

Supplementary Materials and methods

RT-PCR and Real Time RT-PCR analysis

Surgical biopsies of PA operated on at the Neuromed Institute were collected at the time of surgery in a *RNAlater* solution (Ambion[®], distributed by Applera Italia, Milan, Italy) and successively stored at -80°C . Total RNA was extracted with Trizol (Invitrogen, S. Giuliano Milanese, Italy) according to the manufacturer's instructions, and DNase treatment was performed before each retrotranscription step. First-strand cDNA synthesis was performed using 1 μg of total RNA in the presence of random hexamers and 100 U reverse transcriptase (Bioscript, Bioline, distributed by Aurogene, Rome, Italy). RT-PCR experiments for the pituitary factors *Pit-1* and *Tpit*, used as cell-specific markers, were first performed in order to exclude significant contamination of tumour samples by normal pituitary tissue, as previously described (Fratticci *et al.* 2007). Intron-bridging primers for *AIP* and *AHR* (*AIP-F*: CACGTACCAGACGGACCCAT, *AIP-R*: GTCGTA CTTGTTGAGGATGGAA; *AHR-F*: GTATTAGTTGTC ACTACAGATGC, *AHR-R*: AAACCAGATGAATTATCCAGCAG) were designed on the relative Genbank sequences (NM_003977 and NM_001621, respectively), and amplification of *AIP* and *AHR* cDNA was then performed using 150 ng of equivalent RNA, in the presence of 0.5 pM of specific sense and antisense primers, 0.3 mM dNTPs, 1.5 mM MgCl_2 and 0.4 U of BioTaq polymerase (Bioline, distributed by Aurogene, Rome, Italy) in a final volume of 50 μl , for a total of 45 cycles, respectively. Because these preliminary experiments showed universal but variable *AIP* and *AHR* mRNA expression by normal and tumour samples, variations in gene expression was then systematically studied by Real Time RT-PCR based on a Taqman methodology, using an ABI Prism 7700 Sequence Detection System (Applera Italia s.r.l, Monza, Italy), and compared to *β -actin* expression. Ready-to-use gene expression assays were purchased from Applied Biosystems (Applera Italia s.r.l, Monza, Italy), with the following identification numbers: Hs_00610222_m1 (*AIP*), Hs_00354967_m1 (*AHR*) and Hs_99999903 (*β -actin*), respectively. *AIP*, *AHR* and *β -actin* assays were run on the same batch of cDNA and all reactions were performed at least in duplicate on 80 ng of equivalent RNA, as previously described (Fratticci *et al.* 2007). Down-regulation was defined by a *AIP*/ *β -actin* mRNA ratio below the 10^o percentile of values observed in normal pituitary samples.

Genetic analysis

Genomic *AIP* sequencing was performed as previously described (Daly *et al.* 2007), with the informed consent of patients. In addition, specific primers were designed on the *AIP* mRNA sequence (Genbank NM_003977) (Supplementary Table 1 - additional primers designed for shorter RT-PCR fragments are available on request -) and direct cDNA sequencing was performed on purified PCR fragments using the BigDye® Terminator v3.1 Cycle Sequencing kit on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Applied Biosystems, Milan, Italy). In a minority of cases, somatic *AIP* sequencing was performed on tumour DNA, after DNA extraction from frozen surgical biopsies with a commercial kit (Invitex, distributed by Biospa SpA, Milan, Italy).

Supplementary Results

Details of the observed changes in *AIP* sequence

Details concerning the clinical characteristics of patients affected by *AIP* mutations are available in Table 1 in the manuscript file and patients will thus be identified accordingly. The R304X mutation (c. 910C>T) identified in Family 1 has been already reported in two young female cousins with a giant GH-secreting adenoma (see Ref. 4 for clinical details) and then in a 8 years-old nephew presenting with incipient gigantism (Daly *et al.* 2007). Disease penetrance in this family is currently evaluated to 3 out of the 7 parents affected by the mutation. This mutation is the most frequently described to date, being reported in apparently unrelated patients and families in Europe (Daly *et al.* 2007, Verimaa *et al.* 2006, Leontiou *et al.* 2008), though not in the USA (DiGiovanni *et al.* 2007). The somatic R304X mutation identified in a young acromegalic patient unrelated to the Family 1 (Spor 8) was present at an hemizygous state. The Q285fsX17 mutation identified in Family 2 (c854_857delAGGC, Genbank accession number EF06509) was previously reported in two brothers with gigantism and acromegaly, respectively (Daly *et al.* 2007). The mutation was inherited from their father - deceased at 40 years-old from myocardial infarction without obvious clinical features of acromegaly -, as indicated by the mutation identified in the patients' paternal uncle, who is currently unaffected by the disease. The K241E mutation (c.721A>G, Genbank accession number EF066505)

was previously reported in two siblings from an heterogeneous FIPA kindred (Daly *et al.* 2007). At the moment, 2 out of 3 daughters of the prolactinoma patient are unaffected carriers of the mutation. The E174fsX21 truncating mutation (c.517_521delGAAGA, Genbank accession number EF66503) identified in Family 4 and data from the corresponding familial screening have been previously described in details (Naves *et al.* 2007). The corresponding protein is expected to lack the three TPR domains. The Q82fsX7 mutation (c.245_249delAAGGG, Genbank accession number FJ514477) is a new truncating mutation identified in a 15 years-old Bulgarian patient with apparently sporadic gigantism. The mutated protein is expected to lack part of the FKBP and the three TPR domain of the AIP protein. New changes in the AIP sequence were analysed by comparison with 100 controls from Belgium, Italy and France, including a subset of subjects from North African origin. Novel missense mutations were studied according to SNPs databases and compared with ten orthologue sequences in vertebrates (accession numbers in Genbank are available on request). The V195A mutation (c.584T>C, Genbank accession number FJ514478) was identified in a 12-years Brazilian boy (Spor 2) with an apparently sporadic macroprolactinoma and in his mother, who is unaffected at the moment. The corresponding valine residue, located in the first TPR domain, was conserved in all vertebrates, with the exception of *Xenopus Laevi* and *Dario Renio*. The A277P mutation (c.829G>C, Genbank accession number FJ514479) was identified in an Italian 12 years-old boy with apparently sporadic incipient gigantism and in his father, who is currently unaffected. The corresponding alanine residue, located in the third TPR domain, was conserved in all vertebrates. Introduction of a proline residue can be expected to significantly modify the secondary structure of the protein, thereby possibly altering the interaction with AHR. The R128H mutation was identified in a 27 years-old patient from Morocco presenting with an apparently sporadic acromegaly. Interestingly, the histidine residue was observed instead of arginine in all other vertebrates. Three intronic mutations could be identified by genomic DNA sequencing, which included exon-intron junctions. Two of them were contiguous changes (IVS3 c.486+15C>T and c.486+16G>T, respectively) observed in two 15-years-old female patients, both affected by an apparently sporadic GH-secreting adenoma (Spor 4 and 3, respectively). The IVS3 c.486+15C>T change was previously reported at an heterozygous state in a breast cancer sample (Georgitsi *et al.* 2007). The IVS2

c.279+23C>T mutation was observed in an Italian 43-years old female patient with a macroprolactinoma. (Spor 7). Finally, a silent heterozygous somatic D172D polymorphism (c.516C>T, Genbank accession number RS2276020) was identified in a clinically silent GH/PRL-secreting adenoma, operated on in a 44 years-old female patient and subsequently on germline DNA (data not shown). This polymorphism has been previously reported in patients with sporadic pituitary adenomas as well as in controls (Buchbinder *et al.* 2008).

References

Buchbinder S, Bierhaus A, Zorn M, Nawroth PP, Humpert P & Schilling T 2008 Aryl Hydrocarbon Receptor Interacting Protein Gene (AIP) Mutations are Rare in Patients with Hormone Secreting or Non-secreting Pituitary Adenomas. *Experimental and Clinical Endocrinology & Diabetes* **116** 625-628.

Daly AF, Vanbellinghen JF, Khoo SK, Jaffrain-Rea ML, Naves LA, Guitelman MA, Murat A, Emy P, Gimenez-Roqueplo AP, Tamburrano G et al 2007 Aryl hydrocarbon receptor-interacting protein gene mutations in familial isolated pituitary adenomas: analysis in 73 families. *Journal of Clinical Endocrinology and Metabolism* **92** 1891-1896.

DiGiovanni R, Serra S, Ezzat S & Asa S L 2007 AIP Mutations are not Identified in Patients with Sporadic Pituitary Adenomas. *Endocrine Pathology* **18** 76-78.

Ferretti E, Jaffrain-Rea ML, Asteria C, Di Stefano D, Esposito V, Ferrante L, Daniele P, Tiberti C, Gallucci M, Bosman C et al 2001 Two familial giant pituitary adenomas associated with overweight: clinical, morphological and genetic features. *European Journal of Endocrinology* **144** 227-235.

Fratticci A, Grieco FA, Spilioti C, Giangaspero F, Ventura L, Esposito V, Piccirilli M, Santoro A, Gulino A, Cantore G et al 2007 Differential expression of neurogenins and NeuroD1 in human pituitary tumours. *Journal Endocrinology* **194** 475-484.

Georgitsi M, Karhu A, Winqvist R, Visakorpi T, Waltering K, Vahteristo P, Launonen V & Aaltonen LA 2007 Mutation analysis of aryl hydrocarbon receptor interacting protein (AIP) gene in colorectal, breast, and prostate cancers. *British Journal of Cancer* **96** 352-356.

Jaffrain-Rea ML, Di Stefano D, Minniti G, Esposito V, Bultrini A, Ferretti E, Santoro A, Faticanti Scucchi L, Gulino A & Cantore G 2002 A critical reappraisal of MIB-1 labelling index significance in a large series of pituitary tumours: secreting versus non-secreting adenomas. *Endocrine Related Cancer* **9** 103-113.

Leontiou CA, Gueorguiev M, van der Spuy J, Quinton R, Lolli F, Hassan S, Chahal HS, Igreja SC, Jordan S, Rowe J et al 2008 The role of the aryl hydrocarbon receptor-interacting protein gene in familial and sporadic pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* **93** 2390-2401.

Naves LA, Daly AF, Vanbellinhen JF, Casulari LA, Spilioti C, Magalhães AV, Azevedo MF, Giacomini LA, Nascimento PP, Nunes RO et al 2007 Variable pathological and clinical features of a large Brazilian family harbouring a mutation in the aryl hydrocarbon receptor-interacting protein gene. *European Journal of Endocrinology* **157** 383-391.

Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, Raitila A, Tuppurainen K, Ebeling TM, Salmela PI, Paschke R et al 2006 Pituitary adenoma predisposition caused by germline mutations in the AIP gene. *Science* **312** 1228-1230.