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Original Research

Reassessing the role of the p.(Arg304Gln) missense *AIP* variant in pituitary tumorigenesis

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Abstract

Objective: Heterozygous germline loss-of-function variants in *AIP* are associated with young-onset growth hormone and/or prolactin-secreting pituitary tumours. However, the pathogenic role of the c.911G > A; p.(Arg304Gln) (R304Q) *AIP* variant has been controversial. Recent data from public exome/genome databases show this variant is not infrequent. The objective of this work was to reassess the pathogenicity of R304Q based on clinical, genomic, and functional assay data.

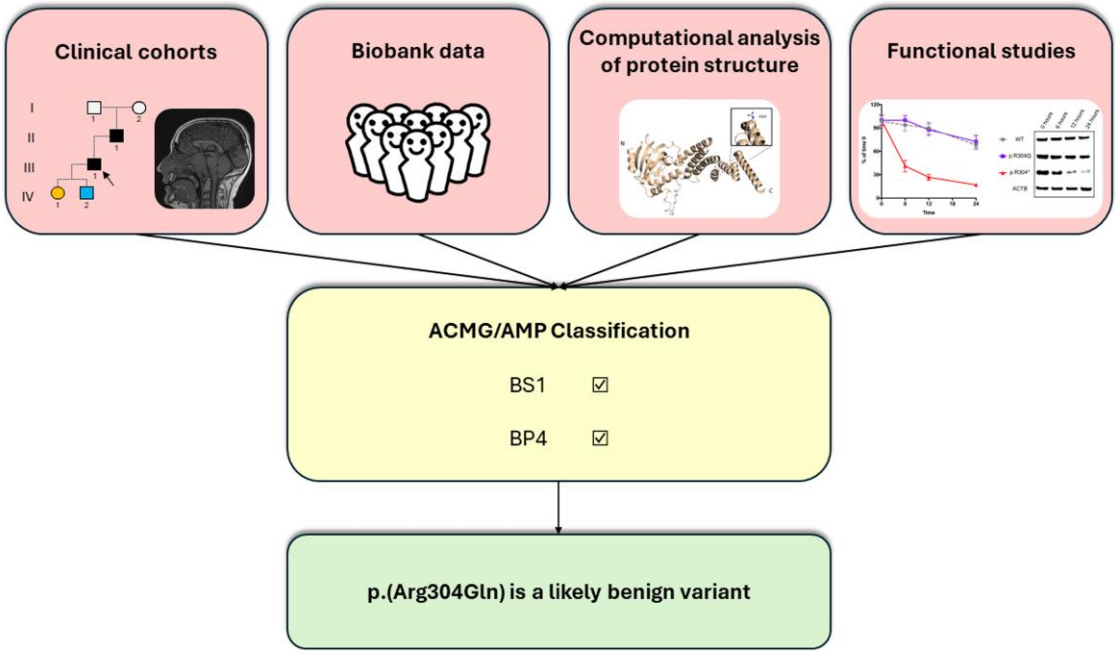
Design: Data were collected on published R304Q pituitary neuroendocrine tumour cases and from International Familial Isolated Pituitary Adenoma Consortium R304Q cases ($n = 38$, R304Q cohort). Clinical features, population cohort frequency, computational analyses, prediction models, presence of loss-of-heterozygosity, and *in vitro/in vivo* functional studies were assessed and compared with data from pathogenic/likely pathogenic *AIP* variant patients (*AIP*mut cohort, $n = 184$).

Results: Of 38 R304Q patients, 61% (23/38) had growth hormone excess, in contrast to 80% of *AIP*mut cohort (147/184, $P < .001$). R304Q cohort was older at disease onset and diagnosis than the *AIP*mut cohort (median [quartiles] onset: 25 y [16-35] vs 16 y [14-23], $P < .001$; median [quartiles] diagnosis: 36 y [24-44] vs 21 y [15-29], $P < .001$). R304Q is present in gnomADv2.1 (0.31%) and UK Biobank (0.16%), including three persons with homozygous R304Q. No loss-of-heterozygosity was detected in four R304Q pituitary neuroendocrine tumour samples. *In silico* predictions and experimental data were conflicting.

Conclusions: Evidence suggests that R304Q is not pathogenic for pituitary neuroendocrine tumour. We recommend changing this variant classification to likely benign and do not recommend pre-symptomatic genetic testing of family members or follow-up of already identified unaffected individuals with the R304Q variant.

Keywords: genetic variant, gigantism, acromegaly, prolactinoma, AIP, FIPA

Graphical Abstract



Significance

Clinicians initiating genetic testing need to understand the principles of investigating a "variant of uncertain significance" and appreciate that this could be a dynamic process. This manuscript details the history of a variant starting as a likely pathogenic variant and ending up as likely benign change. Our approach to variant evaluation included interpretation of allele frequency, computational analyses, prediction models, loss-of-heterozygosity, as well as *in vitro* and *in vivo* functional studies of the p.(Arg304Gln) *AIP* variant. Comparison of the clinicopathological characteristics of pituitary patients with this change to a large cohort of patients with pathogenic/likely pathogenic *AIP* variants further supported the data. Our data will help endocrinologists encountering the p.(Arg304Gln) *AIP* variant and will impact follow-up for individuals carrying the variant.

Introduction

Familial isolated pituitary adenoma (FIPA) accounts for an estimated 2% of all pituitary neuroendocrine tumours (PitNETs).[1] Ten percent of FIPA cases are caused by loss-of-function germline variants in the aryl hydrocarbon receptor interacting protein (*AIP*) gene, which are often associated with an invasive, treatment-resistant phenotype, particularly affecting young patients.[2,3]

The *AIP* gene (RefSeq NM_003977.4) is ubiquitously expressed and conserved amongst species. Approximately 180 sequence variants have been reported in individuals with PitNETs, including common single nucleotide variants, pathogenic or likely pathogenic (P/LP) variants, as well as numerous variants of uncertain significance (VUS).[1,4,5] *AIP* encodes a co-chaperone, with many interacting partners, whose role in various physiological processes is probably tissue and developmental stage-dependent. *AIP* behaves as a tumour suppressor gene in the pituitary, with loss-of-heterozygosity (LOH) studies often displaying loss of the wild-type allele in PitNETs.[6] Over two-thirds of *AIP* disease-causing variants result in truncated proteins that lose their ability to interact with partner proteins, or in a lack of protein expression due to large deletions or promoter defects.[7,8] The P/LP missense variants typically result in misfolding and undergo rapid degradation.[7,8] Interestingly, in other tissues, *AIP* may behave as an oncogene, including AIP in the unique group of proteins that display both tumour suppressor and pro-oncogenic abilities.[9]

The most common mutational hotspot affects the R304 residue of the AIP protein (UniProt O00170). Base pair changes have been observed affecting both bases of the c.910_911 CpG locus, with the transition c.910C > T resulting in the p.(Arg304Ter) nonsense pathogenic variant (p.(R304*), dbSNP rs104894195, abbreviated to R304* in this manuscript), and the transition c.911G > A resulting in an arginine changing to a glutamine, the p.(Arg304Gln) (p.(R304Q), dbSNP: rs104894190, abbreviated to R304Q in this manuscript) missense variant. However, while the pathogenic role of R304* is beyond doubt,[1,3,10-13] the clinical significance of R304Q is questionable. On one hand, several FIPA kindreds and simplex PitNET cases have been described harbouring R304Q.[1,12,14-26] On the other hand, this variant is not infrequently found in the general population as a heterozygous change and has been described in four subjects in homozygosity.[27] The latter observation is unexpected for a pathogenic variant, considering the lethality of complete *AIP* knockout in animal models.[28-30] To further complicate the matter, *in silico* analyses and functional studies have rendered mixed results regarding the likely pathogenicity of R304Q.[8,31-33]

The ambiguous status of R304Q can pose a challenge for the clinician, who needs to consider possible benefits of running potentially unnecessary genetic tests in family members, as well as clinical, biochemical, and radiological follow-up in subjects with a potentially benign variant.[34-36] These decisions have implications for time and costs to the health system and may cause anxiety for individuals and their families. Therefore, we reassessed the pathogenic potential of R304Q, considering published and novel clinical, genetic, computational, and experimental data. By collating these data, we aimed to clarify the role of R304Q in pituitary tumorigenesis.

Patients and methods

Patients, samples, and clinical information

We analysed clinical, genetic, and histopathological data from PitNET patients with the germline *AIP* R304Q variant and compared them to patients in the International FIPA Consortium (IFC) with *AIP* variants classified as P/LP according to the American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG/AMP) guidelines.[34] All IFC cohort participants gave written informed consent approved by the Cambridge East Research Ethics Committee (MREC 06/Q0104/133); this study complies with the Declaration of Helsinki. Inclusion/exclusion criteria, recruitment process, and variant classification methods for the IFC have been reported previously.[3,4] A search (terms: FIPA, familial isolated pituitary adenoma, AIP, Arg304Gln, R304Q, familial acromegaly, familial prolactinoma) was conducted to identify patients with PitNETs with R304Q reported in the literature (completed 01/09/2023). Clinical data were obtained from centres reporting those cases, where available. Gigantism was defined as previously described.[4]

From our IFC cohort, we identified the following:

(i) Six familial and five simplex (simplex being a clinical assertion, defined as a patient with germline variant but no known affected family members at the time of endocrinological diagnosis) R304Q kindreds (11 R304Q kindreds).
(ii) *AIP*mut cohort: 45 familial and 54 simplex; 99 *AIP*mut kindreds with P/LP *AIP* variants, representing 184 patients with pituitary disease. These patients with P/LP *AIP* variants were divided into "non-truncating" (9 families and 22 simplex cases, total 42 subjects) and "truncating" (36 families, 32 simplex cases, total 142 subjects), according to variant effect (Table S1). Prospectively diagnosed patients were omitted from age-related analyses.

LOH analysis by bidirectional Sanger sequencing of *AIP* exon 6 (primers: 5′-GTGTGGAATGCCCAGGAG-3′ and 5′-CAGAAGCATGACGCAGCA-3′) was carried out in DNA extracted from four PitNET samples (Cases 1, 5, 9, and 13) as previously described.[4]

Patients with R304Q and other tumour types

Subjects harbouring the germline R304Q variant were identified in the MI-ONCOSEQ study.[37] An independent patient, who underwent exome sequencing due to young-onset breast cancer, was also included (Table S2).

Genetic and protein structure assessments

We assessed R304Q frequency in the Genome Aggregation Database (https://gnomad.broadinstitute.org),[27] gnomAD v2.1, $n = 139\,782$ (this version does not contain the United Kingdom Biobank [UK Biobank] data) and in individuals from the UK Biobank ($n = 454\,666$; https://www.ukbiobank.ac.uk).[38]

To assess the impact of R304Q on protein structure and folding, the experimental structure of AIP (Protein Data

Bank, PDB:7ZUB) and the AlphaFold three-dimensional AIP protein model were used.[39,40] The structural impact of R304Q was assessed using the in-house algorithm Missense3D.[41] The analysis was then refined manually to assess the impact of the amino-acid substitution on AIP protein folding as well as on AIP interaction with other proteins using available 3D structure of AIP alone and in complex (PDBs 4APO, 4AIF, 2LKN, and 7ZUB).[7,42] *In silico* data on R304Q variant effect were assessed using computational tools (Table S2), including deep-learning methods Eve[43] and AlphaMissense.[44] Protein structures were visualized using Pymol visualization program (http://www.pymol.org/).

UK biobank phenotype assessment

We used self-reported data (fields 20 002 and 20 004), first occurrence, death registry, cancer registry, and Hospital Episode Statistics, including surgical data, to identify individuals with pituitary disease. For hyperpituitarism, we used ICD-10 codes E22.0, E22.8, E24.0, E24.1, E22.1, and E22.9, excluding individuals with E22.2. For hypopituitarism, we used ICD-10 code E23. For pituitary adenoma, we used codes D44.3, C75.1, and D35.2. To identify individuals who had undergone pituitary surgery, we used OPCS-4 codes B01.1, B01.2, B01.4, B01.8, B01.9, B04.1, B04.2, B04.3, B04.4, B04.8, and Z14.1.

Experimental studies

Protein half-life data[8] and interactions of AIP with PDE4A5,[15,17,32,45] PDE4A8,[32] and RET[16,46] were extracted from previous publications. Immunostaining for PDE4A5 and PDE4A8 for samples with R304Q were analysed during the original study,[32] but R304Q data were not reported in that publication. We used previously published experimental data from our *Drosophila melanogaster* model for the rescue experiment regarding R304Q.[29]

Co-immunoprecipitation of R304Q and heat-shock protein family member HSPA8 was performed, as previously described.[8,31] For assessing *Cyp1a1* mRNA expression response of GH3 rat somato-mammotroph cell line with *Aip* knockdown, we used real-time quantitative PCR. We depleted *Aip* from rat somatotrophinoma GH3 cells (ECACC, Porton Down, UK, 87012603) using lentiviral small hairpin RNA (shRNA) with a non-targeting shRNA as control (SirionBiotech, Germany), as previously described.[47] Wild-type, R304* and R304Q *AIP*-containing Myc-tagged pcDNA3.0 plasmids were transfected using Lipofectamine 2000, and transfection efficiency was assessed by co-transfection of the GFP-expressing pDNA vector pZs-Green (Clontech, Mountain View, CA, USA). Forty-eight hours after transfection, the cells were treated for 5 h with dimethyl sulfoxide (DMSO, 10^{-5} M) or 6-formylindolo[3,2-b]carbazole (FICZ, 10^{-8} M, Sigma-Aldrich, UK). Cells were harvested and total RNA extracted using the GeneJet RNA purification kit (Thermo). Reverse transcription was performed using an M-MuLV reverse transcriptase mix (New England Biolabs, UK). Real-time quantitative PCR reactions were prepared with SYBR Select Master Mix (Thermo), and relative expression levels of *Cyp1a1* were quantified using ddCT threshold method[48] and normalized to housekeeping gene *Gapdh* (primers available on request).

Statistics

Statistical analyses were conducted using GraphPad Prism 10.1.1. Categorical variables were compared by the chi-square or Fisher's exact test, as appropriate. Normal distribution was checked using Shapiro–Wilk test. Quantitative variables were expressed as median and interquartile ranges or mean and standard error of the mean, unless otherwise stated. Comparisons were done by means of the independent samples T-test with Welch's correction or ANOVA/Kruskal–Wallis test followed by the Tukey–Kramer/Dunn's *post-hoc* tests, as appropriate. A P value <.05 was taken as significant.

Results and data extracted from previously published data on R304Q

Clinical parameters

Thirty-eight patients with PitNETs harbouring germline R304Q (17 within the IFC, 21 from the literature) and 28 clinically unaffected individuals with R304Q (17 within the IFC, 11 from the literature) were identified (Table S3). This "R304Q cohort" included six kindreds with two affected individuals with pituitary tumours and 26 probands with apparently sporadic presentation ('simplex" cases; Figure 1A and B). We compared their data with 45 families and 54 simplex cases in the *AIP*mut cohort within the IFC (Table 1), comprising 184 patients affected with pituitary disease, 142 with truncating variants and 42 with non-truncating variants (Table S1). We report three novel P/LP *AIP* variants c.85C > T, p.(Gln29*), c.439C > A, p.(Pro147Thr), and c.946_948del, p.(Lys316del).

There were significantly more males in the *AIP*mut compared with the R304Q cohort (Table 1, $P = .012$). The proportion of familial cases in the R304Q cohort was significantly lower than that of the *AIP*mut subgroup (12/38, 32% vs 130/184, 71%, $P < .001$). This significance persisted when comparing the R304Q cohort with the truncating *AIP*mut subgroup (12/38, 32% vs 110/142, 77%, $P < .001$) but not with the non-truncating *AIP*mut subgroup (12/38, 32% vs 20/42, 48%, $P = .174$). In the R304Q cohort, 23/38 (61%) patients had growth hormone (GH) excess including four with gigantism, nine prolactinomas, four clinically non-functioning PitNETs and two Cushing's disease. In contrast, the *AIP*mut cohort was composed of 147 subjects with GH excess (80%, including acromegaly, acromegaly with hyperprolactinaemia, gigantism, and gigantism with hyperprolactinaemia), 20 prolactinomas (10.9%), and 17 (9%) clinically non-functioning PitNETs (13 of these prospectively diagnosed). There was no patient with an *AIP*mut and Cushing's disease. Therefore, the proportion of GH excess patients was significantly different between the two cohorts ($P = .019$).

For R304Q patients, the median age at diagnosis and median age at disease onset were significantly higher than the *AIP*mut cohort (Table 1, $P < .001$ and $P < .001$, respectively). There were significantly more gigantism cases in the GH excess subgroup of the *AIP*mut cohort than in R304Q GH excess cohort (Table 1, $P = .002$).

When omitting prospectively-diagnosed cases, there were significantly more macroadenomas (maximal tumour diameter ≥10 mm) in the *AIP*mut compared with the R304Q cohort (Table 1, $P = .034$). Extrasellar extension did not show a significant difference between R304Q and the non-prospectively diagnosed *AIP*mut cohort (Table 1, $P = .792$). Non-prospectively diagnosed *AIP*mut patients did not require significantly more treatments than R304Q patients (Table 1,

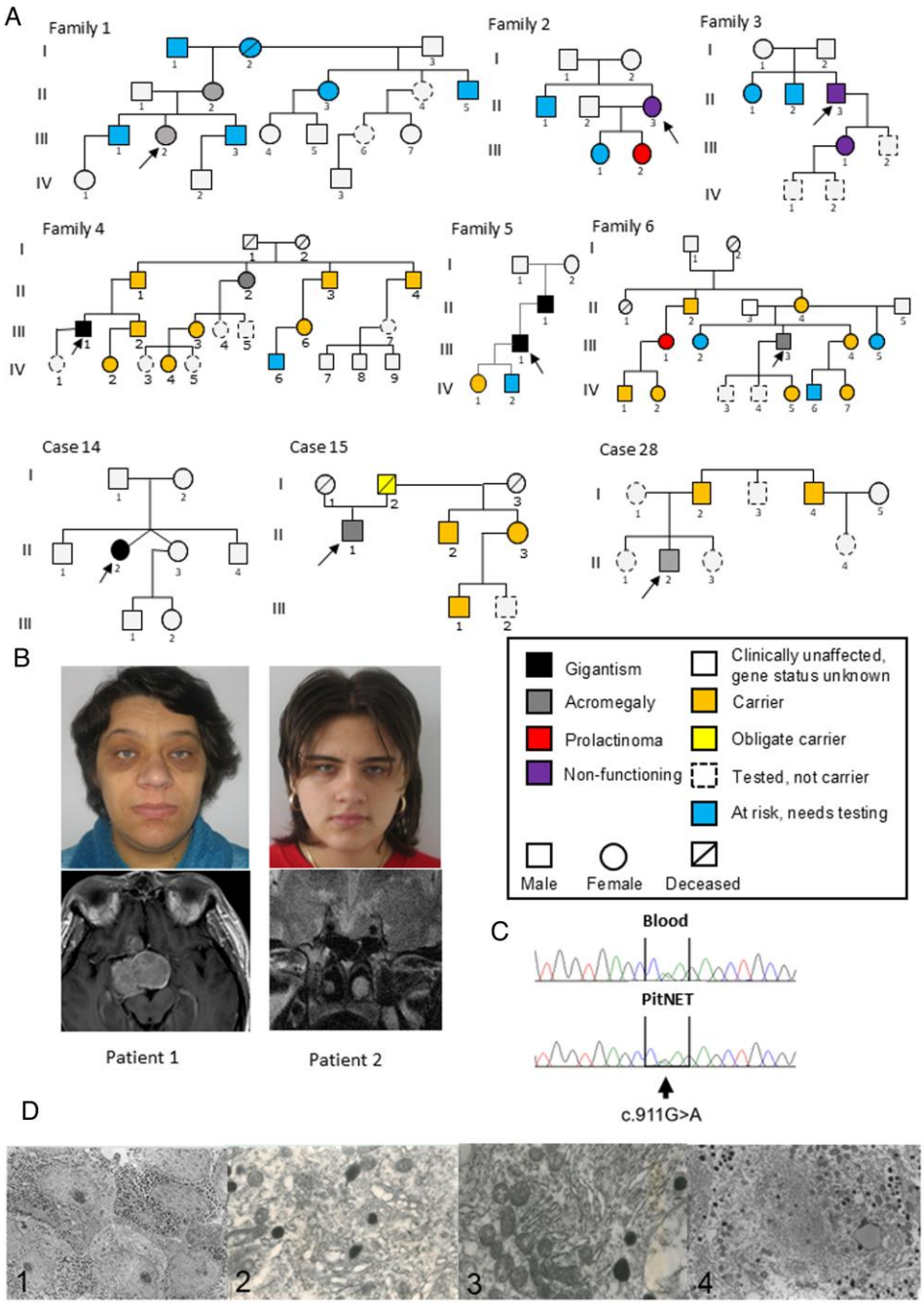


Figure 1. Clinical presentation and genetic findings in R304Q patients from the IFC cohort. A) Family trees of the 6 FIPA families with the *AIP* R304Q variant and of 3 representative simplex patients within the IFC. B) Affected members of Family 1. The proband (Patient 2) was diagnosed with gigantism at age 17 years, caused by a pituitary macroadenoma with cavernous sinus extension. Her mother (Patient 1) was diagnosed with acromegaly at the age of 44 years; her magnetic resonance imaging showed an invasive pituitary macroadenoma (maximum diameter: 46 mm) with cavernous sinus and suprasellar extension, resulting in a visual field defect. Written informed consent for publication of their clinical details and clinical images was obtained from the patients. C) Sanger sequencing of the R304Q surrounding sequence displayed no LOH in DNA extracted from the tumour, compared with germline DNA. D) Electron microscopy images of one somatotrophinoma associated with R304Q (Case 16). Tumour cells have secretory granules that vary from sparse to numerous and vary in size, shape, and electron density (d1). The cells display a prominent Golgi apparatus (d2), with prominent profiles of rough endoplasmic reticulum (d3). The secretory granules vary in size, shape, and electron density. Occasional cells have cytoplasmic fibrous bodies, as seen centrally (d4).

$P = .151$). Comparable results were obtained when considering the GH excess R304Q subgroups vs non-prospectively diagnosed *AIP*mut (mean 2.2 vs 2.5, $P = .429$). There were significantly more affected individuals per kindred between R304Q and *AIP*mut cohorts (1.2 vs 1.9, $P < .001$). Penetrance could not be calculated due to low numbers.

Four PitNET samples from R304Q heterozygote patients underwent LOH analysis (Table S3: Cases 1_F1, 11_F6, 5_F3 and 13). No LOH was detected in any samples (example Figure 1C). Electron microscopy of one R304Q positive somatotrophinoma showed variable numbers of secretory granules and occasional fibrous bodies (Figure 1D); all the samples with

Table 1. Comparison of clinical features of patients with R304Q and pathogenic or likely pathogenic truncating or non-truncating AIP variants.

Clinical feature	*AIP*mut cohort (truncating and non-truncating)	truncating	Non-truncating	R304Q	*P* value truncating vs non-truncating	*P* value R304Q vs all *AIP*mut	*P* value R304Q vs non-truncating	*P* value R304Q vs truncating
Sex F:M	74:110	64:78	10:32	24:14	.019	.012	<.001	.067
Age at disease onset, median, years (IQR)	16 (14-23)	16 (14-23)	17 (13-21)	25 (16-35)	.912	<.001	.002	.001
Age at diagnosis, median, years (IQR)	21 (15-29)	21 (15-31)	21 (16-27)	36 (24-44)	.975	<.001	<.001	<.001
Gigantism, % of GH excess	53.1	56.0	40.5	17.4	.261	.002	.057	.001
Macroadenoma, % of total	92.5	91.3	93.9	78.4	>.999	.034	.090	.074
Extrasellar extension, % of total	76.2	78.0	72.0	72.0	>.999	.792	>.999	.583
Average number of treatments per patient	2.37	2.06	2.47	1.97	.176	.151	.145	.781

*AIP*mut, pathogenic or likely pathogenic *AIP* variant; IQR, interquartile range.

R304Q where data are available showed a sparsely granulated pattern, similar to the majority of tumour samples with P/LP *AIP* variants.

R340Q variant frequency in control populations

The frequency of heterozygous R304Q individuals in gnomAD v2.1 was approximately 0.3%. The frequency in UK Biobank was lower at 0.16% (Table S4). This variant showed a founder effect in Ashkenazi Jews, where it was almost 15-fold higher than in those of European ancestry (Table S4). There were homozygous individuals in Ashkenazi Jews ($n = 2$ gnomAD v2.1 and $n = 1$ UK Biobank), in line with the higher frequency in this population ($P > .9$ for Hardy-Weinberg equilibrium for both cohorts).

Phenotype in cohorts without clinical selection of pituitary disease

*AIP*mut screening of 132 patients with sporadic parathyroid adenomas found two patients with germline R304Q.[49] They had no family history of primary hyperparathyroidism, PitNETs or other endocrine tumours. One R304Q patient's offspring had a normal serum calcium and parathyroid hormone and no pituitary disease. In the MI-ONCOSEQ study (1580 paired germline and tumour samples from cancer patients), eight patients were heterozygous for germline R304Q. They had various tumour types (Table S5). None of their tumours displayed LOH. An additional patient with young-onset breast cancer was identified with a germline R304Q, but breast tumour tissue was not available for study. None of these subjects had personal or family history of PitNET. No other studies investigating *AIP* sequence variation in non-pituitary tumours identified R304Q.[50-52]

We observed in a clinically-unselected population cohort ($n =$ 454 666, UK Biobank study) that heterozygous R304Q individuals ($n = 705$) were not associated with any pituitary disease (0.1% in both groups, $P = .6$; Table 2). Limiting the analysis to individuals who were genetically similar to Ashkenazi Jews, where this variant is most common (3.4%), showed similar results (Table 2). We identified one 54-year-old female individual homozygous for R304Q in UK Biobank. This individual was of Ashkenazi Jewish ancestry and did not report having pituitary disease. UK Biobank data showed the prevalence of R304Q is not significantly higher in patients with pituitary disorders compared with controls (Table 2).

Protein modelling and potential functional effects of R304Q

In silico predictions regarding R304Q variant pathogenicity, using multiple prediction platforms, showed variable results (Table S2). Based on protein modelling, R304Q is unlikely to alter the correct folding of AIP 3D structure (Figure 2A). In line with these findings, we previously demonstrated that multiple P/LP *AIP* variants display significantly reduced half-life *in vitro* compared with wild-type AIP protein.[8] In contrast with the unstable behaviour of the pathogenic R304*; however, the half-life of R304Q was comparable to that of the wild-type protein, (Figure 2H, data from previous study[8]).

Nevertheless, due to its location in the C-terminal α-helix of the protein (Figure 2B), an amino-acid change affecting the highly conserved R304 residue (Figure 2C) may affect AIP's interaction with HSP90/HSP70 or TOMM20 peptides by

Table 2. Clinical characteristics of subjects with or without heterozygous R304Q in 454 665 individuals in UK Biobank.

Clinical characteristics	Heterozygous R304Q individuals $n = 705$	Non R304Q individuals $n = 453\,960$	*P* value
Age at recruitment, year	57.1 (7.8)	57.0 (8.1)	.78
Female	314 (55)	246 255 (54)	.52
Ancestry			$<1 \times 10^{-83}$
European	591 (83.9)	419 210 (92.4)	
Ashkenazi Jews	80 (11.3)	2240 (.5)	
South Asian	1 (0.1)	9537 (2.1)	
Africans	0 (0)	7393 (1.6)	
Others	33 (4.7)	15 580 (3.4)	
Hyperpituitarism	1 (0.1) (patient with acromegaly)	602 (0.1)	.60
Hypopituitarism	2 (0.3)	1236 (0.3)	.71
Pituitary surgery	1 (0.1)	644 (0.1)	1
Pituitary adenoma	1 (0.1)	930 (0.2)	1
Any pituitary disorder	4 (0.5)	2170 (0.5)	.59
Any pituitary disorder in Ashkenazi Jew ancestry	1 (1.2)	21 (0.94)	.54

Data shown as mean (SD) for continuous variable and *n* (%) for the categorical variables.

introducing a neutrally charged residue at codon 304, thus affecting its function (Figure 2D). Based on our previously reported crystal structure studies, however, binding of the R304Q protein to HSP90β, HSP70, and TOMM20 is not predicted to be altered in comparison with the wild-type protein.[7]

A GST pull-down screening for AIP-interacting partners in GH3 cells demonstrated reduced interaction of R304Q with some AIP partner proteins, but the R304Q variant protein conserved important interactions that are impaired by R304*.[8] In particular, quantitative mass spectrometry suggested that the interaction of R304Q with HSPA8 is reduced, compared with R304*, but not lost.[31] To further test these findings, a co-immunoprecipitation experiment of R304Q and HSPA8 was performed, showing this interaction is conserved (Figure 2E).

Phosphodiesterase 4A5 (PDE4A5) is another important interacting partner of AIP. Using the yeast two-hybrid β-galactosidase assay, we demonstrated this interaction is lost for multiple mutant AIP proteins.[15,17,45] By this method, R304Q displays a borderline significant reduced interaction compared with wild-type AIP ($P = .062$).[45] In a different study, the expression of phosphodiesterase 4A4 (PDE4A4) and phosphodiesterase 4A8 (PDE4A8) were analysed in human somatotrophinomas with confocal microscopy and immunofluorescence.[32] *AIP*mut-positive PitNETs were associated with low levels of PDE4A4 and PDE4A8 expression. By contrast, the R304Q variant was found to present with a high expression of PDE4A5 and PDE4A8, as seen in *AIP* wild-type PitNETs (Figure 2F and 2G).[32]

A modified yeast two-hybrid test demonstrated interactions between the RET proto-oncogene isoforms RET51 and RET9 and AIP. Various missense *AIP* variants, including R304Q, were tested with the same method, but none of them impaired the interaction of AIP with the two RET isoforms; the mutant RET protein also displayed intact AIP binding.[16] More recent evidence demonstrated that R304Q prevented RET-dependent apoptosis in the somatotroph GH4C1 cell line and in a primary pituitary cell culture.[46] In contrast, in a mouse model virally injected with R304Q AIP, there were significant decreases in the phospho-PKCδ/β-actin ratio, phospho-PKCδ/PKCδ ratio, and intracellular RET. These R304Q-injected mice developed pituitary hyperplasia and an acrogigantism phenotype, in this instance supporting R304Q pathogenicity.[46]

One of the interacting partners of the AIP-HSP90 complex is AHR. AHR stimulates the transcription of enzymes involved in xenobiotic metabolism in the liver, for example, CYP1A1 and CYP1A2.[53] The effect of *AIP* overexpression on AHR transcription activity was investigated with an *Aip*-knockdown model in GH3 cells. Overexpression of wild-type human *AIP* resulted in a significant increase in AHR transcriptional activity, as reflected in increased *Cyp1a1* mRNA levels (relative expression, empty vector 1 ± 0.07 vs wild-type *AIP* 2.22 ± 0.12; $P < .01$). Overexpression of human R304* *AIP* resulted in reduced *Cyp1a1* expression (1.34 ± 0.15) compared with wild-type *AIP*, whereas the effect of R304Q (1.65 ± 0.20) was intermediate between wild-type (2.2 ± 0.12) and R304* (1.34 ± 0.15), suggesting potential partial loss-of-function (Figure 2I).

Knockdown or knockout of the *CG1847* gene, the orthologue for human *AIP* gene in *D. melanogaster,* induces lethality.[29] The wild-type human *AIP* can rescue the lethality of the *CG1847* knockout, confirming that it is a functional homologue of *AIP*. We have previously shown that while the C-terminally-truncated human *AIP* mutant and the pathogenic p.(C238Y) missense variant did not rescue fruit fly lethality, insertion of the R304Q variant in CG1847 knockout animals resulted in viable fruit flies.[29] These data suggest the protein encoded by R304Q retains a function of the wild-type protein that is required for early development in this animal model.

American College of Genetics and Genomics and Association for Molecular Pathology classification

The ACMG/AMP classification of R304Q in the ClinVar platform (VCV000004893.33) reports conflicting interpretations of pathogenicity, with six entries classifying it as a VUS, four considering it a likely benign (LB) variant, and one classifying it as benign. We applied the ACMG/AMP criteria to the data available for R304Q (Table S6). No ACMG criteria supporting a (likely) pathogenic could be applied; therefore, these criteria are insufficient to classify R304Q as pathogenic or likely pathogenic. When applying criteria for benign variants, one strong (BS1) and one supporting (BP4) criteria are true for R304Q; the supporting criterion BS3 was dubious. Based on BS1 and BP4 and questionable BP4 and BS3, currently R304Q can be classified as likely benign (LB).

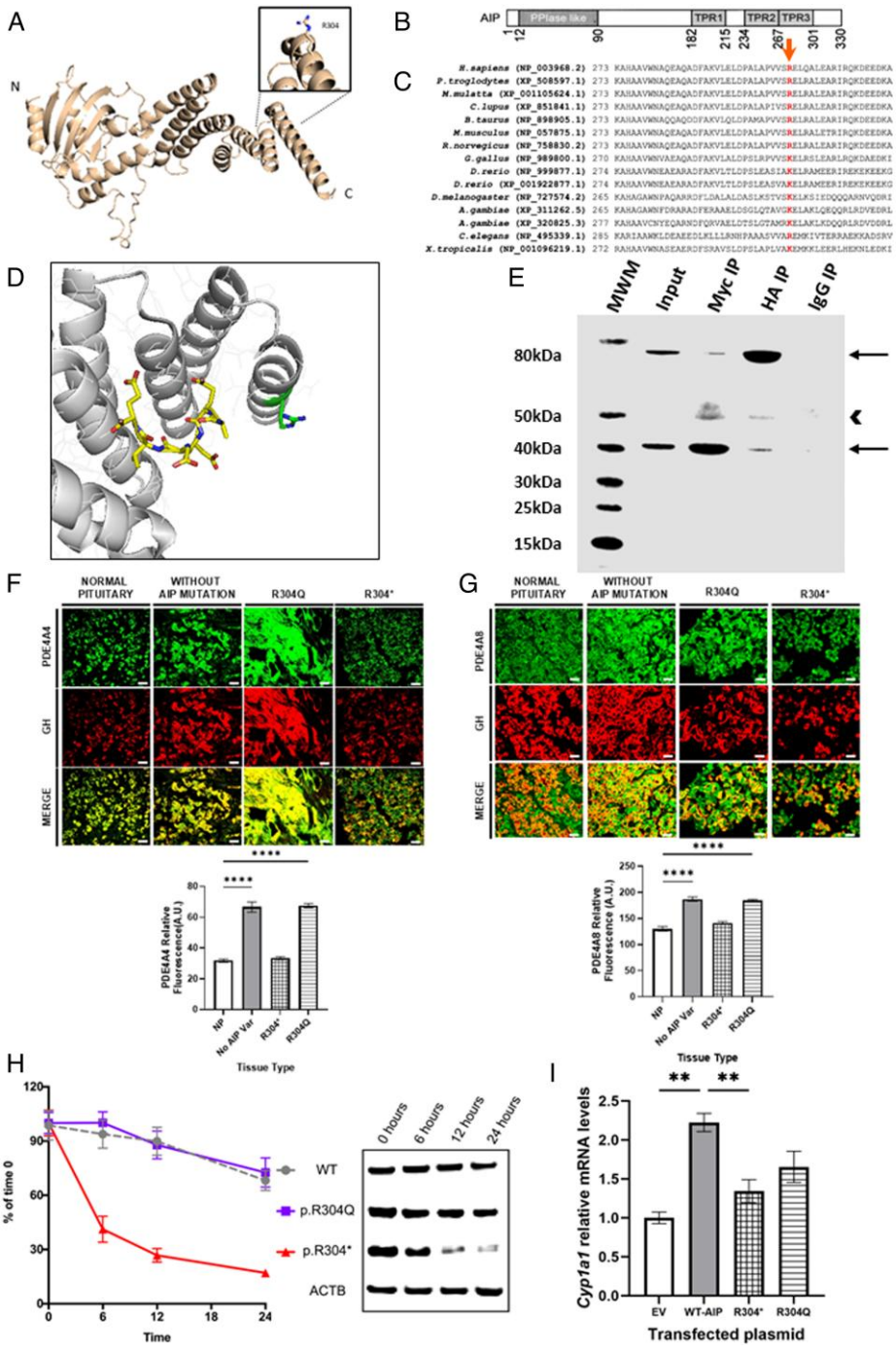


Figure 2. AIP R304Q protein structure and functional assays. A) Three dimensional and B) linear structure of AIP protein with close-up of location of R304Q (insert). The *AIP* co-chaperone protein (330 amino acids) has a peptidyl-prolyl ci-trans isomerase (PPIase)-like domain at the N-terminus, 3 tetratricopeptide domains arranged as antiparallel alpha helices, and a final α−7 helix at the C-terminus. The helical structures enable AIP to interact with numerous partners, including HSP90, AHR, and phosphodiesterases. C) Position 304 is conserved and occupied by the positively charged residues arginine (R) or lysine (K) in the multiple sequence alignment. D) Model of interaction of AIP variant R304Q with Tomm20 protein. AIP is presented in grey, and part of the Tomm20 AQSLAEDDVE-peptide in yellow. Residue Arg304 is presented in green. E) R304Q maintains its interaction with HSPA8. HEK293 cells were co-transfected with pcDNA3.0-Myc-AIP_ R304Q and pSF-CMV-NH2-HA-EKT-NcoI-HSPA8, and immunoprecipitation was performed using anti-Myc or anti-HA mouse antibodies, or mouse IgG. Eluates were resolved by denaturing polyacrylamide gel electrophoresis, followed by Western blot, using anti-Myc and anti-HA antibodies. Top arrow: HA-HSPA8, bottom arrow: Myc-AIP. Arrowhead: heavy chain of mouse immunoglobulins. F) Fluorescence double immunostaining of GH and PDE4A8 in normal pituitary, a tumour without *AIP* variant (no AIP Var), a tumour with R304* variant and a tumour with R304Q variant; scale bar 25 µm. ****$P < .0001$ Kruskal–Wallis followed by Dunn's test. G) Fluorescence double immunostaining of GH and PDE4A4 in normal pituitary, a tumour without AIP variant (No AIP Var), a tumour with R304* variant and a tumour with R304Q variant; scale bar 25 µm. ****$P < .0001$ Kruskal–Wallis followed by Dunn's test. H) Half-life of wild-type AIP and of the AIP variant proteins R304Q and R304*, overexpressed in HEK293 cells. The degradation speed of the R304Q variant ($K = .0118$) was not significantly different to that of the wild-type protein ($K = 0.0145$, $P = .5644$) while the variant R304* showed rapid degradation compared with the wild-type protein ($K = 0.1183$, $P < .0001$). The representative WB images show bands for Myc-AIP wild-type and R304Q (39 kDa), Myc-AIP R304* (35.8 kDa) and ACTB loading control (41.7 kDa); data extracted from.[8] I) *Cyp1a1* relative mRNA levels in *Aip* shRNA-KD GH3 cells treated with 10 nm FICZ for 5 hours (EV: empty vector, $n = 3$; WT-AIP: wild-type AIP, $n = 6$; R304*, $n = 9$; R304Q, $n = 6$). Error bars indicate SEM. ANOVA followed by Tukey–Kramer honest significant difference post-hoc test (**$P < .01$). MWM, molecular weight marker; IP, immunoprecipitation.

Discussion

Determining whether a gene variant is indeed disease-causing or whether it is unlikely to play a role in disease development is challenging, as an ever-increasing amount of sequence data are available. We have previously shown that early diagnosis and careful follow-up of individuals with *AIP*mut can improve their prognosis, as those who develop PitNETs can potentially be treated at an early disease stage.[3,4,54] This precision medicine-based approach requires an accurate classification of gene variants.

R304Q was first reported in two independent patients with acromegaly and Cushing's disease. As it was not detected in 255 controls, it was suggested it was pathogenic.[14] The first FIPA kindred where R304Q was reported consisted of two affected individuals, both with large, young-onset somatotrophinomas (Family 1, Figure 1A and B).[15] In this and other FIPA families subsequently reported, R304Q segregated with pituitary disease.[15,17,26] In the large Danish kindred reported, 14 clinically unaffected family members heterozygous for the R304Q variant have been followed up for 10 years and remain clinically unaffected.[26] Family 5 in the current study has two childhood-onset GH excess patients with no other features suggesting syndromic disease, a scenario that, apart from X-linked acrogigantism cases,[55,56] has only been observed in families with a (likely) pathogenic *AIP* variant. Therefore, R304Q, at the time, seemed to be the cause of their clinical phenotype. Similar circumstances can be considered for the c.47G > A p.(Arg16His) and c.100-18C > T p.? *AIP* variants, which were reported in association with pituitary disease either as potentially pathogenic[51,57-62] or benign variants,[15,29,63-65] but currently the consensus appears to be that they lie in the more benign spectrum.

Notwithstanding these findings, over the years, more general population data have become available, and functional evaluation of R304Q using multiple methods is now possible. Data emerging from these resources show conflicting results. Our systematic analysis of the 38 PitNET patients known to have germline R304Q shows a significantly different phenotype compared with patients with pathogenic *AIP* variants, characterized by older age at onset, more females, lower proportion of GH excess cases and lower proportion of gigantism among them. However, the literature might be biased towards the GH-secreting PitNET and gigantism phenotype, because the indication for *AIP* testing in the first place is young-onset GH and PRL-secreting tumours. In addition, there were significantly more clinically affected members in *AIP*mut kindreds compared with the R304Q cohort. In one case, a patient developed a recurrent corticotroph tumour progression after bilateral adrenalectomy with concurrent germline *AIP* R304Q and somatic *USP8* p.(P720R) variants.[66] This is an atypical phenotype for a pathogenic *AIP* variant, and in this particular case, the tumour-driving change is probably the somatic *USP8* variant.[66] Another consideration is whether these two variants had an additive effect, but this seems unlikely. A further parallel example of this is the *AIP* c.47G > A p.(Arg16His) variant in combination with the truncating *MEN1* c.332del p.(Gly111fs*8).[67] Once again, as the authors concluded, it appears that the *AIP* variant is a bystander as the kindred described had syndromic disease, a phenotype not typical of pathogenic or likely pathogenic *AIP* variants. In our 2015 publication, we studied genotype–phenotype correlation for truncating and missense *AIP* P/LP variants.[4] In this study, R304Q was grouped into the pathogenic missense variant group. However, omitting R304Q patients and recalculating the data, the genotype–phenotype correlation is no longer significant.[3]

When the large gnomAD and UK Biobank general population databases became available, it was noticed R304Q was not rare in the general population. Interestingly, two individuals harbouring R304Q in homozygosis are included within the Ashkenazi Jewish population by gnomAD v2.1 (phenotype unknown) and one in the UK Biobank with no pituitary disease. As complete knockout of *Aip* in mice[28] and its orthologues in *D. melanogaster*[29] and *C. elegans*[30] results in lethality, a homozygous missense change, even if functionally relevant, might result in milder functional damage and therefore be compatible with life. Furthermore, knockout data from animal studies do not always predict the human phenotype, for instance, *BRCA2* variants.[68,69] On the other hand, homozygous non-truncating variants were identified in human subjects leading to a severe, often lethal, phenotype.[70]

LOH has been demonstrated in PitNETs for several truncating *AIP* P/LP variants,[10,15,71-74] and for non-truncating *AIP* P/LP variants,[73,75] but not all the *AIP* missense variants are associated with LOH.[73,75] No loss of the wild-type allele was found in any of the four tumour samples tested in this study, which does not support the pathogenic role of the variant. However, additional mechanisms such as epigenetic changes could contribute to loss-of-function of the normal allele.

An amine group amino-acid, such as arginine, lysine, or histidine at position 304, is a well-conserved feature among tetratricopeptide repeat proteins, and among AIP orthologues in other species, where either arginine or lysine can be found (Figure 2C). Computational methods suggest that R304Q does not alter AIP protein structure but may affect its interaction with other proteins. The majority of the *in silico* assessments performed predicted benign, unknown, or conflicting evidence (Table S2).

We acknowledge the multiple limitations of our study. The relatively small number of patients with the R304Q variant and the lack of detailed clinical data or available tumour tissue are expected caveats of rare genetic disease study. We tried to overcome these problems by pooling cases from multiple centres. Another limitation is that, as the mechanisms driving the tumour suppressor function of AIP are still incompletely understood, the best experimental approach to assess the functional effect of a variant is not clear. Therefore, we used a combination of experimental techniques and *in silico* assessments to strengthen our conclusions.

Rare and common genetic variants are often incorrectly associated with pathogenic and benign effects, respectively.[76] There are common disease-associated variants with a frequency of >1% in certain populations, which may confer an evolutionary advantage to heterozygous individuals, for example, sickle cell trait in malaria-prone areas.[77] Thus, it has been recommended that the term *variant* is used, instead of mutation and polymorphism, with different categories indicating functionality. Classifying tumour-related variants based on functionality has important implications on screening and monitoring. Both the International Agency for Research on Cancer and the ACMG/AMP suggest a 5-tier system of variants: pathogenic, LP, VUS, LB, and benign.[34] Factors such as variant frequency, functional studies and segregation analyses are key when classifying variants. A benign variant would require one stand-alone evidence according to the ACMG/AMP, or at least two strong criteria, but neither

is present for R304Q (Table S6). A LB variant would require one strong and one supporting criteria, or at least two supporting criteria, with one strong (BS1) and one supporting (BP4) criteria applied for R304Q. Therefore, based on ACMG criteria, R304Q is a LB variant.

R304Q could perhaps be a very low penetrance allele at the benign end of the *AIP* variant spectrum contributing to PitNET predisposition in combination with other disease modifier genes or environmental factors. A low penetrance is supported by the fact that R304Q is not over-represented in PitNET cohorts and that PitNETs are not more prevalent in Ashkenazi Jewish populations, which have a significantly higher allele frequency of R304Q compared with Europeans. A possible role for R304Q as a disease modifier is suggested by with PitNETs in some FIPA families. This may indicate that R304Q confers a minor risk for PitNET development but has such a low penetrance that asymptomatic subjects with R304Q do not warrant the same surveillance as that recommended for subjects with pathogenic *AIP* variants.[33] Therefore, the systematic pre-symptomatic genetic testing of family members and/or detailed, long-term clinical follow-up of unaffected individuals with the R304Q *AIP* variant is not currently recommended.

In summary, the pathogenic potential of the missense *AIP* R304Q variant has been reassessed. We consider the data most in support of pathogenicity to be the co-occurrence of this variant in familial gigantism cases, coupled with the acrogigantism phenotype of R304Q-injected mice. Contrary to these data, we consider the strongest data refuting pathogenicity to be R304Q homozygote human subjects and the rescue of CG1847 knockout fruit flies by insertion of the R304Q variant. Our recent data on five infants carrying homozygote non-truncating variants with a severe, in at least two cases lethal, phenotype presenting soon after birth, support the benign nature of the R304Q variant.[70] Gene variant classification should be understood as a dynamic process, liable to frequent re-evaluations that should consider the most recent data from population genomics and novel experimental approaches. Considering the constantly evolving research in human genomics, variant reclassification should be expected to occur more frequently in the future.[78,79]

Conclusions

The pathogenic potential of R304Q has been thoroughly reassessed. Based on clinical data, *in silico* prediction studies, genomic information and *in vitro* and *in vivo* functional data, the position of the *AIP* R304Q variant has shifted over the last 17 years from a pathogenic variant towards the "unlikely to be pathogenic" border of the VUS spectrum, and now to the likely benign ACMG/AMP category (Graphical Abstract). Therefore, we suggest that the pre-symptomatic genetic testing of family members and/or follow-up of unaffected individuals with the R304Q *AIP* variant is not currently recommended.

Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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Conflict of interest: A.R.-O. is now a full-time employee at Ipsen Bioscience (Boston, USA) and he holds stocks from the company.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding authors. UK biobank data is publicly available at https://www.ukbiobank.ac.uk. This research has been conducted using the UK Biobank Resource under Application Number ID 103356.

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