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Pseudohypoparathyroidism and *GNAS* Epigenetic Defects: Clinical Evaluation of Albright Hereditary Osteodystrophy and Molecular Analysis in 40 Patients

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Context: The two main subtypes of pseudohypoparathyroidism (PHP), PHP-Ia and -Ib, are caused by mutations in *GNAS* exons 1-13 and methylation defects in the imprinted *GNAS* cluster, respectively. PHP-Ia patients show Albright hereditary osteodystrophy (AHO) and resistance toward PTH and additional hormones, whereas PHP-Ib patients do not have AHO, and hormone resistance appears to be limited to PTH and TSH. Recently, methylation defects have been detected in few patients with PHP and mild AHO, indicating a molecular overlap between the two forms.

Objectives: The aim of the study was to screen patients with clinically diagnosed PHP-Ia for methylation defects and to investigate the presence of correlations between the molecular findings and AHO severity.

Patients and Methods: We investigated differential methylation of *GNAS* regions and STX16 microdeletions in genomic DNA from 40 patients with sporadic AHO and multihormone resistance, with no mutations in Gsα-coding *GNAS* exons.

Results: Molecular analysis showed *GNAS* cluster imprinting defects in 24 of the 40 patients analyzed. No STX16 deletion was detected. The presence of imprinting defects was not associated with the severity of AHO or with specific AHO signs.

Conclusions: We report the largest series of the literature of patients with clinical AHO and multihormone resistance and no mutation in the Gsα gene. Our findings of frequent *GNAS* imprinting defects further confirm the existence of an overlap between molecular and clinical features of PHP-Ia and PHP-Ib and highlight the necessity of a new clinical classification of the disease that takes into account the recent knowledge on the molecular basis underlying these defects. **(*J Clin Endocrinol Metab* 95: 0000–0000, 2010)**

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Abbreviations: AHO, Albright hereditary osteodystrophy; DMR, differentially methylated region; MR, mental retardation; PHP, pseudohypoparathyroidism; RQ, relative quantification; SDS, SD score; SNP, single nucleotide polymorphism.

Pseudohypoparathyroidism (PHP) refers to a heterogeneous group of rare metabolic disorders characterized by resistance to the action of PTH (1, 2). The two main subtypes of PHP, PHP type Ia and Ib (PHP-Ia, PHP-Ib) are caused by molecular alterations within or upstream of the *GNAS* locus. In particular, heterozygous loss of function mutations in exons 1-13 of the gene encoding Gsα (the α-subunit of heterotrimeric stimulatory G protein) inherited from the mother lead to PHP-Ia, in which Albright hereditary osteodystrophy (AHO), a disorder characterized by a constellation of physical features [short stature, rounded face, brachydactyly, ectopic ossifications, and various degrees of mental retardation (MR)], is associated with end-organ resistance to the action of different hormones that activate the Gs-coupled pathways, primarily PTH, TSH, gonadotropins (reviewed in Refs. 1, 3, and 4) and, more recently reported, GHRH (5, 6). The same mutations inherited from the father lead to pseudo-PHP, in which AHO occurs in the absence of endocrine abnormalities. Most patients with PHP-Ia show a partial deficiency (50%) of Gs activity in red blood cells (7), due to a reduction in its α-subunit mRNA and protein. Although *GNAS* mutations are identified in the majority of patients with PHP-Ia and in their relatives affected with pseudo-PHP, in about 30% patients the molecular diagnosis remains to be ascertained (1).

Besides Gsα, *GNAS* gives rise to several other transcripts, including XLαs, A/B (also referred to as 1A), NESP55, and AS. The complexity of the *GNAS* locus is further reflected by a parent-specific methylation pattern of most of its different promoters (Fig. 1A) (reviewed in Ref. 8). In PHP-Ib patients, AHO is absent, Gsα activity in erythrocytes and fibroblast is normal, and hormone resistance is limited to PTH and TSH, the latter being essentially always subclinical (9, 10). This disorder is caused by the disruption of long-range imprinting control elements in *GNAS* locus. In particular, the most consistent defect is the loss of imprinting at the exon A/B differentially methylated region (DMR), with consequent presumed decreased Gsα transcription in tissues where this protein is derived from the maternal allele only. The familial form of the disease (AD-PHP-Ib) is typically associated with an isolated loss of imprinting at the exon A/B DMR, together with microdeletions disrupting the upstream STX16 gene as the cause of the epigenetic disruption (11, 12). In addition, deletions removing the entire NESP55 DMR have been identified in some AD-PHP-Ib kindreds in whom affected individuals show loss of all the maternal *GNAS* imprints (13). Conversely, most sporadic PHP-Ib cases have *GNAS* imprinting abnormalities that involve multiple DMRs, but the genetic lesion, if any, underlying these defects remains to be discovered (reviewed in Refs. 14 and 15). Interestingly, in both PHP-Ia and familial PHP-Ib, hormonal resistance develops only after maternal inheritance of the mutation/deletion, consistent with the recent finding of tissue-specific paternal imprinting of the Gsα gene in those tissues resistant to hormone action in the two diseases (16–18).

Recently, three independent studies reported methylation defects similar to those observed in PHP-Ib in a total of seven patients clinically diagnosed with PHP-Ia, six of whom displayed mild features of AHO (19–21). In the present study, we analyzed the presence of *GNAS* imprinting defects in a large series of patients with a likely clinical diagnosis of PHP-Ia and absence of mutations in Gsα-coding exons, in whom AHO features varied from mild to severe, and we found that more than half of these patients could be classified at the molecular level as sporadic PHP-Ib.

Patients and Methods

Sequencing analysis of the *GNAS* gene

Genomic DNA was extracted from peripheral blood leukocytes (Nucleon BACC kit; GE Healthcare, Little Chalfont, UK). The Gsα-coding *GNAS* exons (exons 1-13) were then amplified by PCR using the specific primer pairs and conditions previously published (22). Amplification of all exons included each bordering intron region; the following new primer pair for exon 1 was used: *SE*, 5′-CCTCCCGGCCCGCGTGA-3′; and *AS*, 5′-CTGCGGGGCGCCCTTCGA-3′. Direct sequencing of the amplified fragments was then performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Moreover, to rule out deletions in the upstream region of *GNAS*, we performed genotyping by PCR and direct sequencing of a known polymorphic pentanucleotide repeat within the A/B DMR (19, 23), at six polymorphic sites within the NESP55 region (*rs* 1800900, *rs* 1800901, *rs* 1800902, *rs* 1800903, *rs* 1800904, and *rs* 1800905) and eight polymorphic sites within the XL-A/B intronic region (*rs* 35113254, *rs* 2145288, *rs* 6026567, *rs* 12625436, *rs* 7271854, *rs* 6123836, *rs* 6026574, and *rs* 4812042) (24). The primer pairs and conditions used for the specific amplification of these regions are available on request.

Methylation analysis of *GNAS*

Differential methylation of *GNAS* DMRs A/B, AS, XL, and NESP was assessed on bisulfite-treated DNA using highly quantitative analysis based on PCR-pyrosequencing, as previously reported (25). In brief, 1 μg DNA (concentration 50 ng/μl) was treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol, and PCR was amplified. PCR primers for the amplification of the four different *GNAS* exons were specifically designed to amplify the modified DNA (10). For each reaction, a PCR was conducted in 50 μl of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen S.R.L., Milano, Italy), 1 pmol of the forward primer, 1 pmol of the reverse primer, 50 ng of bisulfite-treated genomic DNA, and water. Primer sequences are reported in Supplementary Table 1 (published as supplemental data on The Endocrine

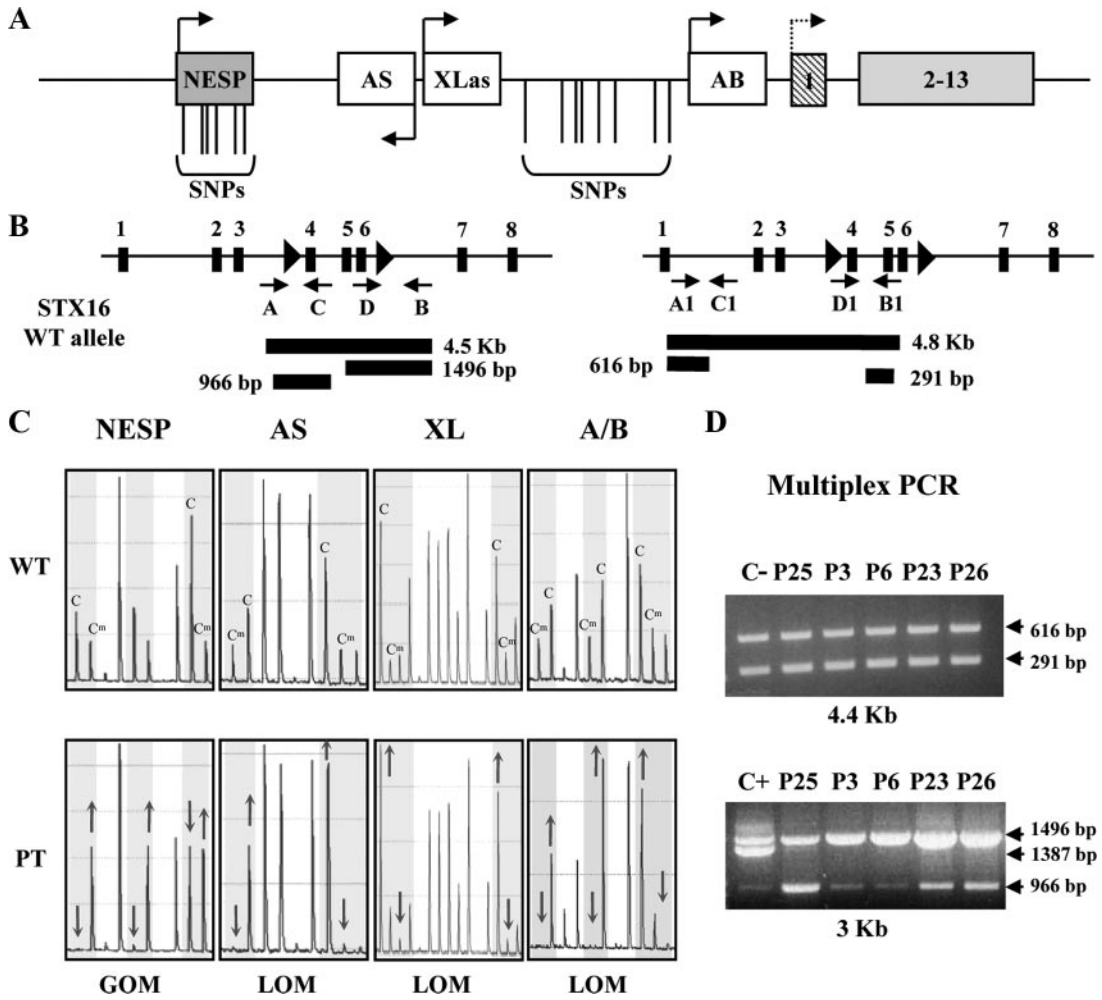

FIG. 1. A, General organization and imprinting of *GNAS*. The maternal and paternal alleles of *GNAS* are shown, and *arrows* indicate the direction of transcription. The alternative first exons and common exons 2-13 are shown as *rectangles*: *white* for the paternally derived ones, *gray* for the maternally derived NESP55, and *striped* for the biallelically transcribed exon 1, *i.e.* the first coding exon for Gsα. *Vertical lines* indicate the position of the SNPs analyzed: in the NESP region, the SNP location partially overlaps with the DMR studied. The diagram is not drawn to scale. B, STX16 gene 3-kb (*left*) and 4.4-kb (*right*) deletions analysis. Exons are depicted as *black boxes*, and direct repeats are shown as *arrowheads*. The 3-kb deleted region lies between the direct repeats and causes the loss of STX16 exons 4-6. Amplification of the *STX16* region with four different primers, located at nucleotides 3606 [primer A (forward)], 4572 [primer C (reverse)], 6455 [primer D (forward)], and 7951 [primer B (reverse)] of AL139349 is shown. When performing multiplex PCR, all these primers are included in the reaction. The amplicons obtained are shown as *black lines*. The expected sizes of wild-type PCR products are 4.5 Kb (primers A and B), 1496 bp (primers D and B), and 966 bp (primers A and C). Obligate carriers and affected individuals show an additional 1387 bp amplicon (primers A and B) that is amplified on the deleted allele (data not shown). The *right panel* shows the 4.4-kb deleted region that lies between exons 1-4. Amplification of this *STX16* region with four different primers at 404, 1020, 4902, and 5193 of AL139349 [primer A1 (forward); primer C1 (reverse); primer D1 (forward); and primer B1 (reverse), respectively] is shown. The expected sizes of wild-type PCR products are 4.8 Kb (primers A1 and B1), 616 bp (primers A1 and C1), and 291 bp (primers D1 and B1). Affected individuals show an additional 417-bp amplicon. C, Methylation analysis of *GNAS* DMRs in patient 3 (PT; as a representative sample) and a normal control (WT). Genomic DNA from each subject included in the study was bisulfite treated, and each *GNAS* DMR was amplified using specific primers. Representative pyrograms for each analyzed locus are shown the figure. The x-axis represents the DMR sequence, whereas the y-axis shows the percentage of incorporated nucleotide for each given position. The *upper panel* shows the normal CpG pattern of methylation in a wild-type subject, whereas the *lower panel* demonstrates methylation alterations (indicated by *arrows*) at the same position in a representative patient. D, STX16 deletion analysis performed by multiplex PCR. Long-range PCR analysis is performed using only primers A and B or A1 and B1 (data not shown). When multiplex PCR is used for the identification of the deletions (4.4 kb, *upper panel*; 3 kb, *lower panel*), normal subjects show two PCR products (1496 bp and 966 bp for 3-kb deletion; 616 bp and 291 bp for 4.4-kb deletion), whereas deleted patients show an additional band of 1387 bp and 417 bp, respectively. Here, we show the results obtained for a normal control (C−), a known patient bearing the 3-kb deletion (C+), and a group of five representative samples: patients 25 (P25) and 26 (P26), with normal methylation pattern; patients 3 (P3), 6 (P6), and 23 (P23) with altered methylation pattern.

Society's Journals Online web site at http://jcem.endojournals.org). One of the forward or reverse primers was biotin-labeled, depending on the proximity to CG sites to be quantified and used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 M NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Westborough, MA), as recommended by the manufacturer. A 0.3 μM

TABLE 1. Clinical characteristics and molecular analysis of patients included in the present study

Pt	Sex	Age 1	Age 2	PTH (pg/ml)	TSH (mIU/liter)	Height (±SDS)	AHO features	Abnormal methylation	Methylation %			
									AB	NESP	AS	XL
1	F	9	*12*	795	6.2	148 (+0.2)	Br/RF	AB/NESP/AS/XL	9	87	5	12
2	M	22	8	380	7.1	113 (−3.1)	Br/SS/RF/Ob/SO/MR	AB/NESP/AS/XL	11	81	13	15
3	F	22	6	256	4.8	99 (−2.5)	Br/SS/RF/Ob/SO/MR	AB/NESP/AS/XL	5	95	8	7
4	F	18	8	401	12.3	111 (−2.6)	Br/SS/RF/Ob/SO/MR	AB/NESP/AS/XL	3	88	13	6
5	F	15	12	83	4.5	138 (−1.8)	Br/SS/RF/Ob/SO/MR	AB/NESP/AS/XL	12	90	9	10
6	F	4	8	391	6.6	112 (−2.0)	SS/RF/Ob/SO/MR	AB/NESP/AS/XL	8	86	12	18
7	M	3	4	90	20.5	92 (−2.2)	Br*/SS/RF/MR	AB/NESP/AS/XL	3	93	7	9
8	M	3	7	95	4.8	107 (−2.7)	Br/SS/RF/Ob/MR	AB/NESP/AS/XL	10	97	15	13
9	M	8	*10*	227	14	140 (+0.7)	Br/RF/Ob/SO/MR	AB/NESP/AS/XL	12	85	11	11
10	M	3	12	203	5.6	130 (−3.2)	SS/RF	AB/NESP/AS/XL	12	90	9	19
11	F	5	13	75	5.3	139 (−3.0)	Br/SS/RF	AB/NESP/AS/XL	4	96	6	7
12	F	2	10	312	16.2	122 (−2.5)	Br/SS	AB/NESP/AS/XL	5	89	8	9
13	F	8	*12*	260	25.6	157 (−0.5)	Br*/RF/Ob	AB/NESP/AS/XL	12	90	9	5
14	F	4	*18*	188	5.2	163 (+0.1)	Br/RF/Ob	AB/NESP/AS/XL	7	93	8	8
15	M	2	*9*	216	14.2	170 (+1.0)	Br/RF	AB/NESP/AS/XL	10	84	15	10
16	M	13	13	256	5.0	137 (−2.2)	Br/SS/RF/Ob/SO	AB/NESP/AS/XL	7	97	8	8
17	F[a]	15	*22*	315	2.2	166 (+0.5)	Br/RF/Ob/SO/MR	AB/NESP/AS/XL	9	88	11	13
18	M	19	*21*	468	1.9	178 (+0.3)	Br/RF/Ob	AB/NESP/AS/XL	8	92	9	12
19	F	13	13	135	1.7	138 (−2.8)	Br*/SS/RF/Ob/MR	AB/NESP/AS/XL	15	91	5	9
20	F	18	*20*	360	7.8	165 (−0.1)	Br/MR	AB/NESP/AS/XL	12	83	18	11
21	M	6	7	80	7.5	107 (−1.9)	Br/SS/RF/Ob	AB/NESP/AS/XL	3	98	5	6
22	F	5	6	107	9.2	102 (−1.7)	Br*/SS/RF/Ob/SO/MR	AB/NESP/AS/XL	9	91	6	15
23	F	14	14	182	2.1	143 (−3.5)	Br/SS/RF/Ob/SO/MR	AB/NESP/AS/XL	13	89	12	8
24	F	7	10	307	2.0	121 (−2.0)	Br/SS/RF/Ob	AB/NESP/AS/XL	8	92	10	7
25	F	13	15	90	15.3	146 (−2.3)	Br/SS/RF/Ob/MR	No	48	42	44	47
26	F	2	*8*	198	5.8	124 (+0.7)	Br/RF/SO	No	38	45	34	47
27	F	12	13	109	5.1	140 (−2.2)	Br/SS/RF/Ob/SO	No	47	40	42	39
28	M	5	*5*	130	7.5	113 (+1.3)	Br/RF/Ob/SO/MR	No	41	39	45	42
29	F	1	*12*	281	10.4	150 (+0.6)	Br/RF/Ob/SO	No	40	45	45	41
30	F	13	13	73	8.8	139 (−2.6)	Br*/SS/RF/Ob/SO	No	43	38	35	40
31	F	11	*22*	452	13.2	168 (−0.3)	RF/MR	No	45	42	44	37
32	F	15	*15*	105	50/88[b]	161 (−0.1)	Br/RF	No	44	40	48	42
33	F	30	30	222	18.1	144 (−2.8)	Br*/SS/RF/Ob/SO/MR	No	43	42	40	44
34	F	10	13	256	6.0	143 (−2.1)	Br/SS/RF/Ob/SO	No	39	42	45	37
35	F	1	*10*	302	1.7	133 (+0.2)	Br/RF/SO	No	46	41	36	40
36	F	12	*12*	95	4.8	152 (+0.6)	Br/RF/SO	No	42	45	39	43
37	F[a]	12	*30*	375	2.5	170 (+1.5)	Br/RF	No	40	43	44	41
38	F	23	*25*	95	4.6	168 (+0.5)	Br/RF	No	46	47	45	48
39	M	10	12	111	2.0	134 (−2.0)	Br/SS/RF/Ob/MR	No	39	41	41	44
40	M	22	22	164	5.8	158 (−3.0)	Br/SS/MR	No	37	42	38	40

F, Female; M, male; Br, brachydactyly, clinically evident; Br*, radiological evidence of brachydactyly; SS, short stature; RF, round face; Ob, obesity defined as body mass index above 30 kg/m^2 in adults or weight above the 97th centile in children; SO, sc ossifications. Age 1 refers to the age (in years) at diagnosis; age 2 is the age at which short stature became apparent (during late infancy or puberty in most nonadult patients) or, in those cases where short stature was absent, age 2 refers to the age at last evaluation and is *italicized*. PTH and TSH refer to the levels at the time hormone resistance was discovered; normal values are 10–65 pg/ml for PTH, and 0.2–4.0 mIU/liter for TSH. Subclinical hypothyroidism, *i.e.* elevated TSH levels with normal free thyroid hormones, is characterized by TSH levels between 4.2 and 10 mIU/liter. Height is expressed in centimeters and always refers to age 2; target height SDS is reported in *parentheses*. The last column (% methylation) shows the mean of three different determinations at each *GNAS* DMR. The degree of methylation is expressed as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines.

[a] Female patients with a history of nonspecific menstrual irregularities because of which combined oral contraceptive pill was started.

[b] For patient 32, instead of TSH, the FSH and LH levels (IU/liter) are shown, respectively.

pyrosequencing primer was then annealed to the purified single-stranded PCR product, and pyrosequencing was performed using the PyroMark MD (Pyrosequencing). Methylation quantification was performed using the provided software. The degree of methylation was expressed for each considered DNA locus as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines. We used built-in controls to verify bisulfite conversion efficiency. Every sample was tested three times for each marker to confirm the reproducibility of our results, and the mean obtained is shown in Table 1.

Detection of the 3-kb and 4.4-kb deletions in STX16

We investigated the presence of STX16 deletions on patients' genomic DNA by both long-range and multiplex PCR tech-

niques, as previously described (10, 11). Briefly, long-range PCR was performed using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen S.R.L.) at the manufacturer's conditions and two different couples of primers located at the boundaries of each deletion and a smaller fragment in case of the presence of a deleted STX16 allele. For multiplex PCR, we simultaneously used two pairs of primers giving raise to PCR products of different length depending on the presence of the deleted allele (Fig. 1B).

mRNA quantitative PCR analysis

Total RNA was isolated from peripheral blood cells using a commercial kit (QIAGEN Rneasy mini kit; QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After digestion of genomic DNA, 1 μg of RNA was reverse-transcribed through the Archive cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For Gsα mRNA relative quantification, as well as for three housekeeping genes (18S, TBP, and UBC), 10 ng of cDNA was then amplified (TaqMan technology) in a final PCR volume of 10 μl using a 7900HT instrument (26, 27). Specific primers and probes for Gsα mRNA are as follows: forward primer, 5′-GCTGCCTCGGGAACAGTAAG-3′; reverse primer, 5′-GCTGCTTCTCGATCTTTTTGTTG-3′; TaqMan probe, 5′-6-FAM-CCGAGGACCAGCGCAACGAGG-TAMRA. Commercial gene expression assays (Applied Biosystems) were used to amplify housekeeping genes, and assays numbers are: 18S, Hs9999901_s1; TBP, Hs00427620_m1;and UBC, Hs00824723_m1.

All quantitative PCRs were carried out in duplicate and confirmed by two independent experiments. The geometrical mean of the three reference genes was used as normalization factor for Gsα mRNA relative quantification (RQ) in 19 patients and in seven normal subjects (used as controls). Gsα was considered differentially expressed if its RQ in a patient was at least decreased of 2.5-fold compared with Gsα averaged level quantified in controls.

Results

Patients

The study included 40 patients (28 females and 12 males), all born from healthy, nonconsanguineous Caucasian parents. In all of them, a likely clinical diagnosis of PHP-Ia was based upon the presence of PTH resistance (*i.e.* hypocalcemia, hyperphosphatemia, and raised serum PTH levels in the absence of vitamin D deficiency) together with at least two of the six AHO manifestations: brachydactyly [shortening of fourth and/or fifth metacarpals defined as the metacarpal sign and/or shortening below -2 SD score (SDS) at the metacarpophalangeal profile pattern in at least one metacarpal bone or distal phalanx (Fig. 2)] (19, 28), short stature (height below the third percentile for chronological age), obesity (body mass index $>$ 30 kg/m^2 in adults and $>$97th centile in children), round face, sc ossifications (either clinically evident or at x-ray), and MR. In particular, in most patients, shortening of the fourth and/or fifth metacarpals was clinically evident, and this was particularly true in all those patients in which brachydactyly was the main clinical sign of AHO (patients 1, 12, 15, 32, 37, 38) (Fig. 2). Mental retardation was defined in case of a history of delayed motor and/or speech milestones or need of extra help in preschool or mainstream school. The age at which the diagnosis was made ranged between 1 and 30 yr. The majority of patients showed either subclinical (n = 21) or clinical (n = 10) hypothyroidism due to resistance to TSH. This was documented by raised serum TSH levels, in the absence of antithyroid antibodies and in the presence of a normal thyroid scan (data not shown). There was no evidence of other endocrine defects in all the other patients but one, in which resistance to gonadotropins was also present (patient 32), documented by elevated LH and FSH levels, together with low estradiol levels and secondary amenorrhea. In the remaining patients, the onset of puberty was regular, and so was the menstrual cycle in females, with the exception of patients 17 and 37, who developed oligomenorrhea. Nevertheless, we point out that at least 14 patients were still pre- or peripubertal at the time of last evaluation, and menstrual irregularities may become apparent during the next few years. Gonadotropin, ACTH, and cortisol levels, as well as estradiol levels in females and testosterone levels in males, were in the normal range (data not shown). Clinical and biochemical details are shown in Table 1. Informed consent was obtained from all subjects involved in the study.

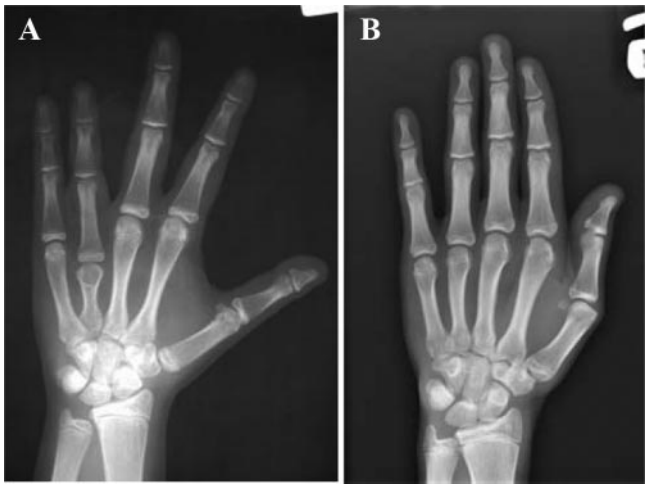

FIG. 2. Left hand x-rays of cases 1 (A) and 7 (B). Although the metacarpal sign is evident in patient 1 (who showed clinically evident brachymetacarpia), patient 7 exhibited shortening of all metacarpal bones and of I, IV, and V distal phalanx according to z-score calculations <2 SDS in comparison with normal subjects.

Molecular analysis

Sequencing analysis confirmed the absence of mutations in Gsα-coding *GNAS* exons. All patients displayed at least one of the known polymorphisms present in Gsα-

coding sequence (C/T at positions 749, 911, and 1469 in exons 5, 7, and 11, respectively; NM000516), excluding the existence of large deletions comprising the gene. Moreover, to rule out deletions in the upstream region of *GNAS*, we analyzed a known polymorphic pentanucleotide repeat within the A/B DMR, as well as six single nucleotide polymorphisms (SNPs) within the NESP55 region and eight SNPs in the XL-A/B intronic region: overall, each patient was heterozygous for at least one of these SNPs in each region, and looking at the polymorphic pentanucleotide repeat as well as at each SNP individually, we found percentages of heterozygosity similar to those reported in the literature (Supplementary Table 2) (19, 23, 24). Moreover, in those patients whose parents' DNA was available, we found concordant SNP patterns (data not shown). Taken together, these results make the presence of deletions in these regions highly unlikely.

Of the 40 patients included in the study, 24 showed methylation defects at *GNAS* DMRs, thus indicating PHP-Ib as the correct diagnosis at the molecular level (Table 1 and Fig. 1C). In particular, all patients showed loss of methylation at exon A/B, a condition today commonly thought to be sufficient to determine PHP manifestations. According to the apparent sporadic form of the disease in our patients, in addition to methylation defects at exon A/B, all had multiple additional methylation changes (loss of methylation at XL and AS DMRs and gain of methylation at NESP DMR). Moreover, none of them showed STX16 deletions as assessed by long-range and multiplex PCRs (Fig. 1D). Finally, parents' DNA from eight patients was available, and the analysis of *GNAS* methylation pattern gave normal results (patients 5, 6, 9, 13, 14, 19, 23, 24; data not shown).

Quantification of the Gsα transcript by real time RT-PCR in 19 of our 40 patients (12 with methylation defects, seven with normal methylation pattern) revealed a reduction of gene expression compared with controls (seven normal subjects, averaged RQ = 6.9 ± 1.34) in 10 of them (patients 2, 7, 8, 14, 15, 16, 18, 22, 31, 33; averaged RQ = 1.38 ± 1.11), whereas an expression similar to the one in normal subjects was observed in nine patients (patients 3, 10, 12, 23, 29, 34, 37, 39, 40; averaged RQ = 5.24 ± 1.73). These results were found regardless of the methylation status or clinical manifestations.

Similarly, no correlation was observed in our study between the presence or absence of methylation defects and the severity of AHO. In fact, as shown in Table 1, our series included patients who displayed the typical AHO features with either broad methylation alterations (patients 2–6, 8, 9, 16, 17, 19, 22, 23) or normal methylation pattern (patients 25, 27, 28, 30, 33, 34, 39), as well as vice versa, patients with only two signs of AHO and either a normal pattern (patients 31, 32, 37, 38) or an abnormal pattern (patients 1, 10, 12, 15, 20) of methylation. Finally, there was no correlation between the methylation pattern and the number or severity of hormonal resistances.

Discussion

Here we report a screening study for methylation defects at the *GNAS* locus in patients with AHO features and hormonal resistance but absence of mutations in Gsα-coding exons. This study, which represents the largest series in the literature with complete clinic and genetic/epigenetic assessment, included 40 patients who displayed the typical features of PHP-Ia, *i.e.* multihormonal resistance in the presence of AHO. In these patients, molecular analysis excluded the genetic defects in *GNAS* coding exons (1–13) usually associated with PHP-Ia, *i.e.* loss of function mutations, large deletions, and paternal unidisomy. Moreover, the observation that all patients were heterozygous for at least one polymorphism in each A/B, NESP55, and XL-A/B intronic region makes the presence of deletions in these regions highly unlikely.

Molecular analysis demonstrated multiple methylation defects at the *GNAS* locus in the absence of STX16 microdeletions in 60% of our patients. This allowed us, at the molecular level, to reclassify those individuals as having sporadic PHP-Ib. Three recent independent reports revealed similar molecular patterns in seven patients previously diagnosed with PHP-Ia and subsequently found to have PHP-Ib (19–21). Six of these patients displayed mild AHO, as suggested by rounded face and slight metacarpal shortening (19, 21). Conversely, in our study no correlation between the presence or absence of methylation defects and the severity of AHO was observed.

The presence and extent of AHO features in the patients included in the present series are key points, many of the individual features of AHO being nonspecific to PHP (1). A classical hallmark of PHP-Ia, *i.e.* sc ossifications, was present in 19 patients, regardless of the methylation defects. As for the bone phenotype, although we followed the most commonly used and accepted radiological criteria for establishing the presence of brachydactyly, in the large majority of our patients this sign was clearly clinically evident, with no real necessity of radiological measurements. As for MR, there are no standardized tests defining it in PHP, and the frequency and severity of this sign are not well established. As in most reported series, we have defined a patient as having MR in case of a history of delayed motor and/or speech milestones or need of extra help in preschool or mainstream school. In general, accordingly with the literature, all of our patients with MR could be classified as having mild/moderate MR, and the

prevalence of MR was similar to that observed in other large reports (47.5% in our series; 65% reported in Refs. 29 and 30). Finally, nonspecific signs such as short stature, rounded face, and obesity were also frequently present in our series, as commonly reported in PHP-Ia. Overall, the large number of patients included in the present study as well as the association of different specific and nonspecific AHO-like features in each individual should exclude the possibility that the phenotype of these patients might be unrelated to GNAS-imprinting defects. The absence of the same features in relatives further strengthens the association between clinical characteristics and molecular findings.

As reported in both PHP-Ia and PHP-Ib, the majority of patients showed either subclinical or clinical hypothyroidism due to resistance to TSH. There was no evidence of other endocrine defects, although it was difficult to draw definite conclusions on gonadotropin resistance in the present series, due to the good proportion of prepubertal females or females just coming through puberty at the time of evaluation. Unfortunately, most of our patients with short stature were not tested for GH deficiency, and we are therefore unable to give additional information on this issue at this moment.

In accordance with the report by de Nanclares *et al.* (19), our data further strengthen the hypothesis that Gsα expression in humans is subject to individual-specific allelic imprinting in a number of tissues larger than that classically thought, *i.e.* the renal proximal tubule and the thyroid. Individual-related variance in Gsα expression in bone (chondrocytes and osteoblasts), central nervous system, and adipose tissue may account for the presence or absence of specific AHO manifestations (31, 32). In particular, as far as bone is concerned, although Gsα has been found to be biallelically expressed in this tissue, we demonstrated a modest maternal predominance of its transcript in one of the human bone samples investigated, further supporting the variability in the degree of allelic bias in selected tissues (31). Recent studies in mice show that Gsα paternal imprinting in the central nervous system leads to a specific defect in the ability of central melanocortins to stimulate sympathetic nervous system activity and energy expenditure, and this could account for obesity in PHP-Ia patients (32). In turn, the variable contribution of the paternal allele in Gsα expression in the thyroid would be responsible for the existence of the broad spectrum of thyroid function alterations observed in these patients, ranging from normal function to neonatal overt hypothyroidism (17, 18).

In conclusion, we report a large molecular analysis of patients with clinical PHP-Ia and absence of mutations in Gsα-coding exons. Molecular evaluation showed that more than 50% of such patients could be classified as sporadic PHP-Ib at the molecular level. It is becoming evident that PHP-Ia and PHP-Ib share more genetic and clinical similarities than previously thought because both diseases end up with silencing or reduction of Gsα transcription in selected tissues. The presence of TSH resistance both in PHP-Ib and PHP-Ia patients firstly supported this hypothesis (9, 10). The incoming data showing that the two diseases also share common epigenetic defects further strengthen this line of evidence and, in our opinion, highlight the need for a new classification of these diseases. Finally, we propose that both mutational analysis of Gsα-coding *GNAS* exons and evaluation of epigenetic defects should be considered in patients with PTH resistance and AHO-like features, regardless of their severity.

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