Expression of aryl hydrocarbon receptor (AHR) and AHR-interacting protein in pituitary adenomas: pathological and clinical implications

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Abstract

Germline mutations of the aryl hydrocarbon receptor (AHR)-interacting protein (*AIP*) gene confer a predisposition to pituitary adenomas (PA), usually in the setting of familial isolated PA. To provide further insights into the possible role of AIP in pituitary tumour pathogenesis, the expression of AIP and AHR was determined by real-time RT-PCR and/or immunohistochemistry (IHC) in a large series of PA (*n*=103), including 17 with *AIP* mutations (*AIP*^mut). Variable levels of *AIP* and *AHR* transcripts were detected in all PA, with a low *AHR* expression (*P*<0.0001 versus *AIP*). Cytoplasmic AIP and AHR were detected by IHC in 84.0 and 38.6% of PA respectively, and significantly correlated with each other (*P*=0.006). Nuclear AHR was detected in a minority of PA (19.7%). The highest AIP expression was observed in somatotrophinomas and non-secreting (NS) PA, and multivariate analysis in somatotrophinomas showed a significantly lower AIP immunostaining in invasive versus non-invasive cases (*P*=0.019). AIP expression was commonly low in other secreting PA. AIP immunostaining was abolished in a minority of *AIP*^mut PA, with a frequent loss of cytoplasmic AHR and no evidence of nuclear AHR. In contrast, AIP overexpression in a subset of NS PA could be accompanied by nuclear AHR immunopositivity. We conclude that down-regulation of AIP and AHR may be involved in the aggressiveness of somatotrophinomas. Overall, IHC is a poorly sensitive tool for the screening of *AIP* mutations. Data obtained on AHR expression suggest that AHR signalling may be differentially affected according to PA phenotype.

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Introduction

Germline mutations of the aryl hydrocarbon receptor (AHR)-interacting protein (*AIP*) gene confer a predisposition to pituitary adenomas (PA; Vierimaa *et al.* 2006), usually in the setting of familial isolated PA (FIPA; Daly *et al.* 2006, 2007, Beckers & Daly 2007). Because mutations are likely to be inactivating and associated with tumour loss of heterozygosity (LOH), *AIP* is viewed as a new pituitary tumour suppressor gene in 11q13 (Vierimaa *et al.* 2006). Most PA associated with *AIP* mutations (*AIP*[mut]) are somatotrophinomas or prolactinomas – although non-secreting (NS) adenomas occur occasionally (Buchbinder *et al.* 2008). The prevalence of AIP mutations in FIPA is 15% overall, but reaches 50% in FIPA kindreds with homogeneous somatotrophinomas (Vierimaa *et al.* 2006, Daly *et al.* 2007). *AIP*[mut] PA occur earlier than their sporadic counterpart and are usually more aggressive (Beckers & Daly 2007, Daly *et al.* 2007). Immunohistochemistry (IHC) has shown AIP to be expressed by PA, with loss of AIP staining in *AIP*[mut] tumours being variably appreciated (Vierimaa *et al.* 2006, Georgitsi *et al.* 2007, Leontiou *et al.* 2008). As such, the clinical application of IHC for AIP as a screening tool remains controversial. Germline AIP mutations are very rare in patients with sporadic PA, although they can be encountered in young acromegalics (Barlier *et al.* 2007, Cazabat *et al.* 2007, Iwata *et al.* 2007, Georgitsi *et al.* 2008*a*). In addition, although variations in AIP expression among sporadic PA have been recently reported according to their functional phenotype (Leontiou *et al.* 2008), their potential relationship with tumour-evolutive features has not been investigated. Thus, the possible role of AIP in PA pathogenesis independently of germline mutations is unknown.

The best characterized function of AIP is to stabilize the AHR, or dioxin receptor, in a cytoplasmic core complex involving AIP, AHR and the heat shock protein Hsp90. Upon activation, AHR moves to the nucleus and heterodimerizes with the Ah receptor nuclear translocator (ARNT) to exert direct transcriptional effects (Petrulis & Perdew 2002). AHR is widely expressed in endocrine tissues, and dioxin-related compounds are endocrine disruptors (Pitt *et al.* 2000, Nishimura *et al.* 2002, Pocar *et al.* 2005). *In vitro* exposure to 2,3,7,8 tetrachlorodibenzo-*p*-dioxin induces GH and prolactin (PRL) secretion from rainbow trout pituitary cells, in part through AHR-mediated transcriptional effects (Elango *et al.* 2006). AIP generally enhances AHR response to exogenous ligands by increasing its cytoplasmic levels, but nuclear localization of non-ligand-bound AHR can be observed in the absence of AIP (LaPres *et al.* 2000). This may be relevant to AIP-related tumourigenesis, since endogenous functions of AHR are being increasingly recognized (Barouki *et al.* 2007, Nguyen & Bradfield 2008). AHR exerts complex modulatory effects on cell cycle regulation and apoptosis (Marlowe & Puga 2005), and genetic models have clearly underscored its involvement in normal ontogeny and in tumourigenesis (Gasiewicz *et al.* 2008). Dioxin is carcinogenic (Popp *et al.* 2006), and AHR can be overexpressed in human tumours (Harper *et al.* 2006, Schlezinger *et al.* 2006). Yet, the effects of dioxin exposure on the incidence of pituitary tumours remain uncertain (Pesatori *et al.* 2008), and the expression of AHR in the normal human pituitary and in PA has not been reported to date.

We wished to further characterize AIP expression and assess AHR expression and cellular localization in a representative series of PA. We also aimed to further characterize *AIP*[mut] PA in order to provide new insights into their pathogenesis and possibly help identify patients who could benefit from *AIP* mutational screening.

Material and methods

Patients and samples

A series of 103 PA have been studied for AIP and AHR expression by real-time RT-PCR ($n = 66$) and/or IHC ($n = 94$). There were 46 GH-, 22 PRL-, 7 ACTH-, 2 TSH-secreting and 26 NS adenomas respectively. Unselected sporadic adenomas were operated on for medical reasons at the Neuromed Institute (Italy). Seventeen FIPA samples were collected in 14 FIPA patients who came from eight international centres (Italy, Belgium, France, Brazil, Spain and Bulgaria), out of which 10 PA were operated on in 8 patients with a germline *AIP* mutation. The study was approved by local ethical committees. Clinical, biological and neuroradiological data, as well as intra-operative findings and pre-operative pharmacological treatment, were systematically recorded. Invasiveness was defined according to pre-operative neuroradiological imaging and intra-operative findings, including macroscopic evidence of dural infiltration. Tumour phenotype was defined on the basis of pre-operative endocrine evaluation and immunohistochemical characterization of hormone secretion. This latter was available in all but two cases and performed with the commercial anti-PRL, anti-GH, anti-FSH, anti-LH, anti-ACTH and anti-TSH antibodies in use in the participating centres (in Italy, polyclonal antibodies from Orthodiagnostic Systems, Raritan, NJ, USA).

Cell proliferation was evaluated in 77 PA by Ki-67 immunostaining with the monoclonal MIB-1 antibody (DBA Italia srl, Milan, Italy), as previously described (Jaffrain-Rea *et al*. 2002). Tumours showing microscopic evidence of contamination by normal pituitary fragments were considered for immunohistochemical studies only. In addition, normal human pituitary glands were collected at autopsy in four patients who died from non-endocrine diseases and divided into frozen and formalin-fixed paraffin-embedded fragments, as previously described (Fratticci *et al*. 2007).

Molecular and genetic analysis

Methodological details on RT-PCR and real-time RT-PCR protocols are provided in the Supplementary Materials and methods, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/. Briefly, after preliminary, RT-PCR experiments allowed the exclusion of tumour samples with potential contamination with normal pituitary cells according to cell-specific transcription factors' criteria and revealed an almost universal *AIP* and *AHR* gene expression; *AIP* and *AHR* transcripts were quantified by real-time RT-PCR based on a Taqman methodology and corrected for *β-actin* expression. Leukocyte genomic *AIP* sequencing (gDNA) was performed in 28 patients, as previously described (Daly *et al*. 2007). Searches for somatic mutations, or LOH in *AIP*[mut] tumours, were performed by direct sequencing of tumour cDNA and/ or DNA (tDNA) in 35 and 6 cases respectively. Procedures for AIP cDNA sequencing are described in the electronic Supplementary Materials and methods. Overall, the *AIP* gene status could be determined in 67 cases. Wherever new changes in *AIP* sequencing were identified, *AIP* variations were considered as *AIP* mutations for statistical purposes and their potential biological significance further analysed in the Supplementary Materials and methods and in the Discussion section.

Immunohistochemistry

Tissue sections were dewaxed in xylene and rehydrated through a descending ethanol series, and antigen retrieval was performed by microwave boiling at 850 W in citrate buffer 0.1 M, pH 6.0. Mouse monoclonal antibodies directed against AIP (clone 35-2) and AHR (clone RPT9, referred to as 'N-mAb') were purchased from Novus (Novus Biologicals LLC, Littleton, CO, USA) and both used at a 1:500 dilution, in 94 and 83 PA respectively. A polyclonal rabbit anti-AHR antibody was also used
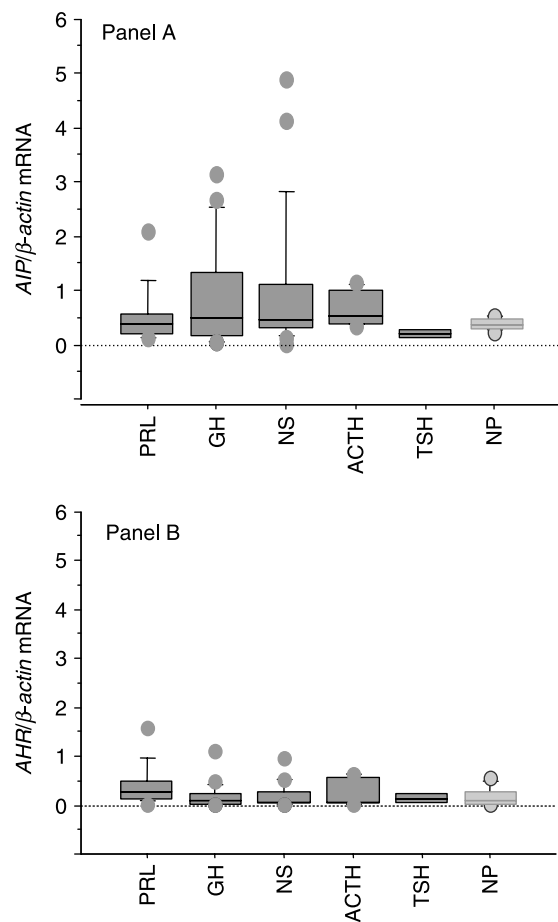

Figure 1 *AIP* and *AHR* gene expression in pituitary adenomas according to tumour phenotype box plots representing *AIP/β-actin* and *AHR/β-actin* mRNA ratios in all prolactinomas (PRL), somatotrophinomas (GH), non-secreting adenomas (NS), corticotrophinomas (ACTH), thyrotroph adenomas (TSH) and in normal pituitary samples (NP) are shown in panels A and B respectively (note the different scales used).

in 61 PA (17 PRL-, 21 GH-, 3 ACTH-secreting and 20 NS respectively) at a 1:50 dilution (sc-5579, referred to as 'C-pAb', Santa Cruz Biotechnology, Santa Cruz, CA, USA). This antibody proved to be more sensitive than the N-mAb in the detection of nuclear AHR in positive control sections (human invasive breast cancer samples and rat liver), and cytoplasmic background could be reduced by introducing a further blocking step with 5% milk in PBS. IHC was performed with a multilink biotinylated antibody and the avidin–biotin peroxidase system according to the manufacturer's instructions (LSAB+ kit, DAKO Cytomation, Milan, Italy). Negative controls were performed omitting the primary antibody. Topographical localization of AIP-expressing cells in normal pituitaries was performed

Table 1 Aryl hydrocarbon receptor-interacting protein (AIP) expression in the whole series of pituitary adenomas (PA) according to patients and tumours characteristics

Phenotype	All	Age (years)			Pre-operative treatment			Invasiveness			SSE		
		<30	≥30	P	Treated	Untreated	P	Invasive	Non-Invasive	P	Present	Absent	P
All PA													
AIP/β-actin mRNA (66)	0.8±0.9	0.3±0.4	0.9±1.0	0.015		N/A	–	0.5±0.7	1.0±1.0	0.0197	0.7±0.5	0.9±1.1	NS
AIP staining (94)	37/94 39.4%	11/32 34.4%	26/62 41.9%	NS			–	14/52 26.4%	22/41 53.6%	0.0090	20/63 31.7%	17/31 54.8%	0.031
GH-secreting													
AIP/β-actin mRNA (20)	0.9±0.9	0.5±0.6	1.0±1.0	NS	0.8±0.9	1.0±1.0	NS	0.3±0.2	1.1±1.0	0.039	0.8±0.6	0.9±1.1	NS
AIP staining (44)	23/44 52.3%	9/19 47.4%	14/25 56.0%	NS	12/19 63.1%	11/25 41.7%	NS	6/22 27.3%	17/22 77.3%	0.0009	8/25 32.0%	15/19 77.7%	0.002
PRL-secreting													
AIP/β-actin mRNA (12)	0.5±0.5	0.1±0.1	0.6±0.5	NS	0.3±0.3	0.6±0.7	NS	0.4±0.3	1.0±0.9	NS	0.5±0.5	–	NS
AIP staining (21)	3/21 14.3%	1/8 12.5%	2/13 15.4%	NS	1/7 14.3%	2/14 14.3%	NS	3/15 20.0%	0/6 –	NS	3/16 18.7%	0/5 –	NS
Non-secreting													
AIP/β-actin mRNA (25)	1.0±1.3	0.3±0.1	1.0±1.3	NS		N/A	–	0.7±1.0	1.0±1.0	NS	1.0±1.03	0.6	–
AIP staining (21)	9/21 42.8%	1/3 33.3%	8/17 47.0%	NS			–	5/14 35.7%	3/6 50.0%	NS	9/19 47.4%	0/1 –	NS
ACTH-secreting													
AIP/β-actin mRNA (7)	0.7±0.3	0.4	0.7±0.3	–		N/A	–	0.4	0.7±0.3	–	0.4±0.1	0.8±0.3	NS
AIP staining (6)	1/6 16.7%	0/1 –	1/5 20.0%	–			–	0/1 –	1/5 20.0%	–	0/2 –	1/4 25.0%	NS
TSH-secreting													
AIP/β-actin mRNA (2)	0.2±0.1	0.3	0.1	–	0.2±0.1	–	–	–	0.2±0.1	–	–	0.2±0.1	–
AIP staining (2)	0/2 –	0/1 –	0/1 –	–	0/2 –	–	–	–	0/2 –	–	–	0/2 –	–

For raw data, the number of studied cases is indicated within brackets. The *AIP/β-actin* ratio was defined according to real-time RT-PCR experiments. AIP staining refers to significant AIP immunostaining (+ diffuse, moderate and ++ diffuse, strong) – as compared with very low or absent staining. SSE, suprasellar extension; N/A, not applicable; NS, non-significant data.
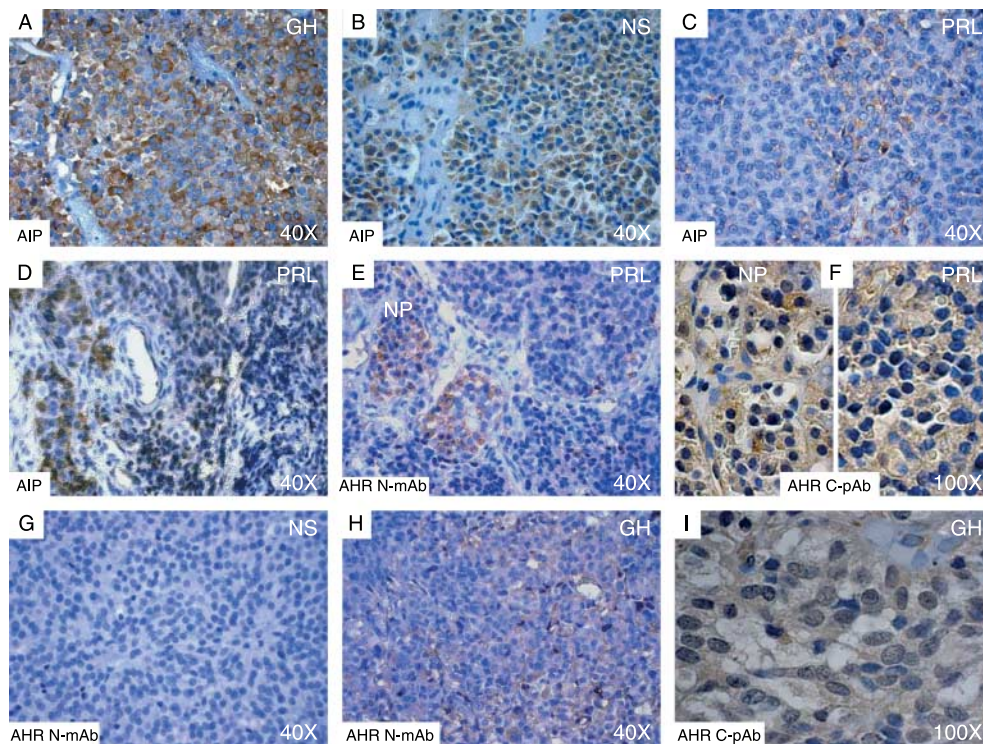
Figure 2 AIP and AHR immunostaining in pituitary adenomas (PA), excluding *AIP*[mut] PA. Shown are representative examples of AIP and AHR immunostaining in different pituitary adenomas (PA), excluding *AIP*[mut] PA. For each image, the PA endocrine phenotype is indicated in the upper right corner, the corresponding antibody in the bottom left corner and magnification in the bottom right corner respectively. AIP immunostaining was quoted $++$ in a somatotrophinoma, $+$ in a non-secreting PA and $+/-$ in a prolactinoma (A, B and C respectively). An example of microprolactinoma displaying AIP and AHR down-regulation (quoted $+/-$) as compared with the adjacent normal pituitary (indicated as NP) is shown in D and E respectively; nuclear AHR staining in this case was observed in the normal pituitary, but not in adenoma cells (F). Most PA had no or weak AHR immunoreactivity (G and H respectively), a few cases displaying nuclear immunostaining, including a non-secreting FIPA without AIP mutation (I).

using pre-diluted mouse mAbs for pituitary hormones (DAKO Cytomation). Semi-quantitative analysis of cytoplasmic AIP and AHR immunostaining was performed at $20\times$ and $40\times$ magnifications and semiquantitatively scored as follows: $-$ (negative); $+/-$ (weak immunostaining and/or scattered positive cells); $+$ (diffuse, moderate immunostaining); $++$ (diffuse, strong immunostaining). In the presence of heterogeneous staining, the areas obtaining the highest score were considered for statistical purposes. Nuclear staining for AHR (C-pAb) was further evaluated at high magnification ($100\times$) in all cases. A double step immunohistochemical study was also performed as previously described (Fratticci *et al.* 2007) to first detect cytoplasmic AIP or the pituitary hormones GH, PRL, ACTH and FSH respectively using 3-3′-diaminobenzidine (DAB) as a chromogen, followed by a second step for the detection of nuclear AHR (C-pAb) using Novored as a chromogen (Vector, DBA Italia), introducing a further blocking step with

milk as indicated hitherto. Photographs of slides were taken using a Zeiss Axioplan 2 microscope and a Leica DFC 320 digital camera.

Statistical analysis

All data are expressed in mean $\pm$ s.d. and statistical analyses were performed using Statview 5.01 software for PC (SAS Institute, Cary, NC, USA). Continuous values were analysed by non-parametric analysis, using Mann–Whitney and Kruskal–Wallis test for 2 and ≥ 3 group comparisons respectively and the Spearman test for correlation studies. Distribution of nominal values was compared by the χ^2-test, logistic regression being used for multivariate analysis. For logistic regression and analysis of subgroups, the immunostaining scores for cytoplasmic AIP and AHR were transformed into binary parameters as follows: significant (scores $+$ and $++$) versus low ($-$ and $+/-$) AIP immunostaining; and

Table 2 Characteristics of pituitary adenomas (PA) with *AIP* mutations and immunohistochemistry (IHC) for aryl hydrocarbon receptor (AHR)-interacting protein (AIP) and AHR

Patients surgery[a]	*AIP* mutation	Country	Sex	Age (years)	Symptoms[b]	Tumour[c]	Hormone values at diagnosis (ng/ml)	Pre-op treatment	Pre-op hormone values (ng/ml)	Pituitary hormones IHC	AIP IHC[d]	AHR IHC[e]
Fam 1[(i,ii)]												
Fam 1A TC	R304X	Italy	F	19	Amenorrhoea–galactorrhoea, visual loss, bitemporal haemianopsia, weight gain and hydrocephalus requiring emergency surgery for ventricular derivation (VD)	Giant-SSE (52 mm), Inv+ (CS)	PRL 99, Post-VD, GH 15.8, IGF-I 420, PRL 32	OCT up to 200 μg t.i.d. CAB 0.5 mg ×2/w	PRL 9.1, GH 16.0, IGF 900	GH, FSH (±), Ki-67, 0.5%	+/− (scattered cells +)	C − N −
Fam 1B 1[rst] TS	R304X	Italy	F	21	Amenorrhoea, weight gain, mild acromegaly, DH 164 cm and headache	Giant-SSE (41 mm), Inv+ (CS)	GH 33, IGF-I 630, PRL 5	None	See values at diagnosis	GH, PRL (±), Ki-67, 6.0%	+/−	C− N ND
2° TS	R304X	Italy	F	22	Evolutive disease and pharmacological resistance	Ma-SSE (27 mm), Inv+ (CS)	*Post-TS1* GH 4.0, IGF-I 400, PRL 7.2	LAN 30 mg /14 d. CAB 0.5 mg ×2/w.	GH 5.8 IGF-I 598 PRL 1.3	GH, FSH, LH (±), Ki-67, 2.7%	+/−	C− N ND
Fam 1C TS	R304X	Italy	M	8	Incipient gigantism	Ma-IS (12 mm), Inv−	GH 48.2, IGF-I 1070, PRL 11.8	OCT-LAR 10 mg/28 d.	GH 65.9 IGF-I 1294 PRL 21.9	GH, Ki-67, 5.0%	+/− (scattered cells+)	C− N−
Fam 2[(i)]												
Fam 2A TS	Q285*fs*17X	Italy	M	21	Gigantism – DH 208 cm, headache and visual fields' defects	Ma-SSE (28 mm), Inv+	GH > 30.0, PRL 7.4, IGF-I N/A	None	See values at diagnosis	GH, PRL (±), Ki-67, 0.7%	+/−	C− N−
Fam 2B 1[rst]TS	Q285*fs*17X	Italy	M	33	Acromegaly – DH 185 cm	Ma-SSE (30 mm), Inv+(CS)	GH 74.0, IGF-I 664, PRL 35.4	OCT-LAR 20 mg/28 d.	GH 3.5, IGF-I 414, PRL 4.4	GH, PRL, FSH (±), Ki-67, 0%	+/− (large areas −)	C− N−
2nd TS	Q285*fs*17X	Italy	M	34	Evolutive acromegaly	Ma-IS (15 mm), Inv+ (CS)	GH 6.0, IGF-I 540, PRL 7.0	OCT-LAR 10 mg/28 d.	GH 19.6, IGF-I 449, PRL 6.7	GH, PRL (±), Ki-67, 0%	−	C−
Fam 3[(i)]												
Fam3A TS	K241E	Belgium	F	53	Visual loss and bitemporal haemianopsia.	Ma-SSE (30 mm), Inv+	PRL 41, IGF-I 50	None	See values at diagnosis	FSH, LH	+	C− N−
Fam 3B TS	K241E	Belgium	M	53	Visual loss	Ma-SSE (34 mm), Inv+ (CS)	PRL 2333, GH 0.2, IGF-I 100	CAB 0.5 mg ×2/w.	PRL 12.5, GH 0.2	PRL	−	C− N ND

Table 2 *continued*

Patients surgery[a]	AIP mutation	Country	Sex	Age (years)	Symptoms[b]	Tumour[c]	Hormone values at diagnosis (ng/ml)	Pre-op treatment	Pre-op hormone values (ng/ml)	Pituitary hormones IHC	AIP IHC[d]	AHR IHC[e]
Fam 4[(iii)] TS	E174*fs*21X	Brazil	M	17	Gigantism, DH 193 cm, headache and visual fields' defect	Giant-SSE (46 mm), Inv+ (CS)	GH 51, IGF-I 778, PRL 50	None	See values at diagnosis	GH, PRL, Ki-67, 0%	Het +, +/−	C+/− N −
Spor 1 TS	V195A	Brazil	M	12	Visual loss and pre-puberal, post-operative resistance to dopamine-agonists	Giant-SSE (40 mm) Inv+ (CS)	PRL 10 561, GH 0.06, IGF-1 147	None	See values at diagnosis	PRL, Ki-67, 0%	+	C− N ND
Spor 2 TS	A277P	Italy	M	12	Incipient gigantism and visual defect	Ma-SSE (27 mm), Inv+ (CS)	GH 13.6, IGF-I 530, PRL 52.5	None	See values at diagnosis	GH, PRL, Ki-67, 15.0%	+/−	C− N−
Spor 3 TS	IVS3 c.468+16G>T	Italy	F	15	Oligomenorrhoea, visual field defects and then pituitary apoplexy	Ma-SSE (20 mm), Inv+ (CS)	N/A	None	N/A	GH, Ki-67, 0.5%	− (in part necrotic)	ND
Spor 4 TS	IVS3 c.468+15C>T	Spain (Ecuador)	F	17	Acromegaly, tall stature, headache and menstrual disturbance	Ma-IS (13 mm), Inv−	GH 54.8, IGF-I 635, PRL ↑	LAN-60 mg i.m. /28 d.	N/A	GH, PRL FSH, LH, ACTH (±)	Het +/−, −	C+/− N ND
Spor 5 TS	Q82*fs*X7	Bulgaria	M	15	Gigantism, headache and hydrocephalus	Giant-SSE (60 mm), Inv+	N/A	None (RxT)	GH 42.3, IGF-I ↑, PRL 60	GH	−	C− N ND
Spor 6 TS	R128H	Belgium (Morocco)	M	27	Acromegaly	Ma-SSE (20 mm), Inv−	GH 58.7, IGF-I 909, PRL N/A	OCT 100 μg t.i.d. CAB 0.5 mg ×2/w	IGF-I 1090	GH	Het + +/+	ND
Spor 7 TS	IVS2 c.279+23C>T	Italy	F	43	Secondary amenorrhoea, headache and transient visual defects	Ma-SSE (18 mm), Inv−	PRL 30, GH 0.2, IGF-I 152	None	See values at diagnosis	PRL, GH (±), Ki-67, 3.0%	+/−	C− N−
Spor 8 [(T)] TS	R304X	Italy	F	17	Visual defects, headache, secondary amenorrhoea and DH 171 cm	Ma-SSE, Inv−	GH 9.0, IGF-I 905, PRL 57	None	See values at diagnosis	GH, Ki-67, 3.0%	+/−	ND

[a(i)]Previously reported in Daly *et al*. (2007), [(ii)]clinical details on Fam1 A and B previously reported in Ferretti *et al*. (2001), [(iii)]previously reported in details in Naves *et al*. (2007), (T) only tumour available.
[b]DH, definitive height.
[c]Ma, macroadenoma; SSE, suprasellar extension; maximal tumour diameter within brackets; Inv−, non-invasive; Inv+, invasive; CS, cavernous sinus.
[d]AIP staining with semi-quantitative score. + + strong, diffuse; + moderate, diffuse; +/− weak or scattered positive cells; − negative.
[e]AHR staining; C, cytoplasmic staining with an N-terminal monoclonal antibody; N, nuclear staining with a C-terminal polyclonal antibody respectively; ND, not done (unavailable material).

Table 3 Characteristics of somatotrophinomas and relationship with the presence of aryl hydrocarbon receptor-interacting protein (*AIP*) mutations

	Group A	Group B	*P*
Patients			
Age	18.7 ± 6.6	40.1 ± 12.5	*P* < 0.0001
Sex	7M/5F	13M/17F	NS
Tumours			
Suprasellar extension	10/14 (71.4%)	16/32 (50.0%)	NS
Invasive	10/14 (71.4%)	12/32 (37.5%)	$\chi^2 = 4.49$, *P* = 0.034
Ki-67, > 3.0%	5/11 (45.4%)	6/29 (20.7%)	NS
Pre-operative treatment	7/14 (50.0%)	13/32 (40.6%)	NS
AIP expression			
AIP/β-actin mRNA (n)	0.18 ± 0.05 *(4)*	1.05 ± 0.98 *(16)*	*P* = 0.072
AIP score $(-; +/-; +; ++)$	2;8;3;1	1;10;15;4	NS
Low AIP score $(+/-, -)$	10/14 (71.4%)	11/30 (36.7%)	$\chi^2 = 4.62$, *P* = 0.031
AHR expression			
AHR/β-actin mRNA (n)	0.01 ± 0.02 *(4)*	0.02 ± 0.03 *(16)*	NS
AHR score (N-mAb) $(-; \pm; +)$	10;2;0	15;12;2	NS
Detectable AHR (N-mAb) $(+/-, +)$	2/12 (16.7%)	14/29 (48.3%)	$\chi^2 = 3.56$, *P* = 0.059
Nuclear AHR (C-pAb)	0/7	2/13	NS

Groups A and B refer to patients and tumours with or without documented *AIP* mutations respectively. *AIP/β-actin* and *AHR/β-actin* mRNA ratios were obtained by real-time RT-PCR (the number of studied tumours is indicated in italicized text within brackets). Semi-quantitative AIP and AHR scores were defined by immunohistochemistry ($++$ diffuse, strong; $+$ diffuse, moderate; $+/-$ weak immunostaining and/or scattered positive cells; $-$ negative), using anti-AIP and AHR (N-mAb) monoclonal antibodies and, in a subset of cases, a polyclonal anti-AHR antibody (C-pAb) recognizing the C-terminal half of AHR respectively. Nearly significant *P* values are indicated in italics; NS, non-significant.

detectable (scores $+/-$ and $+$) versus undetectable AHR immunostaining respectively. The level of significance was set at $P < 0.05$.

Results

Normal pituitary

AIP and *AHR* transcripts were detected in normal pituitary samples, with a significantly lower expression of *AHR* ($P < 0.0001$ versus *AIP*). Accordingly, AIP-immunopositive cells were largely present in the adenohypophysis (see Supplementary Fig. 1A, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/), with topographical studies arguing for a preferential strong and diffuse expression in somatotrophs (see Supplementary Fig. 1B), and to a lesser extent by lactotrophs (data not shown). AHR (N-mAb) immunostaining was less intense, with a roughly similar, although more scattered and diffuse, cell distribution (see Supplementary Fig. 1C). AIP and AHR (N-mAb) immunostaining was confined to the cytoplasm (see Supplementary Fig. 1D and E), although some degree of AHR nuclear positivity was revealed by C-pAb immunostaining (see Supplementary Fig. 1F). Some scattered AIP- and AHR-immunopositive cells were observed in the *pars intermedia*, containing

ACTH-secreting cells (see Supplementary Fig. 1G–I). Double immunostaining with AHR C-pAb revealed co-localization of AHR with AIP (see Supplementary Fig. 1J), nuclear AHR immunostaining being observed in a subset of somatotrophs (see Supplementary Fig. 1K) and a few corticotrophs (data not shown), whereas in lactotrophs, AHR appeared to be mostly perinuclear (see Supplementary Fig. 1L). FSH-secreting cells were virtually negative for nuclear AHR (data not shown).

Pituitary adenomas

The expression of AIP and AHR has been first performed on the entire series of PA. *AIP* and *AHR* transcripts were detected in all PA (Fig. 1), with a markedly lower AHR expression ($P < 0.0001$ versus *AIP*). Accordingly, AIP and AHR proteins were detected in 84.0 and 38.6% of the cases respectively. Marked individual variations were observed in *AIP* gene expression and immunostaining (Fig. 1A; Table 1) – examples of IHC scoring are shown in Fig. 2A–C, including one microprolactinoma with a normal pituitary fragment shown as an internal control (Fig. 2D). The AIP immunostaining score was significantly correlated with AIP expression ($P = 0.0014$) and with the AHR (N-mAb) score ($\chi^2 = 18.1$, $P = 0.006$). However, cytoplasmic AHR
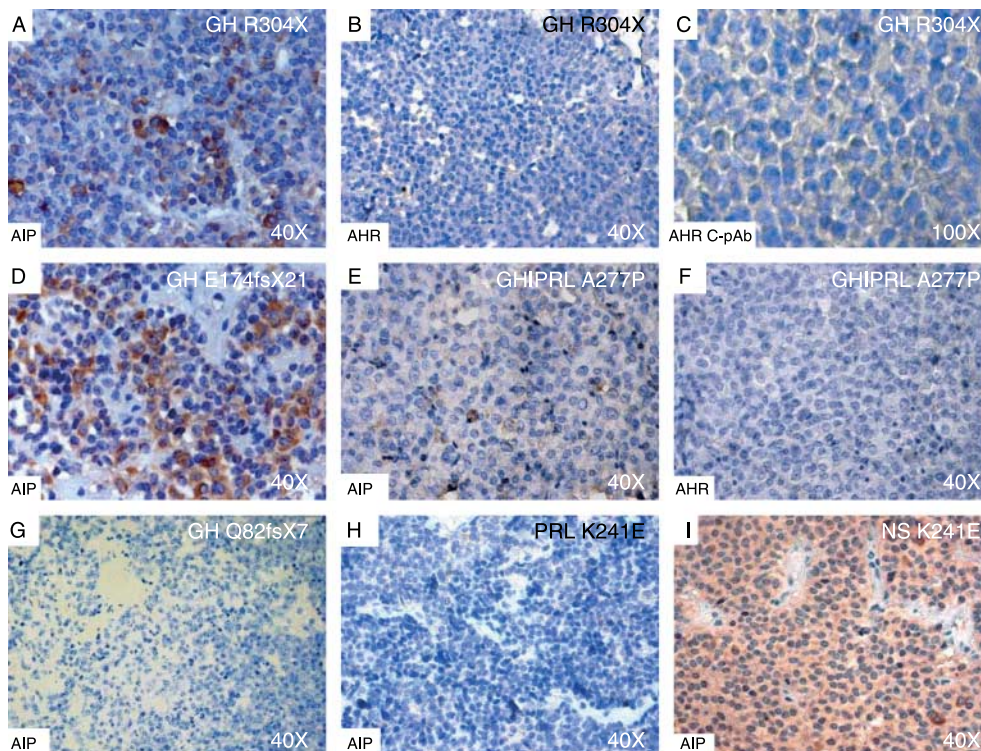
Figure 3 AIP and AHR immunostaining in *AIP*^mut pituitary adenomas. Examples of AIP and AHR immunostaining and relative semi-quantitative AIP evaluation in *AIP*^mut PA with references to Table 2 for case identification. (A) AIP immunostaining in a familial somatotrophinoma (Fam 1A) with a R304X mutation ($+/-$, an area displaying scattered positive cells), with no detectable AHR in the cytoplasm (B) or in the nucleus (C). (D) AIP immunostaining ($+$) in a familial somatotrophinoma (Fam 4) with an E174*fs*X21 mutation. (E) Low AIP immunostaining ($+/-$) and (F) lack of detectable AHR immunostaining in a sporadic somatotrophinoma with a germline A277P mutation (Spor2). (G) Negative AIP immunostaining in a sporadic somatotrophinoma with a Q82*fs*X7 mutation. Differential expression of AIP in two siblings (Fam 3) with a K241E mutation, affected by a prolactinoma (H) (quoted $-$) and a NS adenoma (I) (quoted $+$) respectively.

immunostaining (N-mAb) was generally weak, and nuclear immunostaining (C-pAb) was observed in a minority of cases ($12/61 = 19.7\%$; Fig. 2E–I), including a familial NS PA with normal AIP sequencing (Fig. 2I).

Correlation with clinical characteristics

A series of factors were found to account for the variations in *AIP* mRNA and AIP immunostaining observed on the entire series of PA, including tumour phenotype and aggressiveness (Table 1). Briefly, both were found to be highly variable in somatotrophinomas, generally low in prolactinomas and in other secreting PA, but paradoxically high in a subset of NS adenomas (Fig. 1A; Table 1). *AIP* mRNA was significantly lower in invasive PA ($P = 0.0197$ versus non-invasive), which were also more likely to show a low AIP immunostaining ($\chi^2 = 6.91$, $P = 0.009$ versus non-invasive). Overall, no significant difference in AIP expression was observed according to the *AIP* gene status (data not shown). *AHR* mRNA was poorly

influenced by phenotype (Fig. 1B), but complete loss of AHR immunostaining (N-mAb) was significantly more frequent in invasive (71.4 vs 47.4% in non-invasive, $\chi^2 = 5.2$, $P = 0.022$) and in *AIP*^mut PA (86.7 vs 58.7% in non-mutated PA, $\chi^2 = 3.92$, $P = 0.048$). Nuclear AHR immunostaining (C-pAb) was observed in three somatotrophinomas, three prolactinomas and six NS adenomas respectively, but in none of the *AIP*^mut PA included in this study.

Analysis of PA subgroups

Because the proportion of *AIP*^mut tumours varied according to phenotype, data obtained in all somatotrophinomas, prolactinomas and NS adenomas have been further analysed as distinct subgroups.

Somatotrophinomas

As most *AIP*^mut tumours were somatotrophinomas, data were analysed according to the presence (group A) or the absence (group B) of documented *AIP* mutations

respectively. In group A, *AIP* mutations were identified on patient's leukocyte DNA in six FIPA patients (eight PA) and in five sporadic cases, respectively, and on tumour cDNA and DNA in an additional sporadic case where no leukocyte DNA was available. Individual data are shown in Table 2 (Family 1, 2 and 4; Spor 2–6 and 8). Germline mutations were heterozygous whereas only the mutated allele was detected in cDNA/tDNA where available, thereby indicating somatic loss of heterozygosity in four out of five cases (Fam 1B and C, Fam 2B; Spor 2) and strongly suggesting hemizygosity in the other case (Spor 8). In group B, the lack of detectable *AIP* mutations was confirmed in most cases by sequencing of gDNA ($n=7$), tumour cDNA ($n=12$) and/or tDNA ($n=4$) respectively. Data observed in group A and group B are summarized in Table 3. Somatotrophinomas occurred in group A at a significantly younger age ($P<0.0001$) were more frequently invasive ($P=0.034$) and more likely to display decreased AIP immunostaining ($P=0.031$) than those in group B respectively. Of note, the pattern of AIP immunostaining could differ according to the mutation (Fig. 3A, D, E and G). Similarly, there was a trend towards cytoplasmic AHR (N-mAb) being less likely to be detected in group A (Fig. 3B and F) than in group B ($P=0.059$). Further evaluation with the AHR C-pAb confirmed the lack of nuclear immunostaining in group A (Fig. 3C).

Excluding somatotrophinomas with undetermined *AIP* gene status, *AIP* mutations were estimated to account for *AIP* gene down-regulation in 3 out of 4 cases and for low AIP immunostaining in 10 out of 19 cases respectively. Low AIP immunostaining tended to be more frequent in *AIP*$^{\text{mut}}$ somatotrophinomas than in those with normal *AIP* sequencing (10 out of 14 vs 9 out of 22, $\chi^2=3.20$, $P=0.074$), indicating a sensitivity of IHC in detecting *AIP* mutations of 71.4% and a specificity of 40.9% respectively. According to logistic regression analysis, tumour invasiveness was the only independent predictor of AIP down-regulation in somatotrophinomas ($P=0.019$), whereas young age was the only independent predictor of *AIP* mutations ($P=0.002$; see Supplementary Table 2, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/). Complete loss of AHR (N-mAb) immunostaining was significantly more frequent in invasive than in non-invasive somatotrophinomas (78.9 vs 35.7%, $\chi^2=4.7$, $P=0.012$), while a trend was confirmed in *AIP*$^{\text{mut}}$ somatotrophinomas as compared with those with normal *AIP* sequencing (83.3 vs 52.4.%, $\chi^2=2.76$, $P=0.096$).

Prolactinomas

Both *AIP* mRNA and AIP immunostaining were generally low in prolactinomas (Table 1; Figs 1 and 2C and E). The *AIP* gene status was available in most cases (15 out of 22), according to AIP sequencing performed on gDNA ($n=8$) or tumour cDNA ($n=7$). Germline AIP mutations were identified in three cases (Table 2, Fam 3B; Spor 1 and 7), although no splicing alteration was found by tumour cDNA sequencing in Spor 7. Low AIP immunostaining occurred in prolactinomas with normal AIP sequencing (10 out of 12) as well as in *AIP*$^{\text{mut}}$ (2 out of 3). Excluding *AIP*$^{\text{mut}}$ tumours, low AIP immunostaining was confirmed to be significantly more frequent in prolactinomas than in somatotrophinomas ($\chi^2=16.5$, $P=0.0009$). Cytoplasmic (N-mAb) and nuclear (C-pAb) AHR immunostaining could be detected in 11 out of 21 (52.3%) and 3 out of 17 (17.6%) prolactinomas respectively.

NS adenomas

The mean *AIP* mRNA in NS adenomas was similar to that observed in somatotrophinomas, 42.3% of PA in this group also displaying significant AIP immunostaining (Table 1; Figs 1 and 2B). A germline *AIP* mutation was identified in one FIPA patient (Table 2, Fam 3A), and normal *AIP* sequencing was documented in 11 cases on tumour cDNA ($n=8$) or tDNA ($n=3$) – including all NS occurring in patients aged ≤ 50 years old. Surprisingly, a significant AIP immunostaining was observed in the *AIP*$^{\text{mut}}$ NS adenoma (Fig. 3I), but not in the prolactinoma that was surgically resected in her brother (Fig. 3H). AIP immunostaining was frequently associated with nuclear AHR (55.5 vs 9.0% according to the presence or the absence of significant AIP respectively, $\chi^2=5.09$, $P=0.02$), which in turn was significantly correlated with cytoplasmic AHR ($\chi^2=11.6$, $P=0.0007$). No significant correlation was found between AIP and AHR immunostaining and tumour volume, invasiveness or immunostaining for gonadotrophins.

Discussion

This study shows for the first time that both AHR and AIP are expressed in the normal human pituitary and frequently down-regulated in PA. In normal pituitaries, topographical studies indicated a preferential expression of AIP by somatotrophs, which display a strong and diffuse expression, and to a lesser extent by lactotrophs. These findings are in agreement with recent work by Leontiou *et al*. (2008), which also localized AIP within GH and PRL-containing

secretory granules in normal cells. Scattered AIP-expressing cells were also observed in the *pars intermedia*. AHR was expressed at lower transcriptional levels and with a lower immunostaining intensity than AIP, with scattered AHR-expressing cells being more widely distributed than AIP-expressing cells. AIP and AHR were first observed in the cytoplasm only. However, further evaluation with an antibody directed against the C-terminal part of the AHR protein revealed moderate nuclear immunostaining in a subset of somatotrophs and in some corticotrophs. Differences between cytoplasmic and nuclear staining may reflect variations in protein–protein interactions involving AHR in subcellular compartments, which may in turn influence epitope recognition and modify the sensitivity of the corresponding IHC assays. As DNA binding and basic-helix-loop-helix (bHLH) -interacting domains of AHR are located N-terminal (Fukunaga *et al*. 1995, Fukunaga & Hankinson 1996), the N-mAb epitope of AHR may be masqueraded in its transcriptionally active conformation. Thus, nuclear immunostaining with the C-terminal antibody alone may indicate some degree of endogenous AHR activity. Supporting a role for AHR in pituitary development and/or cell differentiation, binding sites for the pituitary transcription factors Lhx3 and Pit-1 are present in the promoter of the *AHR* gene (Harper *et al*. 2006). Constitutive expression of AHR and ARNT has been shown in the rat hypothalamus (Korkalainen *et al*. 2005). These data suggest a physiological role for AHR in the human hypothalamus–pituitary unit, further extending the spectrum of bHLH transcription factors expressed in the normal pituitary and PA (Jackson *et al*. 1993, Ferretti *et al*. 2001, Huang *et al*. 2002, Fratticci *et al*. 2007).

In agreement with a recent report (Leontiou *et al*. 2008), AIP was found by real-time RT-PCR to be expressed at a transcriptional level in all PA, with a relative overexpression in a subset of somatotrophinomas and NS adenomas. Accordingly, significant AIP immunostaining was observed in about half of somatotrophinomas and NS adenomas, but in ≤15% of other PA. Thus, AIP expression can be either retained or down-regulated in somatotrophinomas and is frequently decreased in prolactinomas, whereas abnormal AIP expression may occur in a subset of NS adenomas.

We first focused our attention on the significance of AIP down-regulation in somatotrophinomas. In these tumours, decreased gene and protein expression of AIP were significantly associated with the presence of aggressive features and, although AIP immunostaining tended to be lower in *AIP*$^{\text{mut}}$ somatotrophinomas, only invasiveness was an independent predictor of AIP loss. On the other hand, young age (<30 years of age) was an independent predictor for the presence of *AIP* mutations, but not for low AIP immunostaining. These findings may have interesting pathogenetic and clinical implications. First, they strongly suggest that AIP down-regulation may be involved in the progression of somatotrophinomas, regardless of detectable germline *AIP* mutations. Although we cannot exclude the presence of large *AIP* genomic deletions, this is unfrequent (Georgitsi *et al*. 2008*b*), and additional mechanisms of *AIP* silencing may be present. Second, such data contrast with those obtained in prolactinomas, where a low AIP expression could be observed at early stages of the disease, suggesting a peculiar role for AIP in somatotrophs. This could explain the large predominance of somatotrophinomas among PA developing in patients with germline *AIP* mutations, as reported in this and other studies (Cazabat *et al*. 2007, Daly *et al*. 2007), and the frequent aggressiveness of *AIP*$^{\text{mut}}$ somatotrophinomas (Daly *et al*. 2007, Iwata *et al*. 2007, Toledo *et al*. 2007, this study). This is consistent with the high expression of AIP in normal somatotrophs, and supported by functional experiments on GH$_3$ cells (Leontiou *et al*. 2008). Third, IHC may not be as suitable as previously suggested for the pre-screening of patients with germline AIP mutations (Georgitsi *et al*. 2007). Indeed, our data support recent evidence that AIP can frequently be detected in *AIP*$^{\text{mut}}$ tumours (Leontiou *et al*. 2008). Such discrepancies may depend on the type of mutation and, to a lesser extent, antibody characteristics. Due to somatic hemizygosity for the mutated AIP allele, as further supported by cDNA sequencing in this study, IHC data will depend on epitope expression by the mutated protein, if actually transcribed. In fact, AIP immunostaining can theoretically be retained unless early stop mutations are present. Early data on IHC were obtained in series where Finnish patients and their distinctive Q14X early stop mutation were largely represented (Georgitsi *et al*. 2007). Although the AIP immunoscore in *AIP*$^{\text{mut}}$ somatotrophinomas can also be low due to mono-allelic gene expression, this is of limited diagnostic interest, since semi-quantitative interpretation of IHC is difficult to standardize and can be confounded readily. Thus, evidence of AIP immunostaining should not discourage *AIP* gene sequencing in acromegalics with a familial history of FIPA or a young age at disease onset. Furthermore, IHC is unsuitable as a pre-screening tool in prolactinomas, due to the frequent loss of significant AIP immunostaining in this group.

The familial AIP mutations reported herein (E174*fs*, K241E, Q285*fs* and R304X) were previously published (Daly *et al.* 2007, Naves *et al.* 2007), whereas seven out of the eight nucleotide changes identified in patients with sporadic PA were undescribed. They consisted of one frameshift (Q82*fs*) and three missense (R128H, V195A and A277P) mutations respectively, three intronic changes (IVS2 c.279 + 23C > T, IVS3 c.468 + 16 G > T and IVS3 c.468 + 15C > T) being also recognized. None was observed in controls and comparison with available SNPs, and orthologue sequences' databases were performed (see the electronic Supplementary Materials and methods). Briefly, the Q82*fs* frameshift mutation is expected to encode for a truncated protein after seven amino acids. None of the novel missense changes has been identified as a SNP to date, the amino acid residues K(241), V(195) and A(277) are highly conserved among species, and loss of the wild-type allele could be verified by cDNA sequencing in the latter case. In contrast, the presence of an arginine or a histidine at position 128 variant occurs variably across mammalian species and may represent an unrecognized human polymorphism, which might explain normal AIP immunostaining in this case. However, the exclusion of this case from *AIP*^mut somatotrophinomas did not significantly alter the statistical results obtained in this group (data not shown). Of note, in the siblings affected by a K241E mutation, AIP immunostaining was present in the NS PA, but absent in the prolactinoma. This may reflect the differential AIP expression in these two phenotypes, regardless of the *AIP* mutation itself, which should not affect the epitope recognized by the mAb used in this study. The novel intronic changes, none of them being encountered in controls, are not expected to induce alterations in *AIP* splicing by *in silico* analysis, and this was confirmed by cDNA sequencing in the prolactinoma associated with a germline change in intron 2. However, the contiguity of the two nucleotide changes in intron 3 and the young age of the affected patients, both with somatotrophinomas with a low or heterogeneous AIP immunostaining, favour their possible role in the onset of the disease and suggest possible molecular implications of intronic changes outside recognized splicing sites (Kleinjan & van Heiningen 2005). The R304X mutation was identified in an additional sporadic somatotrophinoma – a young Italian patient unrelated to the family included in this study, which was previously reported in detail (Ferretti *et al.* 2001, Daly *et al.* 2007). This mutation has been recurrently recognized in Europe (Vierimaa *et al.* 2006, Cazabat *et al.* 2007, Daly *et al.* 2007, Leontiou *et al.* 2008), and R304Q mutations being occasionally

reported also (Georgitsi *et al.* 2007, Leontiou *et al.* 2008). This reinforces the concept of codon 304 as a relative 'hot spot' in the *AIP* gene, although some founder effect concerning the R304X mutation cannot be excluded. Of note, AIP was detected by IHC in all R304X somatotrophinomas studied so far and, where available, electron microscopy showed sparsely granulated somatotrophinomas (Ferretti *et al.* 2001, Leontiou *et al.* 2008).

Another major aim of this study was to investigate for the first time the possible role of AHR in the pathogenesis of PA, especially in *AIP*^mut tumours. Low levels of *AHR* transcripts were found in PA as well as in normal pituitary samples. Cytoplasmic AHR immunostaining was detected in 40.0% of PA, being generally weaker in intensity as compared with normal pituitaries, and significantly correlated with AIP immunostaining. This is consistent with AHR destabilization in the presence of low AIP levels, thereby supporting a role for AIP in AHR cytoplasmic stabilization in the human pituitary, as observed in most tissues. In *AIP*^mut PA, AHR can be further destabilized by a defective interaction with the AIP protein. As the C-terminal half of AIP is critical for interaction with AHR, which is abolished by truncation of the last five amino acids (Bell & Poland 2000), *AIP*-truncating mutations are expected to prevent the constitution of the cytoplasmic complex. The functional implications of each missense mutation would need specific *in vitro* experiments, but a number of single amino acid changes occurring in the TPR region can abolish AIP binding to hsp90 and/or strongly reduce AIP/AHR interaction (Bell & Poland 2000, Petrulis & Perdew 2002). The N-terminal part of AIP may also contribute to the cytoplasmic stabilization of AHR (Kazlauskas *et al.* 2002). Accordingly, none of the *AIP*^mut PA showed nuclear AHR staining, suggesting that increased AHR nuclear signalling is not implicated in AIP-related tumourigenesis. Hence, AHR down-regulation may be involved in *AIP*^mut and/or invasive somatotrophinomas, although we cannot exclude a non-specific bystander effect of AIP down-regulation. In contrast, AHR appears to be up-regulated in a subset of NS overexpressing AIP. Of note, NS but not GH-secreting PA have been reported after Seveso's exposure (Pesatori *et al.* 2008). These findings suggest a differential role of AHR in pituitary cells, and further *in vitro* investigations should help clarifying these issues.

In addition to AHR modulation, AIP is known to interact with a number of cytoplasmic proteins including phosphodiesterases (Bolger *et al.* 2003, de Oliveira *et al.* 2007), thereby possibly modulating

cAMP concentration, survivin, an anti-apoptotic protein (Kang & Altieri 2006), and Ret (Vargiolu *et al.* 2009). Interactions with phosphodiesterases can be altered by AIP mutations (Bolger *et al.* 2003, de Oliveira *et al.* 2007). Possible alterations in cAMP signalling or apoptosis accompanying down-regulation of AIP in somatotrophinomas or increased AIP expression in NS PA should be further investigated.

In conclusion, this study supports the role of AIP in the cytoplasmic stabilization of AHR and shows that reduced AIP and AHR expression are frequently observed in PA, at least at a protein level, with the exception of NS adenomas, in which up-regulation of both proteins can be observed. It suggests a possible role for AIP and AHR down-regulation in the progression of somatotrophinomas, independently from germline mutations, and indicates that IHC is not a sufficiently sensitive tool for the detection of *AIP* mutations in somatotrophinomas, and is unsuitable to this purpose in prolactinomas or NS PA.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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