1 Original Full Length Article

Screening for *GNAS* genetic and epigenetic alterations in progressive osseous heteroplasia: First Italian series

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ABSTRACT

Progressive osseous heteroplasia (POH) is a rare autosomal dominant disorder of mesenchymal differentiation characterized by progressive heterotopic ossification (HO) of dermis, deep connective tissues and skeletal muscle. Usually, initial bone formation occurs during infancy as primary osteoma cutis (OC) then progressively extending into deep connective tissues and skeletal muscle over childhood.

Most cases of POH are caused by paternally inherited inactivating mutations of *GNAS* gene. Maternally inherited mutations as well as epigenetic defects of the same gene lead to pseudohypoparathyroidism (PHP) and Albright's hereditary osteodystrophy (AHO).

During the last decade, some reports documented the existence of patients with POH showing additional features characteristic of AHO such as short stature and brachydactyly, previously thought to occur only in other *GNAS*-associated disorders. Thus, POH can now be considered as part of a wide spectrum of ectopic bone formation disorders caused by inactivating *GNAS* mutations.

Here, we report genetic and epigenetic analyses of *GNAS* locus in 10 patients affected with POH or primary OC, further expanding the spectrum of mutations associated with this rare disease and indicating that, unlike PHP, methylation alterations at the same locus are absent or uncommon in this disorder.

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Introduction

Progressive osseous heteroplasia (POH; OMIM 166350) is a recently described rare autosomal dominant disorder of mesenchymal differentiation characterized by progressive heterotopic ossification (HO) of dermis, deep connective tissues and skeletal muscle. Initially bone formation occurs at birth or during infancy as primary osteoma cutis (OC) and then HO extends progressively into deep connective tissues and skeletal muscle during childhood [1]. Clinical presentation of POH can be extremely variable, thus the severity and morbidity depend on the location and the extent of the HO. Some cases of POH appear sporadic, whereas some are familial.

In 2002, Shore et al. demonstrated that most cases of POH are caused by paternally inherited inactivating mutations of *GNAS*, the complex imprinted locus encoding also for the alpha-subunit of heterotrimeric stimulatory G protein (Gsα) [2]. Recent studies on mouse knockout model and pluripotent mouse ES cells suggest that *GNAS* is a key regulator of adipose-derived mesenchymal progenitor cell commitment and heterozygous inactivation of Gsα enhances osteoblast differentiation [3–5]. Maternally inherited mutations of the same gene lead to pseudohypoparathyroidism (PHP) type Ia (PHP-Ia) and Albright's hereditary osteodystrophy (AHO). In patients with PHP type Ib (PHP-Ib), *GNAS* imprinting defects are predicted to decrease Gs$_\alpha$ expression in tissues where Gs$_\alpha$ is physiologically imprinted, therefore leading to renal parathyroid hormone (PTH) resistance with no or few other clinical manifestations. Moreover, similar imprinting defects have been demonstrated over the last years in some patients with a PHP-Ia phenotype, i.e. hormone resistances plus signs of AHO, further highlighting the phenotypic heterogeneity and overlap among *GNAS*-related disorders [6].

During the last decade, some reports documented the existence of patients with POH showing additional features typical of AHO, such as short stature and brachydactyly, previously thought to occur only in other *GNAS*-associated disorders. Thus, POH is now considered as part of the clinical spectrum of HO disorders caused by inactivating *GNAS* mutations [7–9].

In 2008, Adegbite et al. proposed to classify patients with HO according to the presence or absence of specific characteristics typical of *GNAS*-based disorders (i.e. age of onset of HO, presence and

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location of HO, depth of HO, progression of HO, features of AHO, PTH resistance and *GNAS* mutation analysis) introducing the innovation of a sub-classification of progressive HO. In particular, patients without features of AHO were defined as having POH, while those with multiple AHO features, in the absence or presence of hormone resistance, were defined as having POH/AHO or POH/PHP1a, respectively. As for subjects with non-progressive forms of HO, including AHO, PHP1a and OC, no changes in the definition were made [10].

Here, we report genetic and epigenetic analyses of *GNAS* locus in 10 patients affected with either POH or primary OC, further expanding the spectrum of mutations associated with this rare disease and indicating that, unlike PHP, *GNAS* methylation alterations are absent or uncommon in this disorder.

Materials and methods

Patients

This study includes 10 patients characterized by HO as first-presenting and main clinical feature. They were all born from unrelated parents. Familiar history was negative for HO or hormone resistance in all patients.

In all children the hallmark was the presence of either isolated or multiple erythematous papules, evolving into ossified subcutaneous nodules. A skin biopsy was performed in all patients with the exception of patient 7 who refused the procedure, and confirmed in all cases the presence of ectopic bone (Fig. 1A). The diagnosis of POH or OC was made according to the deepening or not of the ectopic
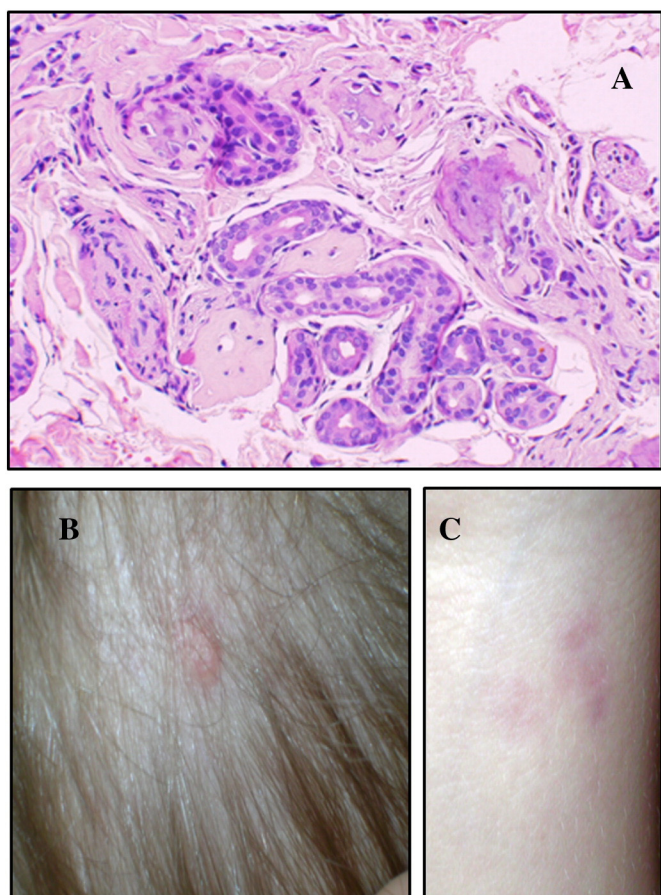

Fig. 1. A. A skin biopsy of a lesion taken from the back of a patient, showing foci of ossification surrounding dermis sweat glands (hematoxylin & eosin 100×). B. Photograph of an ossified nodule on the scalp of a patient. C. Photograph of multiple small skin-colored papules on the back of a patient.

ossification, respectively. Nevertheless, some patients are still too young to exclude further deepening of the lesions.

Endocrinological evaluation of hormone resistances (primarily PTH and TSH) included thyroid function test together with serum calcium, phosphate, PTH, 25-OH vitamin D and 24-h urinary calcium measurement.

Clinical criteria for AHO diagnosis included the presence of at least 2 additional features among brachydactyly (shortening of fourth and/or fifth metacarpals defined as the metacarpal sign and/or shortening below −2SDS at the metacarpophalangeal profile pattern in at least one metacarpal bone or distal phalanx), short stature (height below the 3th percentile for chronological age), obesity (BMI >30 kg/m^2 in adults and >97th centile in children), round faces and mental retardation (motor and/or speech delay or need of extra help in pre-school/mainstream school).

Informed consent was obtained from all patients included in the study.

GNAS DNA sequencing analysis and mutation nomenclature

Genomic DNA was extracted by Nucleon BACC2 genomic DNA purification kit from peripheral blood leukocytes of both patients and parents (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. *GNAS* 1–13 exons and flanking intronic sequences (ENSEMBL ID: ENSG00000087460) were amplified by PCR using previously described specific primers [6,11]. Direct sequencing of amplicons was performed using the AmpliTaq BigDye Terminator kit and 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), as previously described [6,11].

The mutation nomenclature follows the guidelines indicated by the Human Genome Variation Society (HGVS). Nucleotide and protein numbering are based on *GNAS* LRG sequence format created by Leiden Open Variation Database (LOVD at www.lovd.nl/GNAS).

GNAS RNA sequencing analysis

Total RNA was extracted from peripheral blood leukocytes of patients 2, 3 and 6 using Trizol® Reagent (Life Technologies, Paisley, Scotland). cDNAs, synthesized using SuperScript Reverse Transcriptase II (Invitrogen Corp.), were submitted to PCR using forward transcript-specific primers for A/B RNA (ENSEMBL ID: ENST00000477931), 5′-CTGCGTCAGGTGGCTGGC-3′, NESP RNA (ENST00000313949), 5′-GAAGGAGCCCAAGGAGGAGAAGCAGCGGC-3′, and for Gsα RNA (ENSEMBL ID: ENST00000371085), 5′-CCATGGGGCTGCCTCGGGAACA-3′, with common reverse primers for GNAS exons 6 and 10, 5′-CCTTGGCATGCTCATAGAATTC-3′ and 5′-CACGAAGATGATGCCAGTCAC-3′. PCR products were directly sequenced, as reported in GNAS DNA sequencing analysis and mutation nomenclature section. **Q3**

Methylation Specific Multiplex Ligation-specific Probe Amplification (MS-MLPA)

MS-MLPA was used to investigate both the presence of deletions/duplications affecting *STX16* and *GNAS* loci and *GNAS* DMR methylation status (A/B, AS, XL, and NESP). Briefly, about 500 ng of DNA was analyzed by the commercially available kit ME031 MLPA probemix according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). PCR products were separated by capillary electrophoresis using a 3100 Genetic Analyzer (Perkin-Elmer Corp.) with the internal size standard GeneScan 500LIZ (Applied Biosystems, Foster City, CA) and Coffalyser v9.4 (MRC-Holland, Amsterdam, The Netherlands), as previously described [6].

Results

Clinical phenotypes

Clinical and biological characteristics of 10 patients (5 females and 5 males) included in our study are resumed in Table 1. An early age of onset was observed in all cases, except for patient 10 [12]. The spectrum of HO varied from an isolated nodule on the left ankle in patient 7 to papules and nodules scattered all over the body (trunk, limbs and scalp excluding face) in patient 2 (Figs. 1B–C).

Thyroid function tests, serum calcium, phosphate, PTH, 25-OH vitamin D and 24-h urinary calcium were normal in all patients with the exception of patient 4, who was previously described in the mutation update recently published by our group (pt ID 26): POH was first diagnosed at the age of 6 months but she manifested resistance to PTH and TSH over the next 3 years, together with severe weight gain [13]. In particular, mild resistance to PTH was detected in the presence of normal calcium and vitamin D levels and resistance to TSH was accompanied by normal free thyroid hormone levels (PTH: 71.6 mU/L; TSH: 4.8 pg/mL), thus identifying this patient as having POH/PHP-Ia.

Finally, some of our patients also manifested additional clinical features, such as oligohydramnios (pts 1 & 6), multiple angiomas (pt 1), mental retardation (pt 6) and two were born small for gestational age (SGA) (pts 1 & 2).

GNAS molecular analysis

Sequencing analysis of GNAS Gsα-coding exons allowed the identification of 4 different heterozygous loss of function mutations in 6 out of 10 of patients described in the present study (60%). Of these genetic defects, 2 were small deletions causing frameshift, 1 was a nonsense mutation and 1 was a mutation of canonical splice site (Fig. 2). A brief report of these variants is shown in Table 1. Genetic variants c.85C > T (pt 1) and c.568_571del (pts 3, 4 and 5) have been previously described and, in particular, the 4-bp deletion localized in exon 7 is the only mutational hot-spot so far recognized [13]. As for the other two detected mutations, c.554del (pt 2) and c.662 + 2 T > G (pt 6), they are novel to the literature. In silico analysis predicted that the single base deletion c.554del in exon 7 determines the amino acidic substitution Val185Gly with transduction of a truncated protein. The splice site mutation c.662 + 2 T > G in intron 8 likely results in the inclusion of intron 8 in the transcript, with consequent introduction of additional amino acids before a premature stop codon.

Parent's DNA analysis was performed for mutated patients and showed that patients 1, 2, 3 and 6 were sporadic cases, while patients 4 and 5 inherited their genetic defect from the mother and father, respectively. Loss of function mutations in Gsα exons 1–13 inherited from the mother lead to PHP-Ia, while the same mutations inherited from the father may lead to both pseudo-PHP, in which AHO occurs in the absence of endocrine abnormalities, and POH. Thus, the determination of the mutated allele allows planning further investigations aimed to early detect metabolic and endocrinological deficiencies such as hypocalcemia or hypothyroidism in the case of maternal-inherited mutations.

From 3 sporadic patients we collected fresh blood samples for RNA extraction and we performed segregation analysis by RNA Sanger sequencing in order to define that the mutated allele was, as expected by the clinical manifestations, the paternal one. This investigation was possible taking advantage of the fact that GNAS locus is imprinted giving rise to different parent-specific transcripts, the maternally expressed NESP55 (neuroendocrine protein 55) and paternally expressed XLαs (extra-large variant of Gs alpha), AS (also referred to as "Nespas" in mouse) and A/B (also referred to as "1A" in mouse). Gsα, NESP55, XLαs and A/B transcripts are produced using alternative first exons splicing onto a common set of downstream exons (GNAS exons 2–13), therefore, by investigating differentially imprinted GNAS-derived genes for the presence of a specific genetic variant, we were able to define the inheritance of the mutated allele. In particular, in patient 2 we confirmed the presence of the single nucleotide deletion c.554del in the A/B RNA, which is transcribed only from the paternal allele. To further confirm that the mutation rose on the paternal allele of patient 2 we analyzed also NESP RNA, which resulted to have a wild-type sequence. In patients 3 and 6 the paternal A/B RNA was degraded. Interestingly, sequencing of Gsα transcript, that is expected to have biallelic expression in peripheral blood mononuclear cells, showed the presence of the wild-type allele only, supporting the hypothesis of nonsense-mediated decay of the mutated one, as already reported [14,15]. On the contrary, NESP RNA, which is of maternal origin, showed a wild-type sequence, indicating that the paternal allele is necessarily the affected one (Fig. 2).

MS-MLPA analysis was performed in all patients and ruled out, at the same time, the presence of structural rearrangements affecting STX16 and GNAS loci, such as deletions or duplications, and of methylation defects at GNAS DMRs. In particular, no genetic/epigenetic mutations were detected in patients negative for GNAS classical mutations (pts 7, 8, 9 and 10) (Supplementary Fig. 1).

Discussion

In the present study we performed molecular analysis of GNAS locus in 10 patients affected with either POH or primary OC, in order to investigate the presence of causative genetic or epigenetic defects.

Table 1
Clinical and molecular characteristics of patients with HO included in the present study.

Pt ID	Gender	Age	Diag. age	Clinical phenotype	GNAS status		Mutation		Amino acid change	Inheritance pattern	Diagnosis
					Epigenetic	Genetic	Site	Type			
1	F	3.5	0.5	HO/oligohydramnios/SGA/ multiple angiomas	wt	c.85C > T	ex1	NS	p.(Gln29X)	De novo	OC
2	M	6	2	HO/SGA	wt	c.554del	ex7	FS	p.(Val185Glyfs*19)	De novo (♂allele)	OC
3	M	5	1	HO	wt	c.568_571del	ex7	FS	p.(Asp189Metfs*14)	De novo (♂allele)	POH
4	F	3	0.5	HO/Ob/rPTH/rTSH	wt	c.568_571del	ex7	FS	p.(Asp189Metfs*14)	Inherited (♀allele)	POH/PHP-Ia
5	M	12	12	HO	wt	c.568_571del	ex7	FS	p.(Asp189Metfs*14)	Inherited (♂allele)	POH
6	M	9	7	HO/MR/oligohydramnios	wt	c.662 + 2 T > G	IVS8	SP	p.(?)	De novo (♂allele)	POH
7	F	6	5	HO	wt	wt	–	–	–	–	POH
8	M	17	15	HO	wt	wt	–	–	–	–	POH
9	F	4	1	HO	wt	wt	–	–	–	–	OC
10	F	46	42	HO	wt	wt	–	–	–	–	POH

Legend: M (male), F (female), age (years), diag. age (years at diagnosis), HO (heterotopic ossification), SGA (small for gestational age), MR (mental retardation), Ob (obesity), rPTH (parathyroid hormone resistance), rTSH (thyroid-stimulating hormone resistance), wt (wild-type), ♂ (paternal), de novo (no parental inheritance), ex (exon), IVS (intron), NS (nonsense), FS (frameshift), SP (splice site).
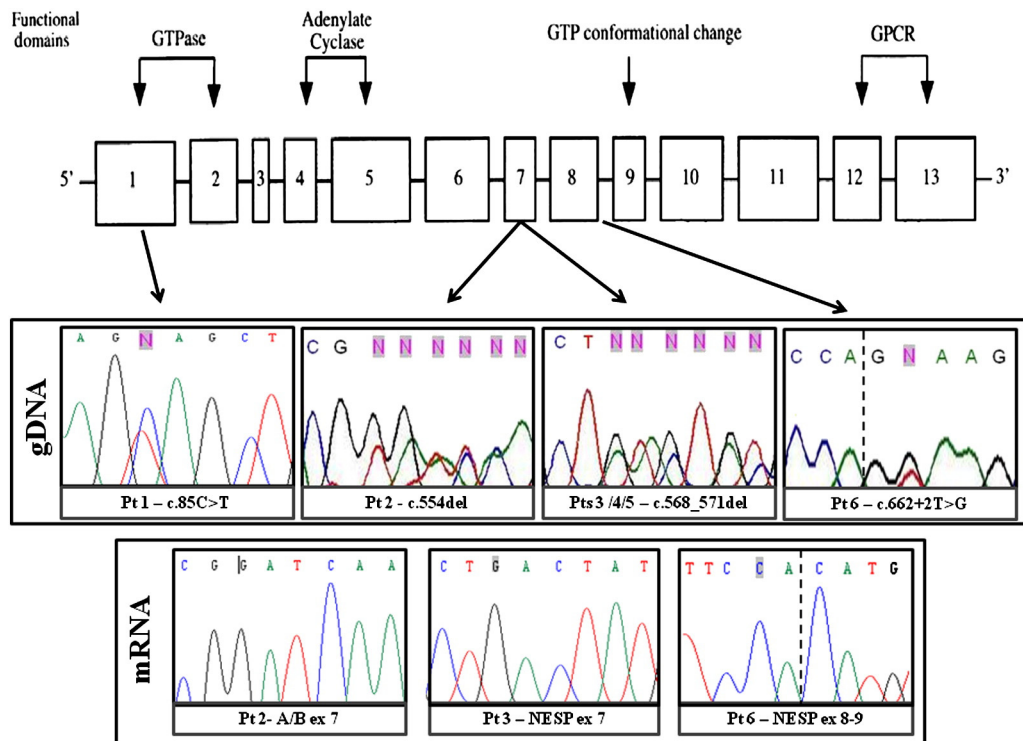
Fig. 2. Schematic illustration of the *GNAS* gene and of mutations detected in patients with HO included in the present study (gDNA, genomic DNA). Above gene illustration encoded functional domains are shown (GTP, guanosine triphosphate; GPCR, G protein coupled receptor).

Progressive osseous heteroplasia is a rare genetic disorder of HO that progresses from skin to deep connective tissues and skeletal muscle. First symptoms are usually noted during infancy and consist of small papules that predate into ossified nodules. The distribution of ectopic bone is variable as it can be widespread or focused in particular areas, especially the arts. Lesions may coalesce to form bony plates. Trauma does not seem to precipitate or exacerbate lesions of POH. Possible complications include ankylosis of involved joints with poor growth of the limb, skin ulceration with possible leakage of bone material and extremely painful infection. Routine laboratory tests are usually normal, unless following the ectopic deposition of bone when levels of alkaline phosphatase and of LDH and CPK may be transiently elevated.

Follow-up of few POH patients beyond adolescence showed a course of slower progression during adulthood [2,16]. However, given the progressive nature of the disease and the insufficiency of data in the literature, it is important to carry out an accurate follow-up as very young individuals with POH/AHO and POH/PHP1a/1c could develop a more complex phenotype over the years. The explanation of this extremely variable phenotypic expression still needs to be investigated and among the possible causes genetic background, epigenetic regulation as well as non-genetic factors can be considered.

Mutational analysis of our patients detected heterozygous loss of function mutations in Gsα coding sequence in 6 of 10 patients, with a mutation rate of about 60%, in accordance with the literature [10]. As expected from the revision of published Gsα mutations associated to POH, our series confirms that mutations are spread all along the gene and that frameshift and nonsense mutations predominate [2,7,9,10,17–23]. We found 2 different small deletions localized in exon 7 (pts 2, 3, 4 and 5), a single base substitution in intron 8 (pt 6) and a nonsense mutation in exon 1 (pt 1), all predicting truncated proteins. According to recent data from the literature, we confirm that exon 1 mutations may be associated with POH, indicating that this disease, like PHP-Ia and PPHP, is mainly Gs alpha-mediated and not XL-mediated as first suggested [7,23]. The specific involvement of Gs alpha in ectopic bone formation is further supported by the observation that, in our PHP-Ia series, patients with mutations in exon 1 showed a higher prevalence of HO with respect to patients affected by mutations in other *GNAS* exons (64.3% vs. 40%) [13].

Our sequencing data strengthen the observation that the same *GNAS* mutation may present with variable expressivity. In fact, although patients 3, 4 and 5 share the same mutation c.568_571del, they exhibit variable degrees of severity based on the extent of progressive HO lesions and PHP/AHO signs.

The investigation of parent's DNA in patients showed that patients 1, 2, 3 and 6 carry de novo mutations, while patients 4 and 5 have inherited mutations. Molecular analysis determined that patient 4 inherited the *GNAS* mutation from her mother. Accordingly, despite POH was diagnosed in this girl at the age of 6 months, she manifested resistance to PTH and TSH over the next 3 years, and she was thus identified as having an intermediate and overlapping form called POH/PHP-Ia.

In order to establish the origin of the mutated allele in patients carrying de novo mutations, we studied the RNA obtained from peripheral blood leukocytes from patients 2, 3 and 6. This analysis defined that in all the investigated cases the mutation occurred on the paternal allele, as expected from the literature for patients with POH [2,10,23].

Finally, we performed MS-MLPA analysis to evaluate, at the same time, the presence of structural rearrangements affecting *STX16* and *GNAS* loci, such as deletions or duplications, and of methylation defects at *GNAS* DMRs. All tested POH/OH patients displayed a normal *GNAS* imprinting status and absence of copy number abnormalities.

As for PHP patients, in the present series POH patients without detectable genetic mutations (pts 7, 8, 9 and 10) were clinically indistinguishable from those with mutations. However, this result does not exclude the presence of genetic alterations in *GNAS* regions not investigated by our assays or in other loci. Moreover, no evident difference was observed among patients harboring different mutations. Thus, neither the presence/absence nor the type or the localization of

mutations allows us to predict a specific phenotype or the severity of progression within the spectrum of *GNAS*-related disorders.

Previous investigations reported that several individuals with paternally inherited *GNAS* mutations were born small for gestational age (measurements at or below the fifth percentile compared to sex-matched normative data) and had a lean phenotype at the time of initial presentation [10]. Data from *Gnas* knockout mouse models support the hypothesis of a critical role played by Gsα in regulating lineage determination towards adipogenic versus osteogenic fate in soft tissue progenitor cells [24]. Our study further confirms these observations, as two patients (pts 1 and 2) had low birth weights. Interestingly, while 9 out of 10 patients displayed a lean phenotype, patient 4, who inherited the mutated allele from her mother, became severely obese few months after birth. Accordingly, this little girl also developed PTH and TSH resistance over the years, supporting the view that only maternally-derived alteration may lead to endocrine manifestations as well as obesity.

Despite this phenotypic heterogeneity, genetic counseling is still an important and delicate matter as: 1) patients have 50% risk of transmitting the mutated allele to their offspring; 2) in the case of paternal inheritance, *GNAS* mutations can either lead to the milder AHO phenotype or to the more severe POH phenotype; 3) genetic diagnosis with prompt identification of the mutated allele in patients showing an overlapping POH/PHP phenotype may avoid unnecessary endocrinological investigations in the case of paternal inheritance, or help to early detect hypocalcemia and/or hypothyroidism in the case of maternally inherited mutations.

Conclusions

In conclusion, our results support the view that POH belongs to a continuum spectrum of HO disorders associated with inactivating *GNAS* mutations and further expand the spectrum of genetic defects associated with this disease. Moreover, we updated the *GNAS* Locus-Specific database (the database is available on the internet at www.lovd.nl/GNAS) with here and elsewhere described mutations associated with POH. Finally, we investigated the imprinting status of *GNAS* DMRs and we observed that, in contrast with what was observed in PHP patients, methylation alterations at this locus are either absent or uncommon in POH/OH.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2013.06.015.

Acknowledgments

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