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**PSEUDOHYPOPARATHYROIDISM TYPE IA AND PSEUDO-  
PSEUDOHYPOPARATHYROIDISM: THE GROWING SPECTRUM OF GNAS  
INACTIVATING MUTATIONS**

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## **ABSTRACT (250 words max)**

**Background** Pseudohypoparathyroidism (PHP) is a rare heterogeneous genetic disorder characterized by end-organ resistance to parathyroid hormone (PTH) due to partial deficiency of the  $\alpha$  subunit of the stimulatory G protein ( $G_{\alpha}$ ), encoded by the *GNAS* gene. Heterozygous inactivating *GNAS* mutations lead to PHP type Ia (PHP-Ia) when maternally inherited or pseudo-pseudohypoparathyroidism (PPHP) if paternally derived. Both diseases feature typical physical traits identified as Albright's hereditary osteodystrophy (AHO), in the presence or absence of multihormone resistance, respectively. *GNAS* mutations are detected in 60-70% of affected subjects and most patients/families harbor private mutations. No genotype-phenotype correlation has been found to date, but these data come from small series of patients. **Methods** We investigated by direct sequencing  $G_{\alpha}$ -coding *GNAS* exons (exons 1-13) in a large panel of PHP-Ia/PPHP patients collected over the past decade in the two Italian referring centers for PHP and we next collected all previously reported mutations of the gene. **Results** Of 48 patients carrying *GNAS* mutations, in 25 cases we detected 17 known alterations while in 23 patients we identified 16 novel mutations. No apparent correlation was found between clinical/biochemical data and results of molecular analysis. Furthermore, we summarized the current knowledge of *GNAS* molecular pathology and reported all known and novel mutations in the *GNAS* LSDB (Locus Specific DataBase). **Conclusion** These results further expand the spectrum of *GNAS* mutations associated with PHP/PPHP and underline the importance of identifying such genetic alterations to supplement clinical evaluation and genetic counselling.

## **KEY WORDS**

Pseudohypoparathyroidism type Ia (PHP-Ia), Pseudo-pseudohypoparathyroidism (PPHP), *GNAS*,  $Gs\alpha$ , Locus Specific DataBase (LSDB).

## INTRODUCTION

Pseudohypoparathyroidism (PHP) is a rare heterogeneous genetic disorder characterized by hypocalcemia and hyperphosphatemia due to end-organ resistance to parathyroid hormone (PTH). [1] Patients affected with its most investigated subtype, PHP type Ia (PHP-Ia) (OMIM 103580), often present with additional hormonal resistances (TSH, GHRH, calcitonin and gonadotropins) and show characteristic physical features collectively termed Albright's hereditary osteodystrophy (AHO) that include short stature, obesity, round face, brachydactyly, heterotopic ossifications and intellectual disability. Patients showing the physical features of AHO without evidence of hormone resistance were described by Albright and colleagues 10 years after their first report of PHP. This syndrome, which was termed pseudo-pseudohypoparathyroidism (PPHP; OMIM 612463) may be present either in kindreds in which PHP is present or as an isolated defect (in this case it is named AHO-like syndrome, OMIM 600430).[1]

PHP exemplifies a quite unusual form of hormone resistance because the underlying molecular defect affects the alpha subunit of the stimulatory guanine-nucleotide binding protein ( $G_{\alpha}$ ), which has a key role in downstream signaling pathway, rather than the hormone receptor itself. G proteins are so called because they bind to guanine nucleotides and belong to a family of proteins (GTP-binding proteins) able to bind and hydrolyze GTP. They are heterotrimers consisting of three distinct subunits ( $\alpha$ ,  $\beta$  and  $\gamma$ ) and the functional properties of each protein mostly depends on the specific  $\alpha$  subunit.[2]

PHP-Ia and PPHP are caused by heterozygous inactivating mutations located within the *GNAS* gene inherited from the mother or the father, respectively. This pattern of inheritance is consistent with tissue-specific imprinting of *GNAS* paternal allele, as demonstrated in selected human organs displaying a parent-of-origin difference in hormone responsiveness.[3,4] The human *GNAS* gene, which encodes also but not only the  $G_{\alpha}$

protein, maps to 20q13 and its cDNA spans a region of about 1.2 kb. The first *GNAS* mutation responsible for PHP-Ia was described in 1990 by *Patten et al.*[5] To date, numerous different mutations have been identified in all of the 13 exons, including exon 3, where mutations would be expected to have little or no clinical consequence as it undergoes physiological alternative splicing.[6] Most of the genetic defects described are associated to a single kindred, though a single mutational hot spot has been so far identified within exon 7.[7-9] Missense mutations and small insertions/deletions predominate, but nonsense mutations and point mutations that alter translation initiation or determinate aberrant mRNA splicing have also been documented. [10-31] In families in which PHP-Ia and PPHP coexist, *GNAS* mutations are detected in all the affected members, i.e. members affected with either PHP-Ia or PPHP.[19,32]

Here we report clinical, biochemical, and molecular analysis of 48 patients affected with PHP-Ia or PPHP carrying heterozygous *GNAS* mutations. In 25 cases we detected 17 already reported mutations while in 23 patients we identified 16 novel mutations. Moreover, a review of all reported mutations was performed in order to look for genotype/phenotype correlations as well as for the existence of novel mutational hot spots. These results further expand the spectrum of *GNAS* mutations associated with PHP/PPHP and underline the importance of identifying such genetic alterations to supplement both clinical evaluation of patients and genetic counseling of their families.

## **MATERIALS AND METHODS**

### **Patients recruitment and evaluation**

This series includes 48 patients (29 females and 19 males), all born from healthy, non-consanguineous Caucasian parents. The inclusion criteria was the presence of a clinical diagnosis of PHP-Ia or PPHP, based upon the presence/absence of PTH resistance (i.e.

hypocalcemia, hyperphosphatemia and raised serum PTH levels) together with at least two of the six AHO manifestations: brachydactyly (shortening of fourth and/or fifth metacarpals) [31], short stature (height below the 3th percentile for chronological age), obesity (BMI >30 kg/m<sup>2</sup> in adults and >97<sup>th</sup> centile in children), round face, subcutaneous ossifications (either clinically evident or at X-ray), and mental retardation.[1] Of the two AHO signs required, either brachydactyly or heterotopic ossifications had to be present in order to define AHO in a given patient. Diagnosis was made at ages ranging between 1 and 34 year-old. The majority of PHP-Ia patients (n=39) also showed resistance to TSH, documented by raised serum TSH levels, absence of anti-thyroid antibodies and presence of a normal thyroid scan (data not shown). Resistance to gonadotropins and/or GHRH was also documented in a subset of patients (8 and 9, respectively), but it is to note that not all patients were screened for these resistances. Clinical details are shown in table 1. Informed consent was obtained from all patients included in the study.

**Table 1** Clinical characteristics of patients carrying *GNAS* mutations included in the present study.

pt ID	gender	age	rPTH	rTSH	Ob	Fr	MR	Br	EO	SS	PHP	ex/IVS	DNA mutation
42	M	18						X	X		Ia	5'UTR+ex1	c.1-10_2delCGCCGCCGCCAT
33	M	2			X	X	X	X			Ia	ex1	c.7T>C
52	M	2				X	X	X			Ia	ex1	c.21_22insT
34	F	2			X	X			X		Ia	ex1	c.85C>T
35a	F	4			X	X	X		X		Ia	ex1	c.85C>T
35b	M	4			X	X	X	X			Ia	ex1	c.85C>T
36	F	2							X	X	PPHP	ex1	c.85C>T
37	F	2			X	X	X	X		X	PPHP	ex1	c.97G>A
38	F	0.5			X	X		X	X	X	PPHP	ex1	c.103C>T
39	M	9				X	X	X	X	X	Ia	ex1	c.103C>T
40a	M	5							X		Ia	ex1	c.103C>T
40b	M	5						X	X		Ia	ex1	c.103C>T
41	M	13				X		X	X		Ia	ex1	c.110delA
3	M	5					X	X			Ia	ex1	c.112delC
5	F	20				X	X	X	X	X	Ia	ex4	c.308C>A
6	M	34			X	X	X	X		X	Ia	ex4	c.311T>C



8	M	4		X	X	X	X		Ia	ex5	c.347_348insT
10	F	2		X	X	X	X	X	Ia	ex5	c.348_349insC
11	M	2			X		X	X	Ia	ex5	c.348_349insC
45	F	1			X	X	X		Ia	ex5	c.366delC
46	F	4		X	X		X		Ia	ex5	c.421_422delTT
12	M	23				X	X	X	PPHP	ex6	c.478G>A
13	F	9		X	X	X	X		Ia	ex6	c.481C>T
14	F	10			X		X	X	Ia	ex6	c.496C>T
47	M	9		X	X	X	X	X	Ia	ex6	C.523_524delTG
17	F	8		X	X		X		Ia	ex7	c.568_571delGACT
18	F	14					X		PPHP	ex7	c.568_571delGACT
20	F	1		X				X	Ia	ex7	c.568_571delGACT
21	M	1		X	X			X	Ia	ex7	c.568_571delGACT
22	F	5		X	X	X	X	X	Ia	ex7	c.568_571delGACT
24a	F	13		X	X	X	X	X	Ia	ex9	c.694C>T
24b	F	13		X	X	X	X	X	Ia	ex9	c.694C>T
25	F	19		X	X		X	X	Ia	ex10	c.728C>T
48a	F	7					X		Ia	ex10	c.742G>C
48b	F	7					X		Ia	ex10	c.742G>C
49	M	12		X	X	X	X		Ia	ex10	c.806A>G
26	F	5		X	X	X	X		Ia	ex11	c.863_864delTG
27	F	2		X	X		X		Ia	ex12	c.1009C>T
28	M	11		X	X		X	X	Ia	ex12	c.1009C>T
50	F	2		X	X			X	Ia	ex12	c.1021_1024delTTCA
51	F	17		X	X	X	X	X	Ia	ex12	c.1021_1022insACGCGTGACCC
											GGGCCAAGTACT
29	F	20				X	X	X	PPHP	ex13	c.1110_1111delTG
53a	F	7			X		X	X	Ia	ex 13	c.1153C>T
53b	M	5							Ia	ex 13	c.1153C>T
43	F	10		X	X		X	X	Ia	IVS1	c.139+1T>A
4	M	7						X	Ia	IVS2	c.212+2delAAGT
44	F	4					X		Ia	IVS3	c.258+1G>A
54	F	9					X	X	PPHP	IVS5	c.435+1G>A

**Legend:** age (years at diagnosis), PTH levels (PTH resistance defined by PTH > 65 mU/L), TSH levels (TSH resistance defined by TSH > 3.9 pg/mL), Ob (obesity), RF (round face), MR (mental retardation), Br (brachydactyly), SC (subcutaneous ossifications), SS (short stature).

### Sequencing analysis of the *GNAS* gene

Genomic DNA was extracted from peripheral blood leukocytes by Nucleon BACC2 genomic DNA purification kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. The DNA obtained was amplified by PCR for *GNAS* 1-13 exons

and flanking intronic sequences (ENSEMBL ID: ENSG00000087460), using specific primers [11,34]. Direct sequencing of the amplicons was performed with the AmpliTaq BigDye Terminator kit and 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), as previously described.[11,34]

### **Mutation nomenclature and literature review**

The mutation nomenclature used in the present paper follows the guidelines indicated by Human Genome Variation Society (HGVS).[35] Nucleotide and protein numbering are based on *GNAS* LRG sequence format created by Leiden Open Variation Database (LOVD at [http://chromium.liacs.nl/LOVD2/home.php?select\\_db=GNAS](http://chromium.liacs.nl/LOVD2/home.php?select_db=GNAS)). LRG reference sequences is not subject to versioning and is annotated with all transcripts and encoded proteins essential for variant description. Nucleotide 1 numbering reflects cDNA numbering with position +1 referring to the A of the translation initiation codon. Mutation description at the protein level considers the initiator methionine as codon 1. We performed an electronic search in public locus-specific databases HGMD (<http://www.hgmd.org/>) and LOVD in order to collect all reported pathogenic variants. Cases were cross-referenced and the correctness of descriptions was checked in order to avoid duplications.

### **Assessment of pathogenicity**

In the present study, in agreement with the view that the molecular basis for PHP type I is represented by  $G\alpha$  haploinsufficiency, we classified all novel nonsense mutations, frameshift mutations and mutations of the first two bases of canonical intron splice sites as deleterious. A nonsense mutation results in a premature stop codon in the transcribed mRNA and in a truncated, incomplete and usually nonfunctional protein product. Moreover, nonsense-mediated mRNA decay has been also associated with *GNAS* nonsense mutations in PHP-Ia.[19] A frameshift mutation (also called a reading frame shift) is a genetic mutation

caused by insertions or deletions of a number of nucleotides that is not evenly divisible by three and, due to the triplet nature of gene expression by codons, it changes the reading frame, resulting in a completely different translation from the original. The earlier in the sequence the defect occurs, the more altered the protein produced is. Nucleotide signals at the splice sites precisely guide the enzymatic machinery in the removal of intronic sequences when the pre-mRNA is processed to form the mRNA so that, when mutated, the intron is retained as part of the final RNA molecule and the translation of its sequence alters the sequence of the protein product.

As for novel missense mutations, we performed an *in silico* analysis using different computer generated algorithms in order to predict the possible effect *in vivo*. Indeed, bioinformatic tools allowed to evaluate the impact of genetic mutations on the protein product according to the structure of the *GNAS* gene and Gs $\alpha$  structural domains. In particular, we used the following algorithms: 1. Polymorphism phenotyping program 2, Polyphen2 (available at <http://genetics.bwh.harvard.edu/pph2/>), an online tool able to predict the impact of an aminoacid substitution on both structure and function of human proteins, based on the combined analysis of protein multiple sequence alignment and protein structural and functional attributes; 2. Sort Intolerant From Tolerant human Protein, SIFT human Protein program (available at [http://sift.jcvi.org/www/SIFT\\_enst\\_submit.html](http://sift.jcvi.org/www/SIFT_enst_submit.html)), that provides predictions for tolerated and non-tolerated mutations at selected amino acids in Ensembl transcripts with an assigned ENSP number, through protein sequence alignments of multiple species.

## **Data analysis**

A paired two-tailed Student's *t* test was used to detect the significance between two series of data.  $p < 0.05$  was accepted as statistically significant.

## RESULTS

### Mutation analysis

Sequencing analysis of *GNAS* Gs $\alpha$ -coding exons identified 33 heterozygous loss of function mutations in 48 patients affected with either PHP-Ia or PPHP (tables 2 and 3, figure 1). These sequence abnormalities were demonstrated to co-segregate with the disorder in the few familial cases included in this series (pts 3, 13, 24a, 24b, 26, 35a, 35b, 38, 40a, 40b, 45, 48a, 48b, 53a and 53b) and to be absent as common polymorphisms in 100 unrelated normal individuals. Of these, 17 were already reported mutations, of which 9 were missense/nonsense point mutations, 6 were small insertions or deletions causing frameshift and 2 were mutations of canonical splice acceptor or donor sites (for a brief description of these known variants see table 2).

**Table 2** *GNAS* mutations described in literature and detected in patients included in the present study.

Pt ID	inheritance	site of mutation	DNA sequence change	amino acid change	Type of mutation	HGMD® accession number
3	♀	ex1	c.112delC	p.(Arg38Glyfs*19)	FS	CD002905
4	n.a.	IVS2	c.212+2delAAGT	p.(?)	SP	CD031052
5	n.a.	ex4	c.308C>A	p.(Ala103Glu)	MS	CM013729
6	n.a.	ex4	c.311T>C	p.(Ile104Thr)	MS	CM002271
8	<i>de novo</i>	ex5	c.347_348insT	p.(Pro116Profs*24)	FS	CI020091
10-11	n.a.	ex5	c.348_349insC	p.(Val118Argfs*22)	FS	CI031096
54	n.a.	IVS5	c.435+1G>A	p.(?)	SP	CS941495
12	n.a.	ex6	c.478G>A	p.(Val160Met)	MS	CM020280
13	♀	ex6	c.481C>T	p.(Arg161Cys)	MS	CM002273
14	n.a.	ex6	c.496C>T	p.(Arg166Cys)	MS	CM930348
17-18-20-21-22	n.a.	ex7	c.568_571delGACT	p.(Asp190Metfs*13)	FS	CD920862
24a-24b	♀	ex9	c.694C>T	p.(Arg232Cys)	MS	CM013732

25	n.a.	ex10	c.728C>T	p.(Thr243Ile)	MS	CM031194
26	♀	ex11	c.863_864delTG	p.(Val288Aspfs*11)	FS	CD002906
27-28	n.a.	ex12	c.1009C>T	p.(Arg337Trp)	MS	CM013736
29	n.a.	ex13	c.1110_1111delTG	p.(Thr370Thrfs*11)	FS	CD002504
53a-53b	♀	ex13	c.1153C>T	p.(Gln385X)	NS	CM056331
<b>Legend:</b> ♀, maternal; ♂, paternal; <i>de novo</i> , no parental inheritance; n.a., parents not available, ex, exon; IVS, intron, MS, missense; NS, nonsense; FS, frameshift; SP, splice site.						

The remaining 16 mutations were novel to the literature and distributed as following (see also table 3):

#### *Mutations in the GTPase domain*

Both exons 1 and 2 encode the functional domain with GTPase activity, so that mutations in this region are expected to affect the ability to bind or hydrolyze GTP.

Seven different novel mutations detected in 11 patients were located in exon 1 (3 frameshift, 2 nonsense and 2 missense). Patient 52 (c.21\_22insT) was found to carry the insertion of a thymine that generates a premature stop codon (p.Lys8X). A *de novo* frameshift mutation detected in patient 41 (c.110delA) causes the substitution of Tyr37Ser and the creation of a premature stop codon. Patient 42 displayed a *de novo* 12-bp deletion in the region encompassing the translation start site (c.1-10\_2delCGCCGCCGCCAT), causing the loss of the ATG initiation codon. Four subjects (pts 35a and 35b, who are brother and sister, and 2 unrelated cases, pts 34 and 36) presented the nonsense mutation Gln28X caused by c.85C>T transition. The nonsense mutation p.Gln35X, due to c.103C>T transition, was found in 4 patients belonging to 3 families (paternal inheritance in pts 40a and 40b, maternal inheritance in pt 38 and *de novo* in pt 39).

As for missense mutations, the first is a c.7T>C transition (pt 33), resulting in the substitution of the hydrophobic amino acid cysteine in position 3 with the hydrophilic

arginine (p.Cys3Arg). This highly conserved amino acidic residue has an important role in membrane anchoring, undergoing conversion to S-palmitoyl cysteine. Protein S-acylation is a major post-translational modification and a well-recognized determinant of protein function and localization. The mutations is predicted to be deleterious according to bioinformatic analysis.

In patient 37 we detected the c.97G>A transition resulting in the substitution at position 33 of an acid aspartic residue (negatively charged) with an asparagine (non polar). According to the bioinformatic prediction this mutation (p.Asp33Asn) is probably damaging and, although this residue is not highly conserved, no known species carries an asparagines at this position.

#### *Mutations in the adenylate cyclase interaction domain*

Patient 45 displayed a single base deletion in exon 5 (c.366delC) which results in the creation of a premature stop codon 11 amino acids downstream Asn122. Patient 46 showed a *de novo* 2-bp deletion in exon 5 (c.421\_422delTT), that determines the creation of a premature stop codon instead of wild-type Phe141.

#### *Mutations in domains involved in protein structure/conformation*

In patient 47 we found a 2-bp deletion in exon 6 with consequent formation of a premature stop codon 14 amino acids downstream Cys175 (c.523\_524delTG). Sisters 48a and 48b maternally inherited a missense mutation (c.742G>C; p.Val248Leu) in exon 10. The pathogenic effect of this mutation was confirmed by computer investigations, who predicted a damaging effect. A last novel missense mutation, c.806A>G, was also detected within exon 10 (pt 49), determining the substitution p.Glu269Gly. A damaging effect of this substitution was predicted and further validated by the high conservation of this residue.

### Mutations in the GPCR interaction domain

Patient 50 had a *de novo* 4-bp deletion in exon 12 (c.1021\_1024delTTCA), with generation of a premature stop codon 5 downstream Phe341. Finally, a *de novo* insertion of 23-bp in exon 12 was detected in patient 51 (c.1021\_1022insACGCGTGACCCGGGCCAAGTACT), also generating a stop codon at Phe 341.

### Intronic mutations

In patient 43 and 44 we detected splice site mutations in intron 1 (c.139+2T>A) and in intron 3 (c.258+1G>A), respectively. The T>A transversion at intron 1 donor splice site likely results in the inclusion of intron 1 in the transcript, with consequent introduction of 154 additional amino acids before a stop codon. Similarly, transition G>A found in patient 44 likely causes the inclusion of intron 3 and the creation of a premature stop codon with no additional amino acids.

**Table 3** Novel *GNAS* mutations detected in patients included in the present study.

Pt ID	inheritance	site of mutation	DNA sequence change	amino acid change	Type of mutation
33		ex1	c.7T>C	p.(Cys3Arg)	MS
52	n.d.	ex1	c.21_22insT	p.Lys8X	FS/NS
34-35a-35b-36	♀ [35a;35b]	ex1	c.85C>T	p.(Gln29X)	NS
37	n.d.	ex1	c.97G>A	p.(Asp33Asn)	MS
38-39-40a-40b	♀[38], <i>de novo</i> [39], ♂[40a;40b]	ex1	c.103C>T	p.(Gln35X)	NS
41	<i>de novo</i>	ex1	c.110delA	p.(Tyr37Serfs*15)	FS
42	<i>de novo</i>	5'UTR+ex1	c.1-10_2delCGCCGCCGCCAT	p.(0?)	FS

43	n.d.	IVS1	c.139+1T>A	p.(?)	SP
44	n.d.	IVS3	c.258+1G>A	p.(?)	SP
45	♀	ex5	c.366delC	p.(Asn122Asnfs*12)	FS
46	<i>de novo</i>	ex5	c.421_422delTT	p.(Phe141X)	NS
47	n.d.	ex6	C.523_524delTG	p.(Cys175Cysfs*14)	FS
48a-48b	♀	ex10	c.742G>C	p.(Val248Leu)	MS
49	n.d.	ex10	c.806A>G	p.(Glu269Gly)	MS
50	<i>de novo</i>	ex12	c.1021_1024delTTCA	p.(Phe341Phefs*5)	FS
51	<i>de novo</i>	ex12	c.1021_1022insACGCGTGACCC GGGCCAAGTACT	p.(Phe341Tyrfs*3)	FS

**Legend:** ♀, maternal; ♂, paternal; *de novo*, no parental inheritance; n.a., parents not available, ex, exon; IVS, intron; MS, missense; NS, nonsense; FS, frameshift; SP, splice site.

## Review of the literature and LOVD update

We next reviewed all *GNAS* loss-of-function mutations associated to PHP-Ia and PPHP published in the two main public locus-specific databases (HGMD and LOVD). To avoid duplications, we cross-referenced all cases and we updated the *GNAS* LOVD database.

From this analysis we identified 132 *GNAS* mutations in PHP/PPHP patients, of which 116 previously reported plus our 16 novel mutations. Using this information we delineated an updated *GNAS* mutational spectrum, composed of 47% point mutations (37% missense, 10% nonsense), 39% frameshift (25% deletions, 14% insertions) and 13% affecting splicing (figure 2). To date, a single case of in-frame insertion has been reported.[12] Considering the distribution of the mutations within *Gsα* coding sequence, exon 1 is the most affected site, harbouring 16% of all *GNAS* mutations, followed by exon 10 (13%), exon 13 (11%) and exons 4 and 5 (9% each). Moreover, also acceptor/donor splice sites show an high mutation rate, with a frequency of 13% in the overall PHP/PPHP population (figure 1).

## Genotype/phenotype correlation



The patients were divided according to the presence of frameshift, missense, nonsense or intronic mutations and subsequently according to the affected functional domain. We then compared these different groups in terms of related clinical parameters (age at diagnosis; calcium, phosphorus, PTH and TSH levels; number of AHO signs). By this approach, neither the mutation type nor its location did correlate with the onset of the disease, the severity of endocrine resistances, or with the presence/absence of specific AHO signs (data not shown).

## DISCUSSION

The present study describes 48 patients affected with PHP-Ia or PPHP and carrying mutations in *GNAS* Gs $\alpha$ -coding exons, who were diagnosed during the past decade in the two referring Italian Centers for these rare disorders, and reports 33 mutations, 16 of which are novel to the literature.

The review of all published *GNAS* mutations (116 previously reported plus our 16 novel mutations) in PHP/PPHP patients confirms that mutations are distributed throughout the entire gene and allowed us to delineate an updated *GNAS* mutational spectrum, with 47% point mutations (37% missense, 10% nonsense), 39% frameshift (25% deletions, 14% insertions) and 13% affecting splicing (figure 2).

Most of the genetic defects here and elsewhere described are private mutations being confined to one or few patients. In our series, the only mutational hot-spot so far recognized, i.e. a 4-bp deletion in exon 7 (c.568\_571delGACT) that removes a defined consensus sequence for DNA polymerase arrest, accounted for 5 unrelated patients (10.4 % of the patients described here). Our observation is in accordance with previous reports, confirming the presence of an hot spot in this region, though with a lower frequency than the one reported by others (up to 35 %) (table 2).[9]

Few additional mutations have been found to recur in unrelated patients, so that the presence of identical *de novo* mutations probably derives from the presence of a common molecular mechanism rather than a founder effect (table 4).

As known, the frequency of a given mutation is influenced by several phenomena, such as the size of the gene, the nucleotide sequence itself, and chemical changes (i.e. cytosine deamination is a frequent cause of C>T transition).[36] Accordingly, in the present series we detected 2 novel mutations in exon 1, c.85C>T and c.103C>T, in 4 patients from 3 different families. Interestingly, also two other alterations have been described in this exon that recurred in more than one kindred. In fact, c.1A>G and c.91C>T mutations were each detected in 3 patients belonging to 2 families.[5,13,14]

Interestingly, we found 5 additional previously undetected mutations in exon 1 (c.1-10\_2delCGCCGCCGCCAT, c.7T>C, c.21\_22insT, 97G>A, c.110delA), strongly suggesting the importance to carefully screen this exon, that is sometimes omitted being difficult to amplify due to the richness in guanosines and cytosines in its boundaries and that we now propose to consider as a new mutational hot spot.

Importantly, the overall high frequency of mutations located in exon 1 further confirms the primary role of Gs $\alpha$  protein deficiency in PHP/PPHP pathology, as exon 1 is the only *GNAS* exon encoding exclusively for Gs $\alpha$ , exons 2-13 being common also to NESP55 and XL $\alpha$ s genes/proteins. As resumed in figure 2, considering both known and novel mutations, exon 1 is the most frequent site of mutation representing 16% of all *GNAS* mutations, followed by exon 10 (13%), exon 13 (11%) and exons 4 and 5 (9% each). This observation is in accordance with the knowledge that these exons encode for crucial Gs $\alpha$  functional domains (figure 1). Also intronic flanking sequences frequently bear mutations, as shown by the high frequency detected in the overall PHP population (13%) and further

confirmed in our series in which we detected 2 novel splice site mutations (c.139+1T>A, c.258+1G>A).

As suggested by previous observations including ours [11], no apparent difference was observed among patients harboring different types of mutations in terms of age at diagnosis (as marker of disease precocity and severity), number of AHO signs and number or severity of hormonal resistances, further confirming the absence of genotype/phenotype correlation in this disease and supporting the hypothesis of  $Gs\alpha$  haploinsufficiency as the molecular mechanism underlying PHP independently of the mutation type/extension/region (table 1).

In conclusion, our results provide an estimate of the prevalence and the distribution of all *GNAS* mutations detected so far in PHP/PPHP patients and highlight both genetic and phenotypic heterogeneity among and within families. Furthermore, the present work provides an updated compilation of mutational and phenotypic data for both diagnostic and research purposes, as a step towards a better understanding of PHP. Indeed, we updated the *GNAS* Locus-Specific database (the database is available on the internet at [http://chromium.liacs.nl/LOVD2/home.php?select\\_db=GNAS](http://chromium.liacs.nl/LOVD2/home.php?select_db=GNAS)) with the hope that our commitment will promote the further contribution from all research scientists, physicians and diagnostic laboratories involved in *GNAS* screening and PHP management.

**Table 4** *GNAS* mutations found associated with PHP/PPHP phenotype in more than 1 kindred and/or affecting the same amino acidic residue (highlighted by black boxes). The 4 nucleotides deletion in exon 7 (in bold) is the more frequent defect so far detected, thus classically considered an hot spot.

exon	DNA sequence change	amino acid change	n°pts	n°families
1	c.1A>G	p.(0)?	3	2
1	c.85C>T	p.(Gln29X)	4	3

1	c.91C>T	p.(Gln31X)	3	2
1	c.103C>T	p.(Gln35X)	5	3
4	c.268delG	p.(Ala90Glnfs*10)	1	1
4	c.269_272delCAAC	p.(Ala90Alafs*9)	1	1
4	c.308C>A	p.(Ala103Glu)	2	2
4	c.308C>T	p.(Ala103Val)	1	1
4	c.311T>C	p.(Ile104Thr)	2	2
5	c.351delC	p.(Pro115Profs*17)	2	2
5	c.346C>T	p.(Pro116Ser)	1	1
5	c.347C>T	p.(Pro116Leu)	2	2
5	c.347_348insT	p.(Pro116Profs*24)	2	2
5	c.348_349insC	p.(Val118Argfs*22)	2	2
5i	c.435+1G>A	p.(?)	3	3
6	c.478G>A	p.(Val160Met)	2	2
6	c.481C>T	p.(Arg161Cys)	2	2
6	c.496C>T	p.(Arg166Cys)	2	2
7	<b>c.568_571delGACT</b>	<b>p.(Asp190Metfs*13)</b>	<b>19</b>	<b>19</b>
9	c.681_682insC	p.(Gln228Profs*3)	1	1
9	c.683A>G	p.(Gln228Arg)	2	2
9	c.684G>(CorT)	p.(Gln228His)	1	1
10	c.728C>T	p.(Thr243Ile)	2	2
10	c.728delC	p.(Thr243Metfs*28)	1	1
10	c.841A>G	p.(Arg281Gly)	1	1
10	c.842G>A	p.(Arg281Lys)	1	1
10	c842insAACAG	p.(Arg281Argfs*X)	1	1
12	c.1109C>T	p.(Arg337Trp)	2	2
13	c.1110_1111delTG	p.(Thr370Thrfs*11)	2	2
13	c.1153C>T	p.(Gln385X)	3	2
13	c.1166T>G	p.(Leu389Arg)	1	1
13	c.1166T>C	p.(Leu389Pro)	1	1
13	c.1177G>A	p.(Glu393Lys)	1	1
13	c.1177G>T	p.(Glu393X)	7	6

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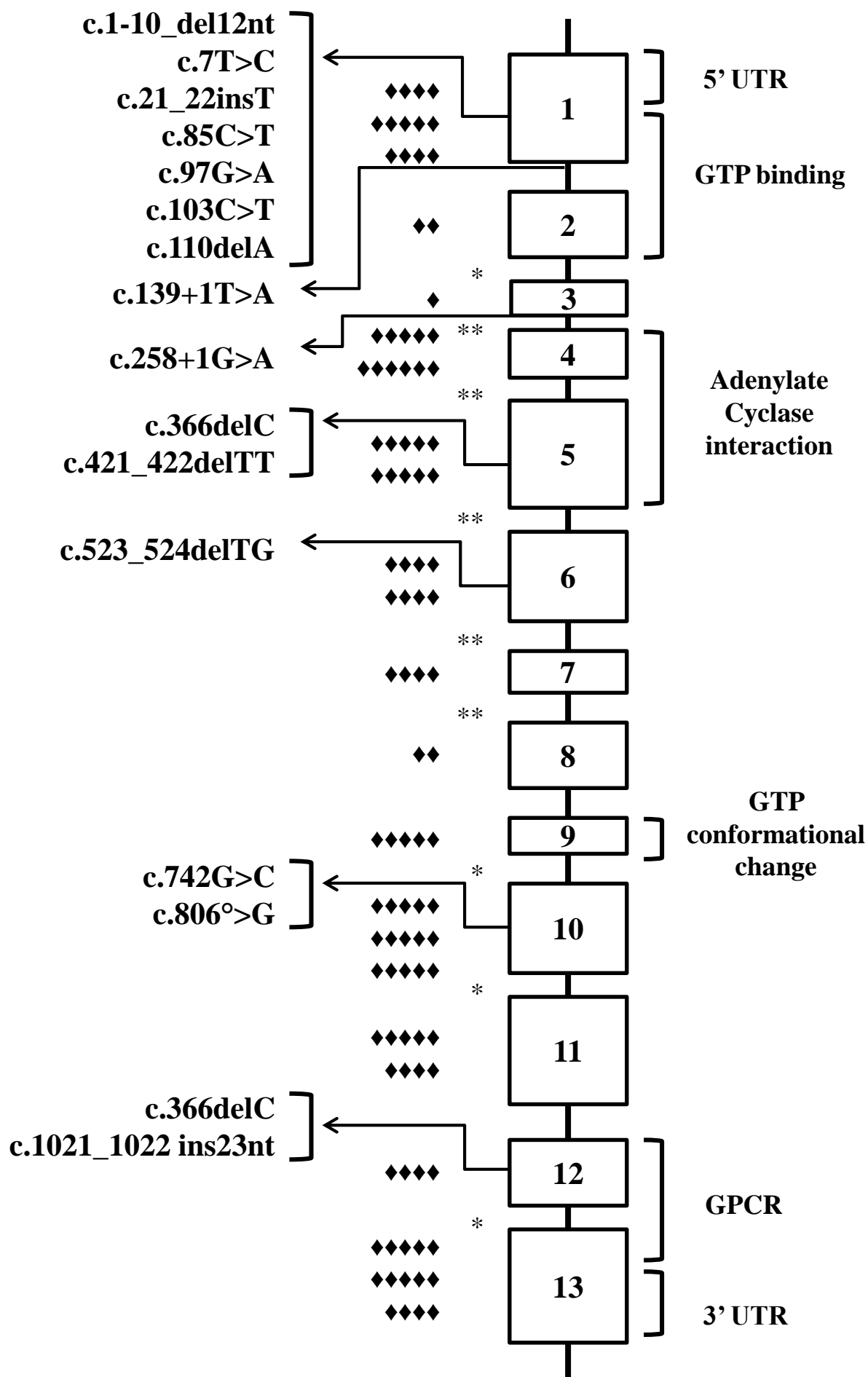
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## FIGURE LEGENDS

**Figure 1** Schematic illustration of the *GNAS* gene and of all mutations found in PHP type A and PPHP phenotypes. On the left the novel mutations found in our series are represented. In the middle of the figure mutations already reported in locus-specific public databases are resumed. Each symbol represents a single mutation, regardless of the number of patients carrying it (♦ mutations in coding exons, \* mutations in introns).

**Figure 2** *Upper panel.* Graph showing the mutational spectrum of *GNAS* Gs $\alpha$ -coding gene. *Lower panel.* The figure shows the distribution of mutations associated with PHP/PPHP within the gene. The white bar represents known and novel mutations found in our series, the grey one indicates mutations collected in locus-specific databases and the black one the combination of all the above. Thus, the black bar is an intuitive representation of the distribution of all the mutations found so far demonstrating that the most affected regions are represented by exons 1 & 10 and the intronic flanking sequences, followed by exons 13, 4 and 5. It is also of notice that the majority of novel mutations found in our patients are located in exon 1.



**Figure 1**

