8
9

10
11 Tahir A, Chahal HS, Korbonits M. 2010. Molecular genetics of the AIP gene in familial pituitary
12
13 tumorigenesis. *Progress in Brain Research*.
14
15

16
17 Toledo RA, Lourenco DM, Jr., Liberman B, Cunha-Neto MB, Cavalcanti MG, Moyses CB,
18
19 Toledo SP, Dahia PL. 2007. Germline mutation in the aryl hydrocarbon receptor
20
21 interacting protein gene in familial somatotropinoma. *J Clin Endocrinol Metab* 92:1934-
22
23 1937.
24
25

26
27 Vargiolu M, Fusco D, Kurelac I, Dirnberger D, Baumeister R, Morra I, Melcarne A, Rimondini
28
29 R, Romeo G, Bonora E. 2009. The tyrosine kinase receptor RET interacts in vivo with
30
31 aryl hydrocarbon receptor-interacting protein to alter survivin availability. *J Clin*
32
33 *Endocrinol Metab* 94:2571-2578.
34
35

36
37 Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, Raitila A, Tuppurainen K,
38
39 Ebeling TM, Salmela PI, Paschke R, Gundogdu S, De ME, Makinen MJ, Launonen V,
40
41 Karhu A, Aaltonen LA. 2006. Pituitary adenoma predisposition caused by germline
42
43 mutations in the AIP gene. *Science* 312:1228-1230.
44
45
46

47
48 Yamada S, Yoshimoto K, Sano T, Takada K, Itakura M, Usui M, Teramoto A. 1997. Inactivation
49
50 of the tumor suppressor gene on 11q13 in brothers with familial acrogigantism without
51
52 multiple endocrine neoplasia type 1. *J Clin Endocrinol Metab* 82:239-242.
53
54
55

1
2
3 Yaneva M, Daly AF, Tichomirowa MA, Vanbellinghen JF, Hagelstein MT, Bours V, Zacharieva
4
5 S, Beckers A. 2008. Aryl hydrocarbon receptor interacting protein gene mutations in
6
7 bulgarian FIPA and young sporadic pituitary adenoma patients. Proc of the 90th Annual
8
9 Meet of the Endocrine Soc:P3-520.
10
11
12
13
14

15 Figure Legends

16 17 18 19 **Figure 1.** *AIP* promoter mutations

20
21 **A)** Family tree (Family VI, Supp. Table S2), filled circles represent patients with gigantism with age of
22
23 onset shown, half filled symbols represents carrier subjects

24
25 **B)** Location of the two *AIP* promoter sequence changes. Numbers in boxes represent exon numbers.
26
27 Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation
28
29 initiation codon in the reference sequence
30
31

32
33 **C)** Schematic representation of the four *AIP* promoter constructs

34
35 **D)** The wild-type (WT) and the c.-220A single mutation showed similar promoter activity measured by
36
37 luciferase assay. The -270_-269AA dibasic mutant and the double -270_-269AA & -220A mutant
38
39 constructs showed decreased promoter activity (***, $P < 0.001$ vs. WT)

40
41 **E)** *AIP* promoter activity after treatment with db-cAMP, forskolin and PMA. The WT and the -220A
42
43 single mutant constructs showed increased promoter activity after treatment with db-cAMP and forskolin
44
45 compared to vehicle treatment (#, $P < 0.05$, ##, $P < 0.01$). Treatments did not affect the promoter activity of
46
47 the dibasic -270_-269AA and double -270_-269AA & -220A mutant constructs. Following db-cAMP and
48
49 forskolin there was a significantly lower promoter activity in the -270_-269AA and the reduced -270-
50
51 269AA & -220A mutant constructs compared to WT (***, $P < 0.001$, **, $P < 0.01$ vs. WT)

52
53 **F)** The PKA inhibitor H89 inhibits the stimulating effect of forskolin on luciferase activity of the WT-
54
55 promoter
56
57
58
59
60

1
2
3
4
5
6
7
8 **Figure 2.** Alternatively spliced AIP transcript in the presence of the 249G>T mutation

9
10 **A)** Family tree (Family 28, Supp. Table S1) showing patients with gigantism (filled square), prolactinoma
11 (striped squares) and carrier subjects (half filled symbols) with age of onset shown

12
13
14 **B)** Schematic representation of splicing in the WT and mutant gene showing the location of the mutation
15 (arrow) and the 32 bp lost from the end of exon 2 (shaded area) followed by a novel stop-codon after 15
16 novel codons (marked with *). Numbers in boxes represent exon numbers. Primers used are shown by
17 arrowheads
18
19
20
21

22
23 **C)** RT-PCR using a patient and control cDNA. Patient cDNA shows an extra band (425bp) that
24 corresponds to the alternatively spliced AIP transcript with the upper band showing the WT transcript
25 (457bp). The identity of these PCR products were confirmed by sequencing
26
27
28
29
30
31
32
33

34 **Figure 3.** Decreased *AIP* mRNA expression in the presence of the 807C>T

35
36 **A)** Family tree showing two patients with acromegaly and an asymptomatic carrier with age of onset of
37 disease (Family XVI, Supp. Table S2). The location of the mutation is shown by an arrow; numbers in
38 boxes represent exon numbers
39
40
41

42 **B)** Conventional RT-PCR with AIP primers on blood-derived cDNA obtained from three control
43 individuals (C1-3) and two subjects (M1&2) carrying the 807C>T AIP mutation. Patients carrying the
44 807C>T showed decreased *AIP* expression
45
46
47
48

49 **C)** Real-time PCR using an AIP primer and probe set to compare the *AIP* levels between control
50 individuals and patients; C (control)
51
52

53 **D)** Wild-type (WT) and mutant minigene constructs
54
55
56
57
58
59
60

1
2
3 **E)** Conventional RT-PCR using vector-specific primers for the minigene constructs showing decreased
4 mutated minigene expression (807T) and intermediate level of expression for the co-expression of WT
5 and mutant minigenes (807CT)
6
7
8

9
10 **F)** Real-time PCR showing an increased AIP expression in the WT minigene construct compared to
11 mutant and co-expression of WT and mutant minigenes. EV, empty vector
12
13
14
15

16 **Figure 4.** Single amino-acid substitutions in AIP
17

18 **A)** A yeast two-hybrid quantitative β -galactosidase assay was used to assess the interaction of AIP with
19 PDE4A5. It shows more than 5-fold difference (activity 0-20% of wild-type (WT)) from WT AIP for
20 missense mutations K103R, C238Y, K241E and R271W as well as for the positive control truncation
21 mutation Q164X. The R16H, V49M, I257V A299V and the R304Q variants show no difference or
22 activity 33-100% of wild-type (less than 3-fold difference) from WT AIP (mean \pm SD, n=3). β -
23 galactosidase activity was measured as described by Guarente (Guarente, 1983). Each mutation was
24 tested in at least two different yeast clones, with identical results (n=3 for each clone).
25
26
27
28
29
30
31
32

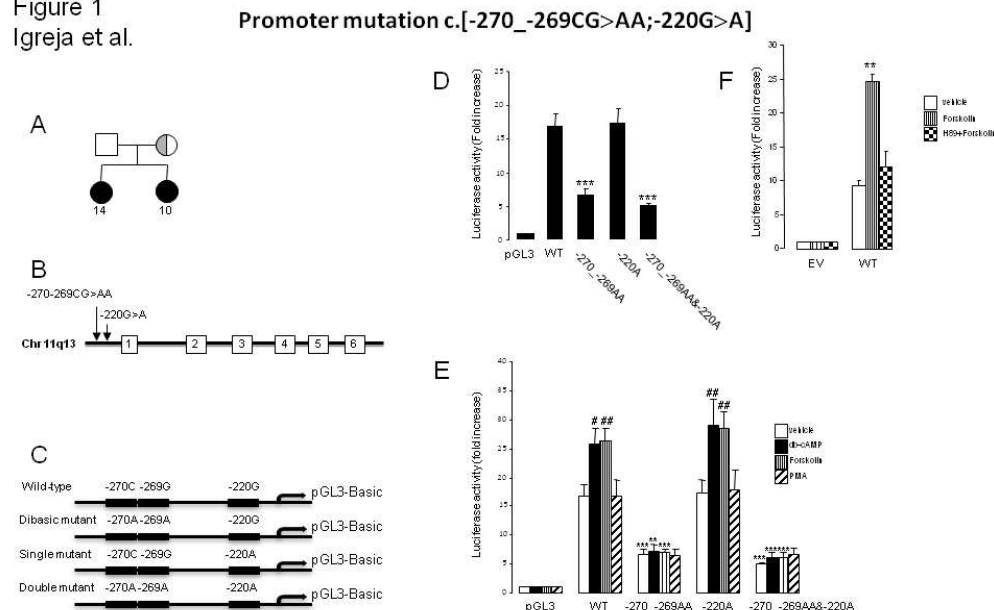
33 **B)** Hypothetical structure of AIP based on the structure of FKBP52 showing the three tetratricopeptide
34 (TPR) domains with three pairs of anti-parallel α -helices and the final extended α -helix, α -7 (courtesy of
35 Prof. David Barford, London, UK). Sequence comparison of human AIP and FKBBP52 is shown in
36 Supp. Figure S1. Amino-acids with reported missense variants are highlighted
37
38
39
40
41

42 **C)** The three TPR domains, each consisting of an A and B helix are shown of several Hsp90 binding
43 proteins including AIP (Table modified from (Hidalgo-de-Quintana et al., 2008)). Numbers in diamond
44 shapes show TPR motif position numbers. Amino-acids marked with blue bold letters are important for
45 the packaging and stability of the α -helices. Amino-acids at position 8 and 20 were shown to be important
46 in helix A and B packaging, while position 27 helps packaging of helix B with helix A of the same TPR
47 domain and helix A of the following TPR domain. Underlined amino-acids are predicted to be involved in
48 the peptide binding pocket of FKBP52. Full-length variants of AIP affecting the TPR domains are shown
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 on the figure (see review (Tahir et al., 2010)). Amino-acids circled with red in the AIP sequence show
4
5 missense variants described (C238Y, K241E, I257V, R271W). Amino-acids marked with orange bold
6
7 italics have been shown to be replaced by stop codon in FIPA patients (K201X, Q239X, K241X, Y268X).
8
9 The amino-acid marked by a red arrow is followed by an in-frame insertion (p.F269_H275dup) in a FIPA
10
11 family. In-frame deletion variants p.Y238del is shown by strikethrough and complex missense &
12
13 in-frame deletion mutation p.[E293G; L294_A297del] is shown by black circle and
14
15 in-frame deletion mutation p.[E293G; L294_A297del] is shown by black circle and
16
17 strikethrough.
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

Figure 1
Igreja et al.



- A) Family tree (Family VI, Supp. Table S2), filled circles represent patients with gigantism with age of onset shown, half filled symbols represents carrier subjects
- B) Location of the two AIP promoter sequence changes. Numbers in boxes represent exon numbers. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence
- C) Schematic representation of the four AIP promoter constructs
- D) The wild-type (WT) and the c.-220A single mutation showed similar promoter activity measured by luciferase assay. The -270_-269AA dibasic mutant and the double -270_-269AA & -220A mutant constructs showed decreased promoter activity (***, $P < 0.001$ vs. WT)
- E) AIP promoter activity after treatment with db-cAMP, forskolin and PMA. The WT and the -220A single mutant constructs showed increased promoter activity after treatment with db-cAMP and forskolin compared to vehicle treatment (#, $P < 0.05$, ##, $P < 0.01$). Treatments did not affect the promoter activity of the dibasic -270_-269AA and double -270_-269AA & -220A mutant constructs. Following db-cAMP and forskolin there was a significantly lower promoter activity in the -270_-269AA and the reduced -270_-269AA & -220A mutant constructs compared to WT (***, $P < 0.001$, **, $P < 0.01$ vs. WT)
- F) The PKA inhibitor H89 inhibits the stimulating effect of forskolin on luciferase activity of the WT-promoter

254x190mm (96 x 96 DPI)

Figure 2
Igreja et al.

Splice mutation c.249G>T, p.G83AfsX15

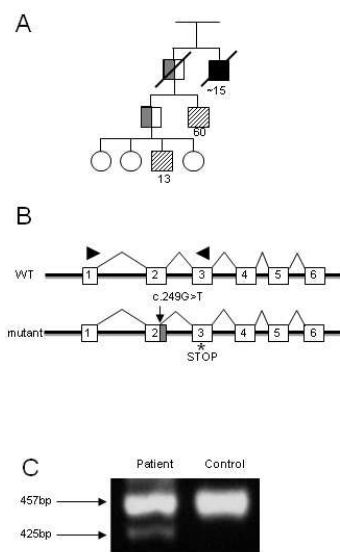


Figure 2. Alternately spliced AIP transcript in the presence of the 249G>T mutation
 A) Family tree (Family 28, Supp. Table S1) showing patients with gigantism (filled square), prolactinoma (striped squares) and carrier subjects (half filled symbols) with age of onset shown
 B) Schematic representation of splicing in the WT and mutant gene showing the location of the mutation (arrow) and the 32 bp lost from the end of exon 2 (shaded area) followed by a novel stop-codon after 15 novel codons (marked with *). Numbers in boxes represent exon numbers. Primers used are shown by arrowheads
 C) RT-PCR using a patient and control cDNA. Patient cDNA shows an extra band (425bp) that corresponds to the alternatively spliced AIP transcript with the upper band showing the WT transcript (457bp). The identity of these PCR products were confirmed by sequencing

254x190mm (96 x 96 DPI)

Figure 3
Igreja et al.

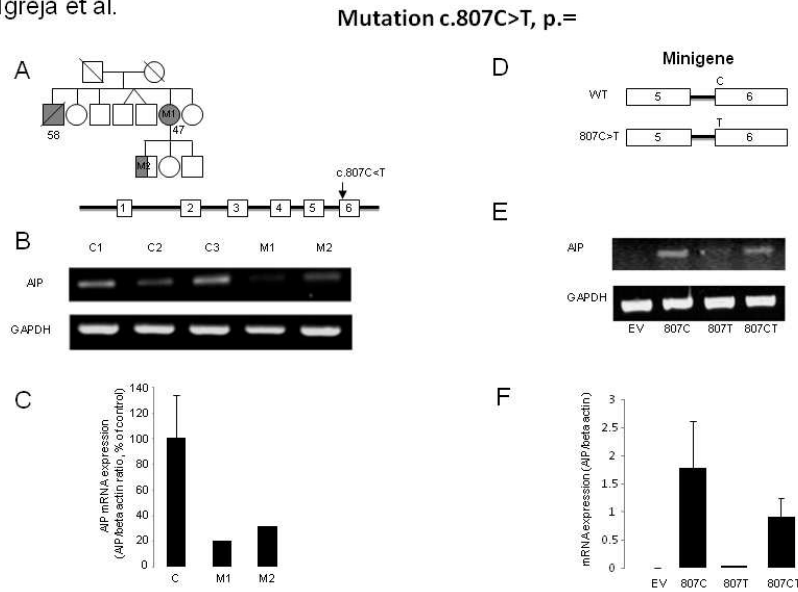


Figure 3. Decreased AIP mRNA expression in the presence of the 807C>T

- A) Family tree showing two patients with acromegaly and an asymptomatic carrier with age of onset of disease (Family XVI, Supp. Table S2). The location of the mutation is shown by an arrow; numbers in boxes represent exon numbers
- B) Conventional RT-PCR with AIP primers on blood-derived cDNA obtained from three control individuals (C1-3) and two subjects (M1&2) carrying the 807C>T AIP mutation. Patients carrying the 807C>T showed decreased AIP expression
- C) Real-time PCR using an AIP primer and probe set to compare the AIP levels between control individuals and patients; C (control)
- D) Wild-type (WT) and mutant minigene constructs
- E) Conventional RT-PCR using vector-specific primers for the minigene constructs showing decreased mutated minigene expression (807T) and intermediate level of expression for the co-expression of WT and mutant minigenes (807CT)
- F) Real-time PCR showing an increased AIP expression in the WT minigene construct compared to mutant and co-expression of WT and mutant minigenes. EV, empty vector

254x190mm (96 x 96 DPI)

Figure 4 Igreja et al.

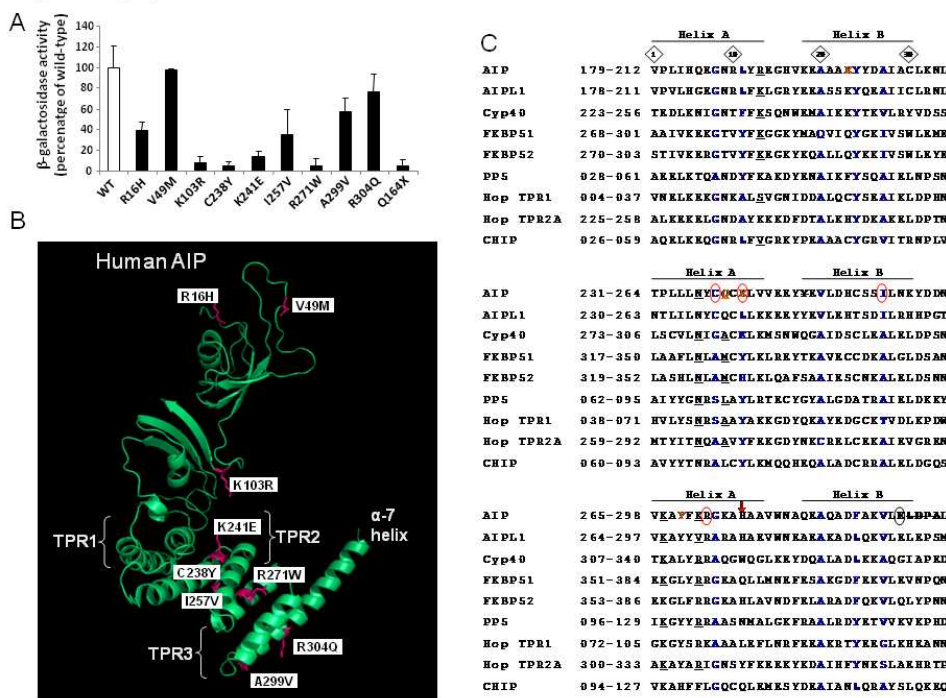


Figure 4. Single amino-acid substitutions in AIP

A) A yeast two-hybrid quantitative β -galactosidase assay was used to assess the interaction of AIP with PDE4A5. It shows more than 5-fold difference (activity 0-20% of wild-type (WT)) from WT AIP for missense mutations K103R, C238Y, K241E and R271W as well as for the positive control truncation mutation Q164X. The R16H, V49M, I257V A299V and the R304Q variants show no difference or activity 33-100% of wild-type (less than 3-fold difference) from WT AIP (mean \pm SD, n=3). β -galactosidase activity was measured as described by Guarente (Guarente, 1983). Each mutation was tested in at least two different yeast clones, with identical results (n=3 for each clone).

B) Hypothetical structure of AIP based on the structure of FKBP52 showing the three tetratricopeptide (TPR) domains with three pairs of anti-parallel α -helices and the final extended α -helix, α -7 (courtesy of Prof. David Barford, London, UK). Sequence comparison of human AIP and FKBP52 is shown in Supp. Figure S1. Amino-acids with reported missense variants are highlighted

C) The three TPR domains, each consisting of an A and B helix are shown of several Hsp90 binding proteins including AIP (Table modified from (Hidalgo-de-Quintana et al., 2008)). Numbers in diamond shapes show TPR motif position numbers. Amino-acids marked with blue bold letters are important for the packaging and stability of the α -helices. Amino-acids at position 8 and 20 were shown to be important in helix A and B packaging, while position 27 helps packaging of helix B with helix A of the same TPR domain and helix A of the following TPR domain. Underlined amino-acids are predicted to be involved in the peptide binding pocket of FKBP52. Full-length variants of AIP affecting the TPR domains are shown on the figure (see review (Tahir et al., 2010)). Amino-acids circled with red in the AIP sequence show missense variants described (C238Y, K241E, I257V, R271W). Amino-acids marked with orange bold italics have been shown to be replaced by stop codon in FIPA patients (K201X, Q239X, K241X, Y268X). The amino-acid marked by a red arrow is followed by an in-frame insertion (p.F269_H275dup) in a FIPA family. In-frame deletion variants p.Y238del is shown by strikethrough and complex missense & in-frame deletion mutation p.[E293G; L294_A297del] is shown by black circle and strikethrough

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

254x190mm (96 x 96 DPI)

For Peer Review

```

1
2
3
4
5 AIP|NP_003968.2| MADI IARLREDGIQKR-VIQEGRGELPDFQDGTKATFHVRTLHSDS---- 45
6 FKBP52|Q02790| MTAEEMKATESGAQSAPLPMEGVDISPKQDEGV LKVIKREGTGTEMPMIG 50
7 *: : *. * . : ** . * . : : * . . : : . : :
8
9 AIP|NP_003968.2| -----EGTVLDDSRARGKPMELIIGKKFKLPVWETIVCTMREG 83
10 FKBP52|Q02790| DRV FVHYTGWLLDGTGKFDSSLDRKDKFSDLGKGEVIKAWDIAIATMKVG 100
11 : ** : * . * . : : : ** : . * : : . * * : *
12
13 AIP|NP_003968.2| EIAQFLCD-----IKHV VLYPLVAKSLRNIAVG-- 111
14 FKBP52|Q02790| EVCHITCKPEYAYGSAGSPPKIPPNATLVFEVELFEFKGEDLTEEEDGGI 150
15 *: : : * . : . * * : : . : . * : *
16
17 AIP|NP_003968.2| -----KDPLEGQRHCCGVAQMREHSSL 133
18 FKBP52|Q02790| IRRIQTRGEGYAKPNEGAI VEVALEGGYKDKLFDQRELRFEIGEGENLDL 200
19 ** * . * . * : * . *
20
21 AIP|NP_003968.2| GH---ADLDALQQNPQPLIFHMEMLKVESPG----- 161
22 FKBP52|Q02790| PYGLERAIQRMEKGEHSIVYLKPSYAFG SVGKEKFQIPNAELKYELHLK 250
23 : : : : : : : : : . * *
24
25 AIP|NP_003968.2| --TYQDPWAMTDEEKAKAVPLIHQEGNRLYREGHVKEAAAKYYDAIACL 209
26 FKBP52|Q02790| SFEKAKESWEMNSEEKLEQSTIVKERGT VYFKEGKYQALLQYKKIVSWL 300
27 : : * * . * . * * : : : : : * . : : * * : * . : : *
28
29 AIP|NP_003968.2| KNLQMKEQPGSPEWIQLDQQITPLLLNYCQCKLVVEEYVEVLDHCSSILN 259
30 FKBP52|Q02790| EYESSFSNEEAQKAQALRLASH---LNLAMCHLKLQAFSAAI ESCNKALE 347
31 : . : : : * ** . * : * : : : : : * . * *
32
33 AIP|NP_003968.2| KYDDNVKAYFKRGKAHAHVWNAQEAQADFAKVLELDPALAPVVSRELQAL 309
34 FKBP52|Q02790| LDSNNEKGLFRRGEAHLAVNDFELARADFQKVLQLYPNNK-AAKTQLAVC 396
35 : * * . * : * : * * : : * : * * * : * * . . . : * .
36
37 AIP|NP_003968.2| EARIRQKDEEDKARFRGIFSH----- 330
38 FKBP52|Q02790| QQRIRRLQAREKKLYANMFERLAEENKAKAEASSGDHPTDTEMKEEQKS 446
39 : * * : : . * : : * : :
40

```

Supp. Figure S1. Alignment of AIP and FKBP52. ‘*’ denotes identical amino acids, while ‘:’ and ‘.’ mark strongly or moderately similar amino acids.

Supp. Table S1. Clinical and genetic data of 32 families with familial isolated pituitary adenoma

Family ID	Country of origin	Clinical diagnosis	Immunostaining of pituitary adenoma	Sex	Age at Diagnosis (yr)	Size of pituitary adenoma	Germline promoter, exon and exon-intron junction sequencing and GeneBank accession number	MLPA	Data for family members
1	UK	Prolactinoma	No surgery	Male	43	Macroadenoma	No mutation	Normal dosage	
		Prolactinoma	No surgery	Male	17	Microadenoma			
		Prolactinoma	No surgery	Female	23	Microadenoma			
2	UK	NFPA	Null cell	Male	60	Macroadenoma	No mutation	Normal dosage	
		NFPA	No surgery	Female	64	Macroadenoma			
3	Brazil	Cushing's disease	Pituitary adenoma	Female	21	Macroadenoma	No mutation	Not done	
		Prolactinoma	Pituitary adenoma	Female	34	Microadenoma			
4	Brazil	Prolactinoma	No surgery	Male	51	Microadenoma	No mutation	Normal dosage	
		Prolactinoma	No surgery	Male	47	Macroadenoma			
		Prolactinoma	No surgery	Female	35	Microadenoma			
5	UK	Acromegaly	GH	Male	24	Macroadenoma	c.662dupC p.E222X	Normal dosage	One carrier free of disease age 80yr
		Acromegaly	GH	Male	28	Macroadenoma			
6	Hungary	Prolactinoma	PRL	Male	51	Macroadenoma	No mutation	Normal dosage	
		Prolactinoma	No surgery	Male	46	Macroadenoma			
7	Germany	Gigantism	GH	Female	23	Macroadenoma	c.490C>T p.Q164X	Normal dosage	One carrier free of disease age 56yr
		Gigantism	No surgery	Male	20	Macroadenoma			
8	USA	Acromegaly	GH, TSH, FSH, LH, occ. PRL	Female	57	Macroadenoma	No mutation	Not done	
		NFPA	N/A	Male	47	Macroadenoma			
9 ¹	UK	NFPA	No surgery	female	17	Microadenoma	No mutation	c.1104-1109_279+578 Ex1_Ex2del EU872273	Five carriers, free of disease at 68, 62, 36, 5 and 2yr. Two subjects at risk tested negative
		Somatomatotrophinoma	GH, PRL	female	29	2 Microadenomas			
		NFPA	occ. GH, PRL	Male	18	Macroadenoma			
		Gigantism	GH, PRL, LH, FSH Sparsely gran.	Male	18	Macroadenoma			

Supp. Table S1. Clinical and genetic data of 32 families with familial isolated pituitary adenoma

Family ID	Country of origin	Clinical diagnosis	Immunostaining of pituitary adenoma	Sex	Age at Diagnosis (yr)	Size of pituitary adenoma	Germline promoter, exon and exon-intron junction sequencing and GeneBank accession number	MLPA	Data for family members
10	Northern Ireland	Acromegaly	GH	Male	20	Macroadenoma	c.910C>T p.R304X AM236344	Normal dosage	Twelve carriers of R304X, free of disease at 70, 69, 66, 56, 40, 38, 38, 35, 29, 26, 24, 2yr, two of these subjects also carry A299V. One unaffected carrier of A299V. Nine subjects at risk tested negative.
		Gigantism	Necrotic tissue	Male	13	Macroadenoma			
		Prolactinoma ?phenocopy	No surgery	Female	30	Microadenoma	c.896C>T, p.A299V EF203235		
11	Sweden	Acromegaly	N/A	Female	31	Macroadenoma	No mutation	Normal dosage	
		Acromegaly	N/A	Male	N/A	N/A			
12	USA	Acromegaly	No surgery	Male	52	Microadenoma	No mutation	Normal dosage	
		NFPA	No surgery	Male	43	Microadenoma			
13	Serbia	NFPA	No surgery	Female	36	Microadenoma	No mutation	Not done	
		Prolactinoma	No surgery	Female	25	Microadenoma			
14	UK	Acromegaly	No surgery	Female	59	Microadenoma	No mutation	Normal dosage	
		Prolactinoma	No surgery	Female	28	Microadenoma			
		Prolactinoma	No surgery	Female	38	Microadenoma			
15	Japan	Acromegaly	GH	Male	59	Macroadenoma	No mutation	Normal dosage	
		Acromegaly	No surgery	Female	30	N/A			
16	Serbia	NFPA	LH,FSH, occ. TSH, PRL	Female	73	Macroadenoma	No mutation	Not done	
		NFPA	N/A	Male	40	Macroadenoma			
17 ^z	Australia	Gigantism	Eosinophil adenoma, sparsely gran.	Male	13	Microadenoma	c.74_81delins7 p.L25PfsX130	Normal dosage	Three carriers, free of disease at 45, 21, 11yr
		Somato- mammotropinoma	Eosinophil adenoma	Male	38	Macroadenoma			
		Prolactinoma	PRL, occ. GH	Female	25	Macroadenoma			
		Acromegaly	Eosinophil adenoma	Male	27	Macroadenoma			

Supp. Table S1. Clinical and genetic data of 32 families with familial isolated pituitary adenoma

Family ID	Country of origin	Clinical diagnosis	Immunostaining of pituitary adenoma	Sex	Age at Diagnosis (yr)	Size of pituitary adenoma	Germline promoter, exon and exon-intron junction sequencing and GeneBank accession number	MLPA	Data for family members
		Acromegaly	Basophil adenoma	Female	32	Macroadenoma			
18	USA	NFPA	occ. PRL TSH, LH, FSH	Female	52	Macroadenoma	c.911G>A p.R304Q EF203236	Normal dosage	no other sample available
		Prolactinoma	No surgery	Female	23	Macroadenoma			
19	Brazil	NFPA	occ. FSH	Male	54	Macroadenoma	No mutation	Normal dosage	
		NFPA	occ. FSH, LH	Male	44	Macroadenoma			
20	Brazil	NFPA	ACTH (silent corticotroph adenoma)	Female	28	Macroadenoma	No mutation	Normal dosage	
		NFPA	N/A	Male	N/A	Macroadenoma			
21	Greece	Acromegaly	GH, occ. PRL	Female	42	Macroadenoma	No mutation	Normal dosage	
		Acromegaly	No surgery	Female	40	Macroadenoma			
22	UK	Acromegaly	GH, densely gran.	Female	36	Macroadenoma	No mutation	Normal dosage	
		Acromegaly	N/A	Female	N/A	N/A			
23	Serbia	Prolactinoma	No surgery	Male	40	Macroadenoma	No mutation	Normal dosage	
		Prolactinoma	No surgery	Female	N/A	Microadenoma			
24	Northern Ireland	Acromegaly	N/A	Male	44	Macroadenoma	No mutation	Normal dosage	
		Acromegaly	N/A	Male	32	Macroadenoma			
25	UK	Acromegaly	N/A	Female	50	Macroadenoma	No mutation	Normal dosage	
		Acromegaly	No surgery	Female	N/A	N/A			
		Gigantism	No surgery	Male	N/A	N/A			
26	Germany	Acromegaly	N/A	Male	N/A	Macroadenoma	No mutation	Normal dosage	
		Acromegaly	N/A	Male	N/A	N/A			
27	UK	Prolactinoma	No surgery	Female	26	Microadenoma	No mutation	Not done	
		Acromegaly	GH	Female	46	Macroadenoma			
28	UK	Prolactinoma	Necrotic tissue	Male	14	Macroadenoma	c.249G>T p.G83AfsX15	Normal dosage	One carrier free of disease at the age of 75yr, six subjects at risk tested negative
		Prolactinoma	No surgery	Male	60	Macroadenoma			
		Gigantism	No surgery	Male	N/A	N/A			

Supp. Table S1. Clinical and genetic data of 32 families with familial isolated pituitary adenoma

Family ID	Country of origin	Clinical diagnosis	Immunostaining of pituitary adenoma	Sex	Age at Diagnosis (yr)	Size of pituitary adenoma	Germline promoter, exon and exon-intron junction sequencing and GeneBank accession number	MLPA	Data for family members
29	Turkey	Acromegaly	No surgery	Female	31	Macroadenoma	No mutation	Normal dosage	
		Gigantism	Eosinophil adenoma	Male	18	Macroadenoma			
30	UK	Gigantism	GH	Female	6	Macroadenoma	c.910C>T p.R304X AM236344	Normal dosage	Two carriers free of disease at 73, 6yr. One subject at risk tested negative
		Acromegaly	N/A	Female	32	Macroadenoma			
		Acromegaly	N/A	Male	17	Macroadenoma			
		Gigantism	No surgery	Male	N/A	Macroadenoma			
		Prolactinoma	PRL	Female	16	Macroadenoma			
31	UK	Acromegaly	GH	Female	17	Macroadenoma	No mutation	Normal dosage	
		Prolactinoma	No surgery	Female	38	Microadenoma			
32	UK	Acromegaly	N/A	Female	23	Macroadenoma	No mutation	Normal dosage	
		Prolactinoma	N/A	Female	25	Macroadenoma			
33	UK	Prolactinoma	No surgery	Female	25	Macroadenoma	No mutation	Normal dosage	
		Acromegaly	GH	Male	51	Macroadenoma			
34	UK	Prolactinoma	No surgery	Female	23	Microadenoma	No mutation	Normal dosage	
		Prolactinoma	No surgery	Female	30	Macroadenoma			
35	UK	NFPA	No surgery	Male	60	Macroadenoma	No mutation	Normal dosage	
		NFPA	N/A	Male	65	Macroadenoma			
36	Russia	Prolactinoma	No surgery	Female	44	Microadenoma	No mutation	Not done	
		Prolactinoma	No surgery	Female	12	Macroadenoma			
37	USA	Acromegaly	GH	Male	70	Macroadenoma	No mutation	Not done	
		Acromegaly	GH	Female	24	Macroadenoma			
38	Northern Ireland	Gigantism	No surgery	Male	19	Macroadenoma	c.910C>T p.R304X AM236344	Normal dosage	3 carriers free of disease at age 68, 63 and 54y. Four subjects at risk tested negative
		Gigantism	GH	Female	13	Macroadenoma			

¹Family previously reported in (Georgitsi et al., 2007; Georgitsi et al., 2008)²Family previously reported in (Pestell et al., 1989)

Igreja et al., *Human Mutation*

6

Nomenclature of cDNA and protein variants follows the Human Genome Variation Society guidelines (www.hgvs.org/mutnomen). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1. Families with AIP mutations are marked with bold letters.

MLPA, Multiplex-ligation dependent probe amplification; GH, growth hormone; PRL, prolactin; TSH, thyroid stimulating hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NFPA, non-functioning adenoma; N/A, not available; occ., occasionally; gran., granulated

For Peer Review

Supp. Table S2. Updated clinical and genetic data of 26 families with familial isolated pituitary adenoma originally reported by Leontiou et al. (Leontiou et al., 2008)

Family ID	Country of origin	Clinical diagnosis	Immunostaining of pituitary adenoma	Sex	Age at diagnosis (yr)	Size of pituitary adenoma	Germline promoter, exon and exon-intron junction sequencing and GeneBank accession number	MLPA	Data for family members
Family I ¹	Mexico	acromegaly	GH sparsely gran.	M	23	macroadenoma	c.713G>A p.C238Y EF643648	Not done	One carrier parent
		acromegaly	GH	M	21	macroadenoma			
		acromegaly	No surgery	M	19	macroadenoma			
Family II ¹	Brazil	gigantism	GH	M	17	macroadenoma	c.70G>T p.E24X EF643644	Normal dosage	Two carriers, one parent and one sibling not affected at the age of 28yr
		acromegaly	GH, PRL	M	24	macroadenoma			
		gigantism	GH	M	15	macroadenoma			
		acromegaly	GH	M	17	macroadenoma			
		gigantism	NA	M	13	macroadenoma			
		gigantism	NA	M	16	macroadenoma			
		gigantism	NA	M	18	macroadenoma			
Family III ²	USA	gigantism	No surgery	M	23	macroadenoma	No mutation	Normal dosage	
		acromegaly	Acidophil tu.	F	21	macroadenoma			
Family IV ²	Serbia	gigantism	GH sparsely gran.	M	20	macroadenoma	No mutation	Exon1-6 deletion	One carrier parent died not due to pituitary adenoma
		gigantism	GH sparsely gran.	F	10	macroadenoma			
Family V ²	USA	acromegaly	GH, PRL sparsely gran.	F	32	macroadenoma	c.241C>T p.R81X EF643647	Normal dosage	Three carriers, free of disease at age 73,40,38yr
		acromegaly	GH, PRL sparsely gran.	M	32	macroadenoma			

Supp. Table S2. Updated clinical and genetic data of 26 families with familial isolated pituitary adenoma originally reported by Leontiou et al. (Leontiou et al., 2008)

Family VI ^{2,3}	Japan	gigantism	GH, PRL sparsely gran.	F	14	macroadenoma	c.[-270_-269CG>AA; -220G>A] EF643645	Normal dosage	One carrier parent free of disease
		gigantism	GH sparsely gran.	F	10	macroadenoma			
Family VII ²	Japan	acromegaly	GH	M	49	macroadenoma	No mutation	Not done	
		acromegaly	GH	M	24	macroadenoma			
Family VIII ²	USA	acromegaly	GH sparsely gran.	M	39	macroadenoma	No mutation	Normal dosage	
			NA	M	38	macroadenoma			
Family IX ²	Sweden	acromegaly	NA	F	71	macroadenoma	No mutation	Normal dosage	
			NA	F	70	macroadenoma			
			NA	M	48	macroadenoma			
			NA	M	14	microadenoma			
Family X ²	UK	gigantism acromegaly	GH sparsely gran.	M	15	macroadenoma	c.805_825dup p.F269_H275dup*	Normal dosage	Four carriers not affected at age 55, 45, 31, 31yr
		gigantism	GH sparsely gran.	M	29	macroadenoma			
		acromegaly	GH sparsely gran.	M	15	macroadenoma			
Family XI	UK	acromegaly prolactinoma	GH sparsely gran.	F	30	macroadenoma	No mutation	Normal dosage	
			No surgery	F	29	macroadenoma			
Family XII	UK	acromegaly	GH sparsely gran.	F	42	macroadenoma	c.910C>T p.R304X AM236344	Normal dosage	no unaffected carriers (five potential carriers were screened and were negative)
		gigantism	GH sparsely gran.	F	24	macroadenoma			
		acromegaly	NA	F	24	macroadenoma			
		acromegaly	GH,PRL	F	27	macroadenoma			
		prolactinoma	PRL	M	25	macroadenoma			
		acromegaly	NA	M	NA	macroadenoma			
		acromegaly	NA	M	NA	macroadenoma			
acromegaly	NA	M	NA	macroadenoma					
Family XIII	UK	acromegaly	No surgery	F	68	macroadenoma	No mutation	Normal dosage	

Supp. Table S2. Updated clinical and genetic data of 26 families with familial isolated pituitary adenoma originally reported by Leontiou et al. (Leontiou et al., 2008)

			GH sparsely gran.	F	45	macroadenoma			
Family XIV	Brazil	acromegaly prolactinoma	GH sparsely gran.	M	41	macroadenoma	No mutation	Normal dosage	
			No surgery	F	25	macroadenoma			
Family XV	Finland	acromegaly	NA	F	54	macroadenoma	No mutation	Normal dosage	
				F	34	macroadenoma			
Family XVI ⁴	UK	acromegaly	no surgery	F	47	macroadenoma	c.807C>T p.= EF643649	Normal dosage	Two carriers not affected at the age of 51 and 29yr
		acromegaly	NA	M	58	macroadenoma			
Family XVII ⁴	UK	acromegaly	NA	F	52	macroadenoma	No mutation	Normal dosage	
			GH sparsely gran.	M	45	macroadenoma			
Family XVIII	UK	acromegaly	GH	M	43	macroadenoma	No mutation	Normal dosage	
			No surgery	M	46	macroadenoma			
Family XIX	Serbia	acromegaly	GH sparsely gran.	M	36	macroadenoma	No mutation	Normal dosage	
			NA	M	50	macroadenoma			
Family XX	UK	acromegaly	GH densely gran.	M	24	macroadenoma	No mutation	Normal dosage	
			NA	F	40	macroadenoma			
Family XXI	UK	acromegaly	GH sparsely gran.	M	35	macroadenoma	No mutation	c.1- ?_993+?del Exon1-6 deletion	Three carriers, 60, 57 and 80yr unaffected
		acromegaly	GH	M	19	macroadenoma			
		acromegaly	GH	M	23	macroadenoma			
Family XXII	Malta	acromegaly	GH	F	53	macroadenoma	No mutation	Normal dosage	
			GH	F	54	macroadenoma			
			GH	M	58	macroadenoma			
Family XXIII	Serbia	acromegaly	GH	F	35	macroadenoma	No mutation	Normal dosage	
			GH	F	36	macroadenoma			
Family XXIV	Romania	acromegaly	GH, PRL	F	17	macroadenoma	c.910C>T	Normal	One carrier free of disease at

Supp. Table S2. Updated clinical and genetic data of 26 families with familial isolated pituitary adenoma originally reported by Leontiou et al. (Leontiou et al., 2008)

		prolactinoma/acromegaly	No surgery	F	26	macroadenoma	p.R304X AM236344	dosage	72yr
Family XXV	Romania	acromegaly	GH, PRL, ACTH; FSH occ.	F	30	macroadenoma	c.911G>A p.R304Q	Normal dosage	No carrier identified as no other family member available for testing
		acromegaly	GH	F	17	macroadenoma	EF203236		
Family XXVI	UK	acromegaly	GH	M	30	macroadenoma	No mutation	Normal dosage	
		NFPA	No positive hormone staining	M	42	macroadenoma			

*This mutation has previously been annotated c.794_823dup, p.A274_H275ins10 (EF643650) (Leontiou et al., 2008) while the current appropriate annotation is c.805_825dup, p.F269_H275dup

¹Family previously reported in (Gadelha et al., 1999; Gadelha et al., 2000)

²Family previously reported in (Soares et al., 2005)

³Family previously reported in (Matsuno et al., 1994)

⁴Family previously reported in (McCarthy et al., 1990)

Nomenclature of cDNA and protein variants follows the Human Genome Variation Society guidelines (www.hgvs.org/mutnomen). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Families with AIP mutations are marked with bold letters.

MLPA, Multiplex-ligation dependent probe amplification; GH, growth hormone; PRL, prolactin; TSH, thyroid stimulating hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; ACTH, adrenocorticotrophic hormone; NFPA, non-functioning adenoma; N/A, not available; occ., occasionally; gran., granulated