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Report

Paternal Uniparental Isodisomy of Chromosome 20q—and the Resulting Changes in *GNAS1* Methylation—as a Plausible Cause of Pseudohypoparathyroidism

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Heterozygous inactivating mutations in the *GNAS1* exons (20q13.3) that encode the α-subunit of the stimulatory G protein (Gsα) are found in patients with pseudohypoparathyroidism type Ia (PHP-Ia) and in patients with pseudo-pseudohypoparathyroidism (pPHP). However, because of paternal imprinting, resistance to parathyroid hormone (PTH)—and, sometimes, to other hormones that require Gsα signaling—develops only if the defect is inherited from a female carrier of the disease gene. An identical mode of inheritance is observed in kindreds with pseudohypoparathyroidism type Ib (PHP-Ib), which is most likely caused by mutations in regulatory regions of the maternal *GNAS1* gene that are predicted to interfere with the parent-specific methylation of this gene. We report a patient with PTH-resistant hypocalcemia and hyperphosphatemia but without evidence for Albright hereditary osteodystrophy who has paternal uniparental isodisomy of chromosome 20q and lacks the maternal-specific methylation pattern within *GNAS1*. Since studies in the patient's fibroblasts did not reveal any evidence of impaired Gsα protein or activity, it appears that the loss of the maternal *GNAS1* gene and the resulting epigenetic changes alone can lead to PTH resistance in the proximal renal tubules and thus lead to impaired regulation of mineral-ion homeostasis.

Pseudohypoparathyroidism type Ia (PHP-Ia [MIM 300800]) is characterized by end-organ resistance to parathyroid hormone (PTH) and several other hormones—such as thyroid-stimulating hormone (TSH)—that mediate their effects through the stimulatory G protein (Gs) (for review, see Levine 1996; Weinstein 1998; Bastepe and Jüppner 2000). Patients affected by PHP-Ia show, in addition to hormone resistance, various developmental defects referred to as "Albright hereditary osteodystrophy" (AHO [MIM 300800]), including a characteristically round face, short stature, obesity, brachydactyly, heterotopic ossifications, and various degrees of mental retardation. PHP-Ia is caused by molecular defects in *GNAS1* (MIM 139320), the gene encoding the α-subunit of Gs (Gsα), as well as several splice variants and antisense transcripts with yet-unknown biological roles (Hayward et al. 1998*a*, 1998*b*; Peters et al. 1999; Hayward and Bonthron 2000; Wroe et al. 2000). Numerous mutations throughout most Gsα-specific exons lead to an ~50% reduction in both Gsα activity and protein in circulating blood cells and fibroblasts. These mutations can also cause AHO without hormonal resistance, a condition termed "pseudo-pseudohypoparathyroidism" (pPHP [MIM 300800]). PHP-Ia and pPHP are typically present within the same kindreds but are never present within the same sibships. Analysis of the parental origin of Gsα mutations in these kindreds has indicated that, although AHO is observed regardless of the parent transmitting the defect, the expression of hormonal resistance requires maternal transmission (i.e., pPHP—AHO only—develops if the mutation is inherited from a male carrier, whereas PHP-Ia—AHO and hormonal resistance—is the phenotype if inheritance is from a female affected by either of these disorders) (Davies and Hughes 1993; Wilson et al. 1994; Nakamoto et al. 1998). Consistent with this mode of inheritance, renal PTH resistance in mice with an ablation of the maternal exon 2 of *Gnas*, genotype +/m−, but not in mice with ablation of this

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exon from the paternal allele, genotype +/p−, shows renal PTH resistance (Yu et al. 1998).

As do individuals affected by PHP-Ia, patients affected by pseudohypoparathyroidism type Ib (PHP-Ib [MIM 300800]) show PTH-resistant hypocalcemia and hyperphosphatemia but typically have no additional endocrine abnormalities. Furthermore, unlike PHP-Ia/pPHP, PHP-Ib is not associated with AHO, and the PTH resistance in PHP-Ib appears to be limited to the proximal tubular cells of the renal cortex (Yamamoto et al. 1988; Farfel 1999). An autosomal dominant form of PHP-Ib has been linked to a chromosome 20q13.3 region that comprises *GNAS1,* and the phenotypic expression of PTH resistance has been shown to be dependent on maternal transmission of the genetic defect (Jüppner et al. 1998) (i.e., the mode of inheritance for hormonal resistance is identical to that observed in kindreds with PHP-Ia/pPHP). Furthermore, several sporadic and familial cases of PHP-Ib have been demonstrated to carry *GNAS1* methylation defects (Liu et al. 2000), making it likely that mutations in a cell- or tissue-specific regulatory element of *GNAS1* are responsible for PHP-Ib.

We now report a patient, K-1, who presented with renal resistance to PTH but showed no evidence of AHO. Analysis of K-1's genomic DNA revealed a duplication of the paternal copy of chromosome 20q, with loss of the maternal homolog (patUPD20q). Informed, written consent was obtained (Massachusetts General Hospital Institutional Review Board form 92-7338).

K-1 was born after 41 wk of an uneventful gestation; maternal age at delivery was 39 years. Besides transient hypoglycemia and neonatal hyperbilirubinemia requiring 60 h of phototherapy, K-1 had right-lambdoid synostosis, which required surgical correction at age 13 mo. K-1 was furthermore noted to have developmental delay during infancy—he sat at age 16 mo, walked at age 19 mo, and said "mama" at age 24 mo—and therefore received speech, occupational, and physical therapy starting at age 2 years. The karyotype was normal 46,XY. K-1's mother's height is 185.4 cm (+3.65 SD), and his father's height is 188.0 cm (+1.7 SD). He has a sister 3 years older, of normal stature, and in good health; her calcium, phosphorus, and PTH have been normal. There is no family history of any calcium disorders or of hypothyroidism.

At the age of 5 years 3 mo, K-1 presented to another hospital, with a tonic-clonic seizure, and was found to be hypocalcemic and hyperphosphatemic, with an elevated level of serum PTH (table 1). PHP was diagnosed, and treatment with calcium carbonate (initially 164 mg/kg/d) and 1,25 dihydroxy vitamin D (initially 17 ng/kg/d) was initiated. After 2 mo of therapy, total serum calcium was normal, but PTH remained elevated, which prompted adjustments in medication. At that time, TSH was also found to be slightly increased, with a low-normal T4 (neonatal T4 was 12.7 μg/dl [normal range 10–28 μg/dl]); therefore, treatment with thyroxine (1.7 μg/kg/d) was initiated. Calcitonin was measured on two occasions and was found to be 22 pg/ml and 31 pg/ml (normal range <8.0 pg/ml).

Table 1

Laboratory Findings for K-1, on Diagnosis and 2 mo after Initiation of Therapy with Calcium and $1,25(OH)_2D_3$

Treatment	Serum and Urine Levels[a]		
	On Admission	After 2 Mo	Reference Range
Calcium (mmol/liter)	1.80	2.28	2.10–2.65
Phosphorus (mmol/liter)	2.62	2.07	.8–1.45
PTH (pg/ml)	113	243	11–54
TSH (μIU/ml)	ND	6.4	.4–5.0
Thyroxine (μg/dl)	ND	4.9	4.5–12.5
$25\text{-}OHD_3$ (ng/ml)	41	ND	10–60
$1,25(OH)_2D_3$ (pg/ml)	50	ND	16–46
Urinary calcium/creatinine[b]	ND	<.001	<.2

[a] ND = not determined.
[b] Measured in spot urine.

At age 5 years 11 mo, when K-1 presented to one of our institutions (i.e., A.H.L.'s), his height was 133.0 cm (+3.6 SD), his weight was 30.4 kg (+3.4 SD) and his head circumference was 54.7 cm (+2.0 SD). Apart from irregularly and widely spaced teeth and upturned, cracked fingernails, no obvious abnormalities were revealed by the physical exam. He did not have thyromegaly. Deep tendon reflexes were slightly hyperactive. His coordination and balance, as well as his range of motion, particularly in the trunk, were moderately limited, but his neurological examination was otherwise normal. At age 6 years, K-1 did not have any features of AHO; his height and weight were above the normal range, and x-rays of both hands showed normal bone age (on the basis of the standard of Greulich and Pyle), without evidence for short metacarpals. According to his parents, K-1's language and reading comprehension improved significantly during the year following initiation of treatment with calcium, calcitriol, and thyroxine; in contrast, his remaining neurological and physical signs did not improve. Currently, he is in first grade and is reported, by his teachers, to be reading at a third-grade level.

To obtain insight into the molecular defect responsible for the form of PHP evidenced by K-1, we searched in genomic DNA, both from K-1 and from his immediate family, for possible deletions/rearrangements in the chromosome 20q region that encompasses *GNAS1*. Mutations in this gene have been implicated in different forms of PHP. The analysis of microsatellite markers (Jüppner et al. 1998; Bastepe et al. 1999) amplified from leukocyte and fibroblast DNA indicated loss of heterozygosity (LOH) for K-1, with a lack of maternal contribution at

eight fully informative markers that are distributed throughout chromosome 20q (fig. 1*A*). Informative markers located in 20p showed normal Mendelian inheritance, and the analysis of at least one fully informative marker for each of the other chromosomal arms revealed no additional abnormalities (data not shown). Together with the karyotype analysis, which did not indicate a large 20q deletion (data not shown), these findings led to the conclusion that K-1 has patUPD20q.

GNAS1 has been previously demonstrated to exhibit parent-specific methylation and expression (Hayward et al. 1998*a,* 1998*b;* Hayward and Bonthron 2000; Liu et al. 2000). To assess the pattern of methylation in this gene, blood-leukocyte genomic DNA from K-1 and from his healthy sister and parents was incubated with a combination of methylation-sensitive and methylation-insensitive restriction enzymes. Southern blot analyses using probes specific for four differentially methylated *GNAS1* regions revealed abnormalities in the patient's genomic DNA, at all four loci (fig. 1*B*), whereas the healthy family members showed a methylation pattern identical to that previously described for healthy individuals (Hayward et al. 1998*a,* 1998*b;* Hayward and Bonthron 2000). The methylation abnormalities in K-1 were documented for at least one other site in each differentially methylated region, by a different methylation-

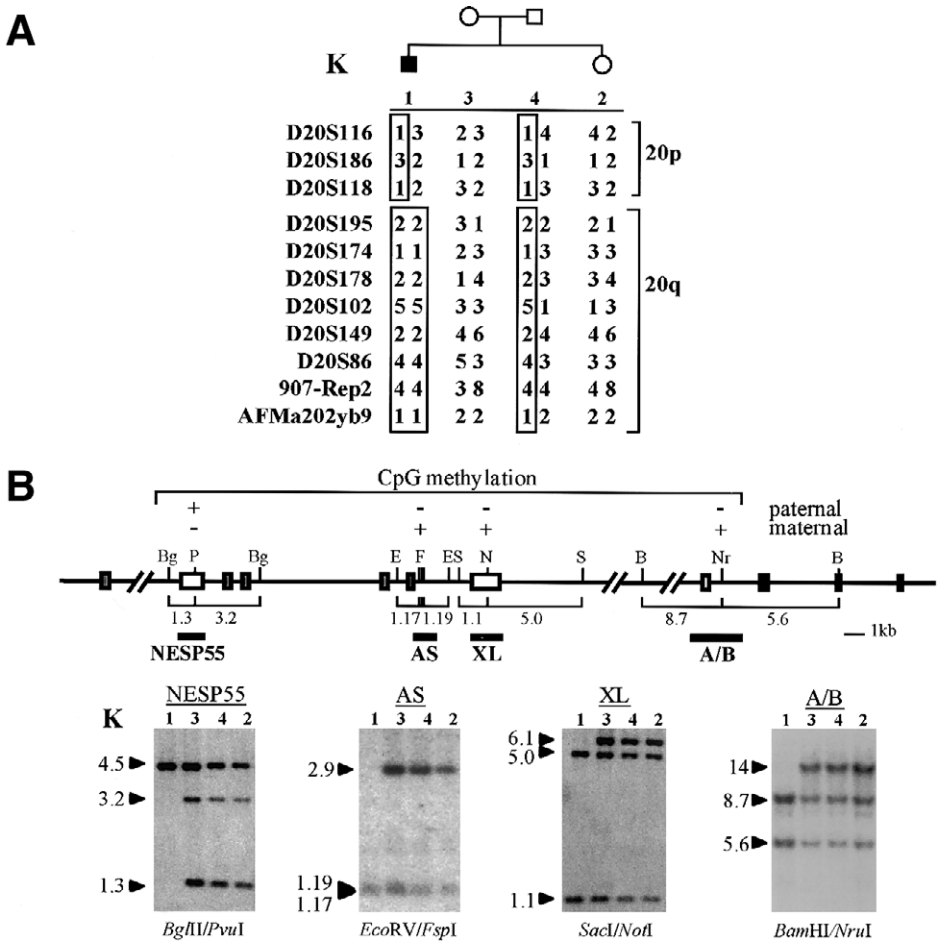

Figure 1 *A,* Haplotype analysis of genomic DNA from K-1 and his immediate family (K-2 = sister; K-3 = mother; K-4 = father), across chromosome 20. The fully informative markers on 20q reveal LOH for K-1, with a lack of maternal contribution. *B,* Schematic presentation of *GNAS1* gene (*top*) and allele-specific methylation of *GNAS1,* in K-1 and his relatives (*bottom*). Exons encoding sense transcripts are depicted by white boxes; those encoding antisense transcripts are depicted by gray boxes. Thicker lines indicate probes used in Southern analysis: NESP55 (nucleotides 317–1705; GenBank accession number AJ009849), XLαs (nucleotides 80–1693; GenBank accession number AJ224868), AS (nucleotides 11646–13156; GenBank accession number AJ251760), and A/B (nucleotides 28580–31035; GenBank accession number AL121917). Normal methylation status of the parental alleles is marked by a plus sign (+) or a minus sign (−). Exons 1–3, encoding portions of Gsα, are depicted by black boxes; exons 4–13 are not included. Combinations of restriction enzymes—Bg = *Bgl*II; P = *Pvu*I; E = *Eco*RV; F = *Fsp*I; S = *Sac*I; N = *Not*I; B = *Bam*HI; Nr = *Nru*I (note that the small internal fragments of the *Eco*RV/*Fsp*I digest were not transferred)—are indicated below autoradiographies, and the sizes (in kb) of the expected DNA fragments are indicated.

sensitive restriction enzyme (data not shown). Furthermore, results indistinguishable from those obtained with blood-leukocyte DNA were obtained with fibroblast DNA from K-1 and his parents (data not shown). Thus, consistent with the haplotype analysis, which provided evidence for paternally inherited DNA only, K-1 shows a lack of a maternal methylation pattern within *GNAS1* (i.e., the regions around exons AS, XL, and A/B are not methylated at all, whereas the region around exon NESP55 is methylated on both alleles).

To rule out the possibility that a Gsα-protein defect/deficiency is responsible for the observed PTH resistance, we investigated fibroblasts derived from K-1 and from his healthy parents. Fibroblast cultures from K-1, his mother, and his father showed similar basal cAMP accumulation—1.21 ± 0.23, 0.92 ± 0.05, and 1.08 ± 0.29 pmol/well, respectively. On challenge with either PTH(1-34) or isoproterenol (both 1 μM), the cAMP level in K-1's fibroblast cultures increased 5.7- and 48-fold, respectively, and these increases were almost identical to the findings for the parents' cells (5.0- and 46-fold, respectively, for the mother, and 5.0- and 34-fold, respectively, for the father; fig. 2*A*). In addition, western blot analysis of cell lysates, performed with a Gsα-specific antibody (Yu et al. 1998), revealed no evidence for reduced Gsα-protein level in K-1's fibroblasts (fig. 2*B*).

Uniparental disomies (UPDs) can lead to disease either through the unmasking of recessive traits or, as observed in some human disorders, through disrupted expression of imprinted genes that is due to unbalanced parental contribution (Kotzot 1999; Tilghman 1999; Nicholls 2000; Robinson 2000). *GNAS1* and its murine homolog give rise to several alternatively spliced transcripts that are derived from either the paternal or the maternal allele (Hayward et al. 1998*a,* 1998*b;* Hayward and Bonthron 2000; Liu et al. 2000; Wroe et al. 2000). Furthermore, data from mice with either +/p− or +/m− ablation of *Gnas* exon 2 indicate that Gsα transcripts, which show broad biallelic expression, are derived, in some tissues (such as the renal cortex), from the maternal allele alone (Yu et al. 1998). We thus propose that the mechanism underlying hormonal resistance in K-1 involves silencing of Gsα transcription from the two normal paternal alleles in proximal renal tubules and that the resulting lack of Gsα protein leads to PTH resistance in that tissue.

A *GNAS1* methylation pattern similar to that observed in K-1 was recently reported elsewhere for some PHP-Ib cases (Liu et al. 2000). In that study, all investigated patients (11 sporadic cases and 2 familial cases) showed a complete loss of methylation at exon A/B, which suggested that a loss of methylation at the A/B locus and the consequent active transcription from this promoter could be involved, directly or indirectly, in the tissue-specific silencing of Gsα transcription. The loss of exon A/B methylation in patient K-1, albeit due to the lack of the maternal *GNAS1* gene rather than to a mutation, could thus explain the kidney-specific PTH resistance. Notably, two of the patients with PHP-Ib who have been reported by Liu et al. revealed a more generalized methylation defect, which involved, besides exon A/B, the other differentially methylated *GNAS1* regions—namely, NESP55, XL, and the promoter of AS (Liu et al. 2000). It thus appears likely that a complete lack of maternal-specific methylation, which is indistinguishable from the epigenetic changes observed in the patUPD20q described here, can also be caused by mutations in regulatory regions of the maternal *GNAS1* allele.

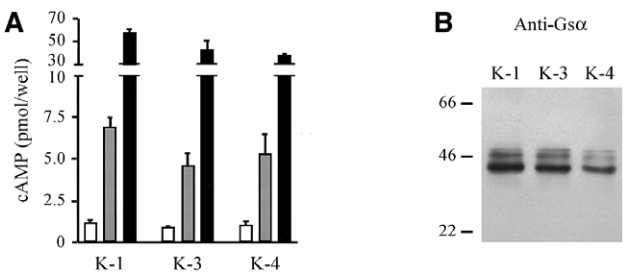

Figure 2 *A,* Basal cAMP accumulation (*white bars*), and accumulation of this second messenger after challenge with 1 μM of either PTH(1-34) (*gray bars*) or isoproterenol (*black bars*), in fibroblasts derived from K-1 and his parents, K-3 and K-4. The data represent the mean ± SEM of three independent experiments. *B,* Assessment of Gsα-protein levels by western blot analysis. Equal amounts of protein were loaded in each lane and were separated by 9% SDS-PAGE. After transfer to nitrocellulose, detection of Gsα protein was achieved with an anti-Gsα antibody and a horseradish peroxidase–labeled second antibody. The positions of protein size markers (in kD) are indicated. The findings are representative of four independent experiments with similar results.

The elevation of TSH with a low-to-normal T4 level, which is comparable to that typically observed in patients with PHP-Ia (Levine 1996; Weinstein 1998), suggests that K-1 also had mild resistance to TSH. Thus, paternal silencing of Gsα transcription may also take place, at least partially, in the thyroid gland, which correlates with the observation in kindreds with PHP-Ia/pPHP in which TSH resistance is subject to paternal imprinting (Levine 1996; Weinstein 1998). It is furthermore plausible that the observed elevation of calcitonin reflects resistance in calcitonin-responsive tissues. On the other hand, the absence of AHO in K-1 probably indicates that expression of the intact Gsα protein is not impaired in the growth plates; this finding contrasts with the findings in patients with PHP-Ia or pPHP, who express a mutant Gsα transcript from the maternal or paternal *GNAS1* allele, respectively (Levine 1996; Bastepe and Jüppner 2000).

It is uncertain whether the craniosynostosis observed in K-1 is related to the documented patUPD20q and to an associated functional impairment of yet another imprinted gene in this chromosomal region. The neuronatin-

encoding gene, *NNAT*, which maps to the chromosomal region 20q11.2-q12 (Dou and Joseph 1996), has been shown, in the mouse, to be methylated on only the maternal allele and to exhibit exclusive paternal expression (Kikyo et al. 1997). Similar findings (i.e., lack of *NNAT* methylation on both paternally derived copies of chromosome 20q) were observed in K-1 (data not shown). Nonetheless, the duplication of the paternal *NNAT* is unlikely to provide an explanation, since the equivalent genetic modification in the mouse was not found to be associated with significant abnormalities (Kikyo et al. 1997). Alternatively, K-1's craniosynostosis may be the result of an inherited recessive mutation unmasked because of isodisomy. Identification and clinical characterization of patients with a genetic abnormality either the same as or similar to that in K-1 will help address these questions.

Several different scenarios have been proposed to explain UPD formation (Kotzot 1999; Robinson 2000), including gametic complementation, "trisomy rescue," and compensatory, mitotic duplication in monosomic gametes. In addition to these mechanisms that function to correct aneuploidy, postfertilization early-mitotic errors that occur in initially normal gametes also can give rise to UPD (Kotzot 1999; Robinson 2000). The advanced maternal age (39 years) in the case described in the present study suggests that possible meiotic nondisjunction resulted in an aneuploid female gamete. However, the findings with several different markers revealed a partial UPD involving only the long arm of chromosome 20, thus strongly suggesting a postzygotic somatic recombination event as the cause of patUPD20q in K-1.

UPD has been reported for most other chromosomes, but only few previously reported patients have shown duplications involving chromosome 20. For example, one of these cases, a child with severe pre- and postnatal growth retardation and hyperactivity, was shown to have a complete maternal UPD of chromosome 20 (matUPD20; combined hetero- and isodisomy) and a small marker chromosome (Chudoba et al. 1999). No abnormalities of mineral-ion homeostasis were noted in either this patient with matUPD20 or a parthenogenetic chimera reported elsewhere (Strain et al. 1995). In accordance with current evidence indicating that Gsα transcripts are derived in the renal cortex predominantly from the maternal *GNAS1* allele, these data suggest that a complete lack of the paternal chromosome 20 is not associated with PTH resistance. However, because the AS transcript (as well as the XLαs and A/B splice variants) is derived from the paternal *GNAS1* allele only, some of the abnormalities in cases of matUPD20 may be attributable to the complete silencing of these gene products. Only a single preliminary report has described a patient with paternal UPD involving the entirety of chromosome 20 (Spinner et al. 1994). Karyotype analysis in the latter case revealed mosaicism for trisomy 20 in several tissues, in addition to a terminal rearrangement at the distal short arms of the two paternal chromosome 20 homologs. On the basis of the reported cytogenetic and microsatellite data, this patient would be predicted to show laboratory abnormalities similar to those which we have observed in K-1, but no details were provided (Spinner et al. 1994). The scarcity of reports suggests a severe, possibly lethal, nature for complete paternal UPD20.

In conclusion, it appears that at least three different *GNAS1*-related genetic mechanisms can lead to renal PTH resistance (fig. 3): (i) heterozygous—maternal mutations in exons encoding the Gsα transcript (as observed in PHP-Ia); (ii) heterozygous—maternally derived mutations in regulatory elements, which are associated with loss of methylation at one or more exons (as observed in PHP-Ib); and (iii) paternal UPD of the chromosomal region comprising *GNAS1*, with the resulting loss of the maternal-specific methylation pattern (as observed in

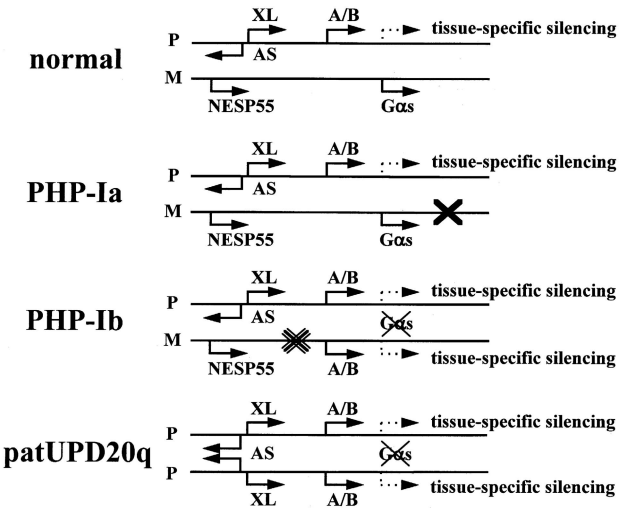

Figure 3 Genetic mechanisms that are predicted to underlie the different forms of PHP. In healthy individuals (normal), the paternal allele (P) gives rise to sense and antisense transcripts (*solid-shaft arrows*) that are derived from the nonmethylated promoter regions upstream of exons XL, AS, and A/B, whereas the transcript that encodes NESP55 is derived from the maternal allele (M). Note that Gsα transcripts are derived in proximal renal tubules and possibly in other tissues from the maternal allele and not from the paternal allele (*dotted-shaft arrows*). In patients with PHP-Ia, mutations within 1 of the 13 Gsα-specific exons on the maternal allele (thick X) lead to either a complete lack of Gsα protein or a functionally impaired protein. In contrast, patients with PHP-Ib are presumed to carry a mutation in regulatory regions of the maternal *GNAS1* allele (light X, over "Gαs") that causes loss of imprinting at exon A/B and that leads to impaired Gsα expression in some tissues (tripled X). Lack of a maternal *GNAS1* allele—as in K-1, who has patUPD20q—is predicted to result in a lack of Gsα transcripts in the renal cortex that leads to PTH-resistant hypocalcemia and hyperphosphatemia.

K-1). The presence of AHO provides an important criterion for establishing the diagnosis of PHP-Ia. However, clinical findings cannot be used to distinguish between PHP-Ib and patUPD20q. For genetic-counseling purposes and to potentially gain additional insights into the role of distinct *GNAS1* regions in hormonal resistance, it will therefore be important both to assess the methylation status of different *GNAS1* exons in patients with PHP and to search in these individuals for the presence of UPD in the telomeric end of chromosome 20q.

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Electronic-Database Information

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Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PHP [MIM 300800])

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