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Aryl Hydrocarbon Receptor-Interacting Protein Gene Mutations in Familial Isolated Pituitary Adenomas: Analysis in 73 Families

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Context: An association between germline aryl hydrocarbon receptor-interacting protein (*AIP*) gene mutations and pituitary adenomas was recently shown.

Objective: The objective of the study was to assess the frequency of *AIP* gene mutations in a large cohort of patients with familial isolated pituitary adenoma (FIPA).

Design: This was a multicenter, international, collaborative study.

Setting: The study was conducted in 34 university endocrinology and genetics departments in nine countries.

Patients: Affected members from each FIPA family were studied. Relatives of patients with *AIP* mutations underwent *AIP* sequence analysis.

Main Outcome Measures: Presence/absence and description of *AIP* gene mutations were the main outcome measures.

Intervention: There was no intervention.

Results: Seventy-three FIPA families were identified, with 156 patients with pituitary adenomas; the FIPA cohort was evenly divided between families with homogeneous and heterogeneous tumor expression. Eleven FIPA families had 10 germline *AIP* mutations. Nine mutations, R16H, G47_R54del, Q142X, E174frameshift, Q217X, Q239X, K241E, R271W, and Q285frameshift, have not been described previously. Tumors were significantly larger ($P = 0.0005$) and diagnosed at a younger age ($P = 0.0006$) in *AIP* mutation-positive *vs.* mutation-negative subjects. Somatotropinomas predominated among FIPA families with *AIP* mutations, but mixed GH/prolactin-secreting tumors, prolactinomas, and nonsecreting adenomas were also noted. Approximately 85% of the FIPA cohort and 50% of those with familial somatotropinomas were negative for *AIP* mutations.

Conclusions: *AIP* mutations, of which nine new mutations have been described here, occur in approximately 15% of FIPA families. Although pituitary tumors occurring in association with *AIP* mutations are predominantly somatotropinomas, other tumor types are also seen. Further study of the impact of *AIP* mutations on protein expression and activity is necessary to elucidate their role in pituitary tumorigenesis in FIPA. (***J Clin Endocrinol Metab* 92: 1891–1896, 2007**)

PITUITARY ADENOMAS OCCUR relatively frequently based on autopsy and radiological series, while recent clinical data suggest a prevalence of approximately one case per thousand of the population (1, 2). Tumorigenesis of sporadic adenomas has been attributed to genetic and molecular abnormalities involving *gsp*, pituitary tumor transforming gene, and a pituitary derived truncated form of fibroblast growth factor receptor-4 (3–6). Pituitary adenomas due to hereditary causes are uncommon and can occur in the setting of multiple endocrine neoplasia-1 (MEN1) and Carney complex (CNC), due to mutations in the genes encoding menin (*MEN1*) and the R1a regulatory subunit of protein kinase A (*PRKAR1A*), respectively (7–9). However, *MEN1* and *PRKAR1A* mutations are an infrequent cause of sporadic pituitary tumors (10). Interest has also focused on isolated familial somatotropinomas (IFSs), which were thought to be linked to a locus close to that of *MEN1* on chromosome 11q13 (11). Vierimaa *et al.* (12) recently reported that inactivating mutations of the gene encoding aryl hydrocarbon receptor interacting protein (*AIP*) on chromosome 11q13.3 occurred in patients with pituitary tumors (mainly acromegaly) in the familial and sporadic settings. Recently, we described familial isolated pituitary adenomas (FIPA) in 64 families with two or more pituitary tumors in patients without *MEN1* or *PRKAR1A* mutations or clinical/biochemical features of MEN1/CNC that included a broader tumor phenotype than IFS (13). To address the potential role of *AIP* mutations in families having the FIPA phenotype, we undertook a genetic screening program involving both the original FIPA cohort and newly identified families.

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Abbreviations: AhR, Aryl hydrocarbon receptor; AIP, AhR-interacting protein; CNC, Carney complex; FIPA, familial isolated pituitary adenoma; FKBP-PPI, FK506-type binding protein type peptidyl-prolyl cis-trans isomerase; hsp90, heat shock protein 90; IFS, isolated familial somatotropinoma; MEN1, multiple endocrine neoplasia type 1; PRKAR1A, R1a regulatory subunit of protein kinase A; TPR, tetratricopeptide repeat.

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Patients and Methods

This was an international study of *AIP* mutations in families having the FIPA phenotype performed across nine countries (Belgium, France, Italy, United States, Spain, Brazil, Argentina, The Netherlands, and Czech Republic). The clinical characteristics of the original FIPA cohort, involving 64 families (138 affected individuals), have been described previously (13). Clinical, biochemical, and genetic studies excluded MEN1 and CNC in all cases. Families with affected individuals that had the same tumor type throughout were termed "homogeneous," and the remaining families had different or "heterogeneous" pituitary tumors among affected subjects.

From the original FIPA cohort, 51 families took part in the current study of *AIP* mutations. In addition, 22 new, previously undescribed FIPA families without MEN1 or CNC were identified and included in the study. Relevant data on demographics and clinical characteristics were collected for each affected member of each family, including age at diagnosis, tumor size, and if available, pituitary hormone immunohistochemistry. Age at diagnosis and mean maximum tumor diameter in the FIPA group overall and for AIP mutation-affected subjects only were calculated as means, medians, 95% confidence intervals, and SD. In families in which a mutation in *AIP* was noted, genetic analysis for this mutation was offered in other affected and unaffected family members; clinical, hormonal, and radiological (magnetic resonance imaging) assessment of individuals that were positive for an *AIP* mutation was also offered. The study was conducted in accordance with the guidelines in The Declaration of Helsinki, approved by the Ethics Committee of the University of Liège, and all subjects provided informed written consent in their own language for the genetic analyses performed during the study.

Statistical analyses

Statistical analyses were performed using GraphPad Instat for Macintosh (GraphPad Software, San Diego, CA). The Mann-Whitney test for univariate analyses, with a two-sided P value, compared data from continuous variables (*e.g.* age at diagnosis and maximum tumor diameter) from subgroups of patients with and without *AIP* mutations. Sex distribution and the proportions of patients with microadenomas and macroadenomas in the *AIP* mutation-positive and negative groups were analyzed using the Fisher's exact test, with a two-sided P value. A P value of <0.05 was considered significant for all analyses.

AIP genetic analysis

Genomic DNA was isolated from blood samples from at least one affected member of each FIPA family. The structure of *AIP* was based on Ensembl sequences ENST00000279146, ENSG00000110711, and ENSP00000279146. The primers used for the analysis of the *AIP* exonic and flanking intronic sequences are as reported by Vierimaa *et al.* (12). Each 25 μl PCR reaction contained 150 ng genomic DNA, 1 μM each primer, 1.5 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 8.3), 200 μM dNTPs, and 1.25 U FastStart *Taq* polymerase (Roche, Vilvoorde, Belgium). PCR conditions were 95 C for 10 min, followed by 30 cycles of 30 sec at 95 C, 30 sec at 68 C, and 20 sec at 72 C. PCR products were sequenced using ABI3100 and BigDye Terminator v3.1 technology (Applied Biosystems, Foster City, CA). A total of 100 blood samples from non-FIPA subjects in Belgium and France were analyzed to assess for polymorphisms in the *AIP* sequence.

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Accession numbers

The accession numbers in GenBank for the novel *AIP* mutations reported in this study are: EF066502 (R271W), EF066503 (E174frameshift), EF066504 (delG47-R54), EF066505 (K241E), EF066506 (Q142X), EF066507 (Q217X), EF066508 (Q239X), EF066509 (Q285frameshift), and EF066510 (R16H).

Results

Genetic screening

A total of 156 subjects were identified among 73 families with the FIPA phenotype (see supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Eleven of 73 (15.1%) FIPA families were found to have 10 different germline mutations in the *AIP* gene (Fig. 1). Of these, nine *AIP* mutations in 10 families have not been reported to date. The characteristics of FIPA families that had *AIP* mutations are detailed in Table 1. There were three novel mutations that led to premature stop codons: Q142X (c.424C>T), Q217X (c.649C>T), and Q239X (c.715C>T). In addition, one three-member family that had a previously described R304X mutation (12) was identified (c.910C>T). Three missense mutations, R16H (c.47G>A), R271W (c.811C>T), and K241E (c.721A>G), were identified in four FIPA families; R271W was found in two two-member families (Table 1). One two-member family had an in-frame G47_R54del (c.138_161del24) mutation. A frameshift deletion, E174frameshift (c.517_521delGAAGA), that led to a stop codon after 21 incorrect amino acids was identified in a family with three affected members. A second frameshift mutation in a two-member family, Q285frameshift (c.854_857delAGGC), was followed by a stop codon after 17 incorrect amino acids.

Characteristics of FIPA cohort

Demographic details and the phenotypic patterns of tumors seen are outlined in Tables 2 and 3, respectively. Briefly, families were divided equally (n = 78 each) among homogeneous and heterogeneous FIPA patterns; two-member homogeneous prolactinoma (n = 18) and somatotropinoma (n = 14) families were the most frequent. All but one heterogeneous FIPA family had at least one member with a prolactinoma or a somatotropinoma. Mean age at diagnosis was significantly lower in subjects with *AIP* mutations (n = 26 subjects) as compared with those without *AIP* mutations (n = 130 subjects) (25.7 ± 11.3 *vs.* 38.8 ± 16.8 yr, respectively; $P = 0.0006$). Mean maximum tumor diameter was significantly larger in the group with *AIP* mutations (24.6 ± 10.7 mm) than those without (14.5 ± 10.1 mm; $P = 0.0005$). Although the proportion of patients with macroadenomas was higher in the *AIP* mutation-positive group (88.5%) as compared with the *AIP* mutation-negative group (71.2%), this difference did not reach statistical significance.

AIP mutation screening in FIPA families

Family members of subjects with pituitary adenomas and *AIP* mutations were contacted whenever possible and underwent genetic screening. Subjects that were positive for an *AIP* mutation were offered clinical assessment and hormonal screening. A total of 45 apparently unaffected relatives were screened, and nine individuals (mean age 39.7 yr; range 16–71) from five different families were found to be positive for mutations in *AIP*. These asymptomatic subjects did not have signs or symptoms suggestive of pituitary tumors, while hormonal and radiological screening was unremarkable.

Discussion

This study involving an extensive cohort of 73 families having the FIPA phenotype has identified a total of 11 families having 10 mutations in the *AIP* gene; nine of these mutations were previously undescribed. The current study extends our

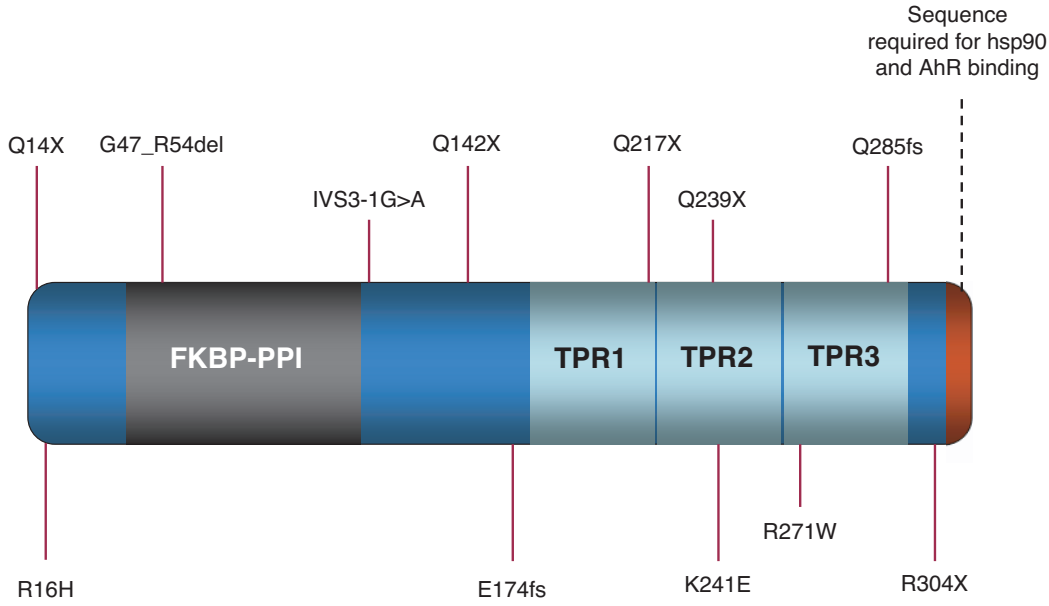

FIG. 1. Representation of *AIP* protein sequence with the position of gene mutations noted in the FIPA cohort and other studies indicated. The FKBP-PPI domain (amino acids 29–121) is shown in *gray*, and the three TPR domains (amino acids 189–296) are in *light blue*. The final carboxy-terminal amino acids that are necessary for interactions of *AIP* with hsp90 and AhR are shown in *orange*.

TABLE 1. Disease characteristics in 11 FIPA families having *AIP* mutations

AIP mutation	No. of affected members	Relation between members	Disease type	Age at diagnosis (yr)	Tumor characteristics	Preoperative hormonal profile	Immunohistochemistry
R16H	2	a. First cousin	Acromegaly	46	Micro	⇑GH, ⇑PRL	Not operated
		b. First cousin	Acromegaly	N/A	Micro	⇑GH/IGF-I	Not operated
G47_R54del	2	a. Brother	Acromegaly	28	Macro, invasive	⇑GH/IGF-I	N/A
		b. Brother	Acromegaly	25	Macro, invasive	⇑GH/IGF-I	N/A
Q142X	4	a. Brother	Gigantism	17	Macro	⇑GH	N/A
		b. Brother	Acromegaly	29	Macro	⇑GH	+GH
		c. Sister	Acromegaly	17	Macro	⇑GH	N/A
		d. Daughter of b.	Prolactinoma	N/A	Micro	⇑PRL	Not operated
E174fs	3	a. Brother	Acromegaly	17	Macro, invasive	⇑GH/IGF-I/⇑PRL	+GH/+PRL
		b. Sister	Acromegaly	25	Macro, invasive	⇑PRL/slight ⇑IGF-I	Not operated
		c. Aunt	Acromegaly	35	Macro, invasive	⇑GH/IGF-I/⇑PRL	Not operated
Q217X	2	a. Brother	Acromegaly	29	Macro, invasive	⇑GH/IGF-I, ⇑PRL	+GH, +PRL
		b. Sister	Acromegaly	24	Macro	⇑GH/IGF-I, ⇑PRL	+GH
Q239X	2	a. Father	Gigantism	14	Macro	⇑GH	N/A
		b. Son	Gigantism	15	Macro	⇑GH/IGF-I	+GH
K241E	2	a. Brother	Prolactinoma	40	Macro, invasive	⇑PRL	+PRL
		b. Sister	Nonsecreting	53	Macro, invasive	Hypopituitarism	+LH/αSU
R271W	2	a. Father	Acromegaly	42	Macro	⇑GH	N/A
		b. Son	Acromegaly	29	Macro	⇑GH/IGF-I	+GH
R271W	2	a. Mother	Acromegaly	22	Macro	⇑GH	+GH, −PRL
		b. Son	Prolactinoma	10	Macro	⇑PRL	N/A
Q285fs	2	a. Brother	Acromegaly	32	Macro, invasive	⇑GH/IGF-I, ⇑PRL	+GH, +FSH
		b. Brother	Gigantism	20	Macro, invasive	⇑GH	+GH, +PRL
R304X	3	a. Sister	Acromegaly	19	Macro, invasive	⇑GH/IGF-I, ⇑PRL	+GH
		b. Sister	Acromegaly	21	Macro, invasive	⇑GH/IGF-I	+GH/PRL
		c. Nephew of a.	Incipient gigantism	9	Macro	⇑GH/IGF-I	Not operated

N/A, Not applicable; PRL, prolactin; αSU, α-subunit.

understanding of the type of tumors associated with *AIP* mutations in the familial setting and increases the number of known *AIP* mutations associated with FIPA from three to 12. The clinical characteristics of this larger FIPA cohort are in line with our previous data indicating a relative predominance of prolactinomas and somatotropinomas in FIPA, and an early age at diagnosis, particularly in subjects with somatotropinomas (13). Furthermore, in this study we note that tumors in patients with *AIP* mutations have a significantly larger mean diameter than those in *AIP* mutation-negative patients, which could reflect a more aggressive disease profile. In the study by Vierimaa *et al*. (12) the pituitary tumors seen in families with *AIP* mutations were somatotropinomas or mixed GH/prolactin-secreting tumors. We found that while the majority of FIPA families with *AIP* mutations had somatotropinomas or mixed GH/prolactin-secreting tumors, one family included a subject with a nonsecreting tumor. This nonsecreting tumor was immunohistochemically negative for both GH and prolactin, and occurred in conjunction with a prolactinoma in the other affected family member. Hormonal patterns at diagnosis in "somatotropinoma" subjects with *AIP* mutations in FIPA families showed that 13 had GH hypersecretion, and eight had elevated GH and prolactin. The three subjects with prolactinomas had only hyperprolactinemia at diagnosis, while the subject with the nonsecreting tumor had hypopituitarism. An identical mutation (R271W) was associated with somatotropinomas in two adults in one family, and with a somatotropinoma (prolactin immunohistochemistry negative) and a macroprolactinoma (in a 10-yr-old child) in another family. Some heterogeneity in immunohistochemical patterns was also evident, with tumors from seven somatotropinoma patients having GH positivity only, four showing GH and prolactin staining, and one stained for GH and FSH.

Vierimaa *et al*. (12) undertook an extensive and detailed study of multiple genes to assess linkage to pituitary adenomas occurring in a familial setting, finally identifying *AIP* as being associated with pituitary adenomas in large, well-described kindreds in Finland. In that study an *AIP* mutation was identified in one family from Italy, but two other families with IFS from Germany and Turkey had normal *AIP* sequences (12). Our

TABLE 2. Demographic description of the FIPA cohort and the subgroup having *AIP* mutations

	AIP mutation positive	*AIP* mutation negative	*P* value
No. of families	11	62	
No. of subjects	26	130	
Sex			
Males, n (%)	15 (57.7)	57 (43.8)	NS
Females, n (%)	11 (42.3)	73 (56.2)	NS
Median age (yr) at diagnosis (mean ± SD)	24.5 (25.7 ± 11.3)	36.0 (38.8 ± 16.8)	0.0006
Median maximum tumor diameter (mm) (mean ± SD)	24.0 (24.6 ± 10.7)	10.0 (14.5 ± 10.1)[a]	0.0005
Macroadenomas, n (%)	23 (88.5)	89 (71.2)[a]	NS

NS, Clinically nonsecreting adenoma.
[a] Tumor size classification was not present for five individuals in the *AIP* mutation negative group.

TABLE 3. Tumor types in homogeneous and heterogeneous FIPA families overall and in the subgroup of families with *AIP* mutations

Tumor type	Affected members per family	No. of families in FIPA cohort	No. of affected subjects in FIPA cohort	No. of families with *AIP* mutations	No. of affected subjects with *AIP* mutations
Homogeneous FIPA families					
Prolactinoma	2	18	36	0	0
Somatotropinoma	2	14	28	6	12
	3	2	6	2	6
Cushing's disease	2	2	4	0	0
NS-adenoma	2	1	2	0	0
Gonadotropinoma	2	1	2	0	0
Homogeneous FIPA total		**38**	**78**	**8**	**18**
Heterogeneous FIPA families					
Prolactinoma-somatotropinoma	2	8	16	1	2
	4	1	4	1	4
Prolactinoma-NS-adenoma	2	8	16	1	2
	3	1	3	0	0
Somatotropinoma-NS-adenoma	2	6	12	0	0
Prolactinoma-somatotropinoma-NS-adenoma	3	2	6	0	0
Prolactinoma-gonadotropinoma	2	3	6	0	0
Somatotropinoma-prolactinoma-Cushing's disease	4	1	4	0	0
Somatotropinoma-prolactinoma-gonadotropinoma	3	1	3	0	0
Somatotropinoma-gonadotropinoma	2	1	2	0	0
Somatotropinoma-thyrotropinoma	2	1	2	0	0
Prolactinoma-Cushing's disease	2	1	2	0	0
NS-adenoma-Cushing's disease	2	1	2	0	0
Heterogeneous FIPA total		**35**	**78**	**3**	**8**

NS, Clinically nonsecreting adenoma.

data from screening a large, diverse population indicate that *AIP* mutations occur in about 15% of families in the FIPA cohort. The majority of FIPA families had normal germline *AIP* sequences, even those with three or four affected subjects. In particular, of the 16 FIPA families with homogeneous presentation of acromegaly (IFS), half were negative for *AIP* mutations, indicating that this gene does not readily explain IFS in its entirety. Other, as yet unidentified, genetic mutations may be involved in producing the FIPA clinical phenotype. The evidence to date suggests that mutations in *AIP* may be linked to the expression of a variety of tumor types. Although somatotropinomas predominate among FIPA families with *AIP* mutations, both pure GH and mixed GH-prolactin secretion and immunohistochemical staining occur commonly, even within the same family. Heterogeneous expression of tumors in FIPA tumor, including prolactinomas or nonsecreting adenomas, can occur in association with *AIP* mutations. The FIPA cohort contained few patients with less common pituitary tumors such as Cushing's disease and only one patient with a TSH-secreting adenoma; these were negative for *AIP* mutations. Therefore, it remains to be seen whether *AIP* mutations can also occur in families with Cushing's disease or TSH-secreting adenomas.

A Q14X mutation was the one most frequently seen in the Finnish patients studied, and both familial and sporadic cases were associated with this germline mutation; tumor analysis indicated loss of heterozygosity at the *AIP* locus (12). One other mutation, IVS3–1G>A, was reported in a sporadic case of acromegaly. In the current study these mutations were not identified in our international series of FIPA families. This, allied with the recent report of the absence of these mutations in sporadic pituitary tumor patients treated in the United States, suggests that these mutations may be characteristic of the Finnish population (14). This would not be unusual in terms of clinical genetics because Finland is known to be relatively genetically homogeneous and subject to founder effects (15). The role of extensive genealogic analysis such as that undertaken by Vierimaa *et al.* (12) to identify distant links among various affected families is important. We describe an Italian FIPA family with an R304X mutation (c.910C>T), the same mutation reported in an apparently unrelated family elsewhere in Italy (12). Further studies may highlight whether specific patterns of *AIP* mutations occur among specific geographical or cultural groups.

The impact of reported mutations in *AIP* on protein expression and function remains to be determined. Data on the structural components of AIP from *in vitro* studies provide some indicators in that regard. AIP is a protein of 330 amino acids in length, and contains conserved domains that include three tetratricopeptide repeat (TPR) domains and a FK506 binding protein-type peptidyl-prolyl cis-trans isomerase (FKBP-PPI) domain that is analogous to a related domain found in immunophilin proteins. Although the function of the FKBP-PPI domain remains to be determined fully, the importance of the "carboxy half" of AIP (residues 154–330) has been well established (16). Mutation studies of the third TPR domain have revealed that it is necessary for interactions with both heat shock protein 90 (hsp90) and the aryl hydrocarbon receptor (AhR) (17). Point mutations of the third TPR domain in murine AIP, including Y268A, G272D, G272E, A284T, and F288A, lead to an AIP that cannot coimmunoprecipitate hsp90; of these, Y268A and G272D cannot coimmunoprecipitate AhR (18). A further mutation, K266A, also abrogated hsp90 binding but retained AhR binding, albeit at a decreased level (19). Other studies that removed the last 32 amino acids from the C-terminal of AIP also prevented hsp90 binding, while the removal of the last 17 amino acids at the C-terminal led to rapid AIP turnover within COS-1 cells (20). Alanine replacement of any of the final four amino acids or

deletion of the final five amino acids at the C terminus of AIP prevents AhR binding (19).

In families with mutations that led directly to stop codons (Q142X, Q217X, Q239X, and R304X), the mutated gene would not encode the third TPR domain, the carboxy terminal amino acids, or both correctly (see supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Two other frameshift mutations (Q285fs and E174fs) also led to premature stop codons 17 and 21 amino acids downstream, respectively, and the loss of the sequences coding for the hsp90 and AhR interaction sites on AIP. The G47_R54del mutation, which read in-frame thereafter, would be expected to delete a series of amino acids within the FKBP-PPI domain, which could interfere with the enzymatic function of this region. In FIPA families with missense mutations of *AIP*, the functional impact is somewhat more difficult to predict. R271W, K241E, and R16H were not found in 100 non-FIPA individuals screened for *AIP* polymorphisms. Two unrelated FIPA families had an R271W mutation in *AIP*. This arginine is highly conserved across species, including the mouse, and forms part of the critical third TPR domain. As noted previously, mutation studies in this region in the mouse are known to abrogate hsp90 or AhR binding, or both (17). Given the sequence identity between the human being and mouse in this important region, it appears reasonable to suggest that R271W could interfere with the interaction of AIP and hsp90/AhR in these subjects. Both K241 and R16 are conserved amino acids across a variety of species; however, the impact of such mutations on the structural and functional status of AIP remains to be determined.

In conclusion, the current study shows that *AIP* mutations occur in 15% of families with the FIPA phenotype. *AIP* mutations that may abrogate expression or function of *AIP* protein could impact subsequent AhR responses to cellular and environmental signals, although *AIP* modulates a variety of other cellular signals (*e.g.* phosphodiesterases, cAMP) that may be involved in tumorigenesis. Experimental studies to assess AIP protein expression, receptor interactions, and xenobiotic responses will be useful in determining the precise effect on pituitary tumorigenesis of the multiple *AIP* mutations now identified.

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