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J Bone Miner Res. 2015 May ; 30(5): 906–912. doi:10.1002/jbmr.2408.**TSH elevations as the first laboratory evidence for pseudohypoparathyroidism type Ib (PHP-Ib)[†]****Angelo Molinaro^{1,2}, Dov Tiosano³, Rieko Takatani¹, Dionisios Chrysis⁴, William Russell⁵, Nikolas Koscielniak⁶, Marie-Laure Kottler⁷, Patrizia Agretti², Giuseppina De Marco², Petteri Ahtiainen⁸, Marta Christov¹, Outi Mäkitie⁹, Massimo Tonacchera², and Harald Jüppner¹**¹Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA²Endocrinology Unit, Department of Clinical and Experimental Medicine, University of Pisa, University Hospital of Pisa, Pisa, Italy ³Division of Pediatric Endocrinology, Meyer Children's Hospital, Rambam Health Care Campus, Haifa, Israel ⁴Department of Pediatrics, Medical School, University of Patras, Patras, Greece ⁵Division of Pediatric Endocrinology and Diabetes, Vanderbilt University School of Medicine, Nashville, TN, USA ⁶Program in Occupational Therapy, Washington University School of Medicine, St. Louis, MO, USA ⁷Centre Hospitalier Universitaire de Caen, Department of Genetics, Reference Centre for Rare Disorders of Calcium and Phosphorus Metabolism, F-14000 Caen, France ⁸Central Finland Central Hospital, Jyväskylä,

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Correspondence: Harald Jüppner, Endocrine Unit, Massachusetts General Hospital, Boston, MA, USA, hjueppner@partners.org.**Authors' Role:**

- Angelo Molinaro: performed most of the genetic analysis of *GNAS* and *STX16 loci*, performed DNA extraction from patients with idiopathic hypothyroidism, collected data about pediatric and adult patients with idiopathic hypothyroidism, wrote the first draft of the manuscript.
- Dov Tiosano: identified, visited and collected data about patient 148/II-1; helped write the manuscript.
- Rieko Takatani: performed part of the genetic analysis and provided the instruments for data analysis of methylation and copy number changes.
- Dionisios Chrysis: identified, visited and collected data about patient 130/II-1; helped write the manuscript.
- William Russell and Marta Christov: identified, visited and collected data about patient 147/II-1; helped write the manuscript.
- Nikolas Koscielniak: Collected clinical and laboratory information.
- Marie-Laure Kottler: Conceived the idea that TSH-resistance may develop in some patients before PTH-resistance together with H. Jüppner; performed the initial MLPA and MS-MLPA; helped write the manuscript.
- Patrizia Agretti and Giuseppina De Marco: performed genetic analysis of TSH receptor in patients with idiopathic hypothyroidism; performed DNA extraction from patients with idiopathic hypothyroidism.
- Petteri Ahtiainen and Outi Mäkitie: identified, visited and collected data about patient 149/II-1; helped write the manuscript.
- Massimo Tonacchera: identified, visited and collected data about patients with idiopathic hypothyroidism and performed genetic analysis of TSH receptor in them; helped write the manuscript.
- Harald Jüppner: principal investigator, conceived the idea that TSH-resistance may develop before PTH-resistance, wrote final version of the manuscript.

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Abstract

Hypocalcemia and hyperphosphatemia because of resistance towards parathyroid hormone (PTH) in the proximal renal tubules are the most prominent abnormalities in patients affected by pseudohypoparathyroidism type Ib (PHP-Ib). In this rare disorder that is caused by *GNAS* methylation changes, resistance can occur towards other hormones, such as thyroid-stimulating hormone (TSH), that mediate their actions through G protein-coupled receptors. However, these additional laboratory abnormalities are usually not recognized until PTH-resistant hypocalcemia becomes clinically apparent. We now describe four pediatric patients, first diagnosed with subclinical or overt hypothyroidism between the ages of 0.2 and 15 years, who developed overt PTH-resistance 3-20 years later. Although anti-TPO antibodies provided a plausible explanation for hypothyroidism in one of these patients, this and two other patients revealed broad epigenetic *GNAS* abnormalities, which included loss of methylation (LOM) at exons AS, XL and A/B, and gain of methylation at exon NESP55, i.e. findings consistent with PHP-Ib. LOM at *GNAS* exon A/B alone led in the fourth patient to the identification of a maternally inherited 3-kb *STX16* deletion, a well-established cause of autosomal dominant PHP-Ib. Although *GNAS* methylation changes were not detected in additional pediatric and adult patients with subclinical hypothyroidism (23 pediatric and 39 adult cases), hypothyroidism can obviously be the initial finding in PHP-Ib patients. One should therefore consider measuring PTH, along with calcium and phosphate, in patients with unexplained hypothyroidism for extended periods of time to avoid hypocalcemia and associated clinical complications.

Keywords

Epigenetics; pseudohypoparathyroidism; hypothyroidism; PTH; *GNAS*

Introduction

Pseudohypoparathyroidism (PHP) is characterized by resistance to parathyroid hormone (PTH) in the proximal renal tubules, which leads to hypocalcemia, hyperphosphatemia, and impaired 1,25(OH)₂ vitamin D production. PHP type Ia (PHP-Ia) is caused by maternally inherited, heterozygous inactivating mutations affecting those *GNAS* exons encoding the alpha-subunit of the stimulatory G protein (G α)¹⁻³. In addition to PTH-resistance, PHP-Ia patients present with features of Albright's Hereditary Osteodystrophy (AHO) and frequently with resistance to thyroid-stimulating hormone (TSH), growth hormone-releasing hormone (GHRH), calcitonin (CT), or other hormones that mediate their actions through G α -coupled receptors.

In contrast to PHP-Ia, patients affected by PHP type Ib (PHP-Ib) typically present only with PTH-resistant hypocalcemia and hyperphosphatemia, and these individuals usually show no evidence for AHO. Several forms of PHP-Ib can be distinguished. The most common form of autosomal dominant PHP-Ib (AD-PHP-Ib) is caused by maternally inherited,

heterozygous deletions in the *STX16* gene, which is located approximately 220 kb up-stream of *GNAS* exon A/B⁴⁻⁶, or by deletions within *GNAS*⁷⁻⁹. These deletions are associated either with a loss of methylation at *GNAS* exon A/B alone^{4-6,9} or a loss of all maternal methylation imprints^{7,8}. Patients affected by the sporadic form of PHP-Ib present with laboratory abnormalities and epigenetic *GNAS* changes that are largely indistinguishable from those observed in AD-PHP-Ib due to deletions within *GNAS*¹⁰⁻¹⁵. The lack of methylation at exon A/B (also referred to as exon 1A) results in biallelic expression of this presumably non-coding transcript, which leads through unknown mechanisms to reduced expression of *Gsa*. The *Gsa* promoter does not undergo parent-specific methylation thus allowing biallelic expression in most cells. However, in certain tissues, such as proximal renal tubules, thyroid, paraventricular nucleus of the hypothalamus, brown fat, ovaries and pituitary, *Gsa* is derived predominantly from the maternal allele, leading to deficiency of this ubiquitously expressed signaling protein and thus hormonal resistance, if this parental allele carries a mutation¹⁶⁻²⁰. The mechanism(s) that silences *Gsa* expression from the non-methylated paternal allele is unknown, but seems to involve a protein(s) that is particularly abundantly expressed in those tissues where *Gsa* is derived predominantly from the maternal allele. Furthermore, production of this unknown factor appears to vary, since PTH-resistance in the proximal renal tubules develops only later in life in humans and mice^{5,11,21} and it is known to vary considerably even among individuals with the same maternally inherited *STX16* deletion^{22,23}.

PHP-Ib patients can present with resistance towards hormones other than PTH that mediate their actions through G protein-coupled receptors. For example, TSH elevations have been observed in some PHP-Ib patients, but these elevations are usually only mild and are typically detected after symptomatic hypocalcemia has been observed^{3,11,22,24-26}. We now describe four patients, who had been diagnosed first with hypothyroidism, yet PTH-resistance became clinically apparent only with a significant delay, in one case not until twenty years later. Silencing of paternal *Gsa* expression thus can be similarly efficient in the thyroid as in the proximal renal tubules.

Materials and Methods

Genetic and epigenetic studies

To assess copy number and methylation status, respectively, for the *GNAS-STX16* region, multiplex ligation-dependent probe amplification assay (MLPA) and methylation-specific multiplex ligation-dependent probe amplification assay (MS-MLPA) were performed following the instructions of the kit's manufacturer (MRC-Holland B.V. Willem Schoutenstraat 6, 1057 DN Amsterdam, the Netherlands). Amplicons were submitted for capillary electrophoresis to the Massachusetts General Hospital DNA Core Facility. Copy number was assessed by comparing for each amplicon patient data to the mean of at least three healthy subjects. Information about the *GNAS* methylation status was obtained by comparing the peaks obtained from reactions using a methylation-sensitive endonuclease with those obtained without enzymatic digestion.

The PCR to search for the 3-kb *STX16* deletion was performed with QIAGEN Taq DNA polymerase and the other reagents supplied with the same kit following the manufacturer's

protocols; the following PCR primers were used: “a”, 5'-TTGGCAGATAACTGCTGTGG-3'; “b”, 5'-GGAAGAGCTAAGAGAACAAG-3'; “c”, 5'-GGTGGAGCAGAACACACTGA-3'; “d”, 5'-CCACCTGTGGCATCATGTTA-3'. Cycler program: denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by an additional elongation step at 72°C for 10 min.

Analysis of microsatellites markers D20S86, 907-rep2, 261P9-CA, 806-CA, 543J19-TTA, and D20S171 was performed by the Center for Human Genetic Research of the Massachusetts General Hospital.

All biochemical results were obtained in the clinical laboratory of the referring hospitals.

Pediatric PHP-Ib patients presenting first with an elevated TSH level

The male patient 130/II-1 was conceived through *in vitro* fertilization and was born by Cesarean section after 26 weeks of gestation because of extreme intrauterine growth retardation and severe premature contractions. Neonatal screening for hypothyroidism on the 3rd day of life was normal. On the 70th day of life his TSH levels were measured again, as is routinely done before discharge from the neonatal intensive care unit, and it was found to be elevated to 10.6 $\mu\text{U/ml}$ (normal range: 0.5-6.0) with an FT4 of 0.67 ng/dl (normal range: 0.95-2.10) consistent with overt hypothyroidism. He was therefore referred to D.C., who initiated treatment with L-thyroxine. Ultrasonography revealed a normal thyroid gland; anti-thyroglobulin and anti-thyroperoxidase antibodies were negative. At the age of 17 months his total serum calcium level was normal (10.4 mg/dl; normal range for this age: 8.9-10.1), while his serum phosphate level was at the upper end of the age-appropriate normal range (5.9 mg/dl; normal range for this age: 4.6-5.6). At the age of 3.5 years, he was hospitalized because of a urinary tract infection and the laboratory work-up at that time revealed significant, albeit asymptomatic hypocalcemia (7.0 mg/dl; normal range at this age: 8.9-10.1) and hyperphosphatemia (6.4 mg/dl; normal range at this age: 3.9-5.3), with an elevated PTH (291 pg/ml) and a normal 25OH vitamin D level (31 ng/ml). He had no dysmorphic features and his development was age-appropriate. Oral calcium (1,000 mg/day) and calcitriol (0.25 $\mu\text{g/day}$) were started; dosages of both medications were adjusted over the time. His linear growth remained appropriate for his target height.

The female patient 148/II-1 was born at term; her neonatal TSH screening was normal. When investigated at the age of 6 months for obesity and developmental delay by D.T., she was found to have elevated TSH of 13.2 $\mu\text{U/ml}$ (normal range: 0.4-5.5) with an FT4 of 1.25 ng/dl (normal range: 0.85-1.46), and treatment with L-thyroxine was started; anti-thyroglobulin and anti-thyroperoxidase antibodies were absent and ultrasonography revealed a normal thyroid gland. At that time she had a normal serum calcium level (10.5 mg/dl; normal range at this age: 8.0-11.0) with an elevated serum phosphate (7.5 mg/dl; normal range at this age: 4.9-6.1); no PTH level was measured. At the age of 3 years, she showed no clinical evidence for hypocalcemia, but was found to have an elevated PTH level (668 pg/ml) with low calcium (7.9 mg/dl; normal range at this age: 8.9-10.1) and elevated phosphate (8.5 mg/dl; normal range at this age: 3.9-5.3); treatment with 1-alpha vitamin D (1 $\mu\text{g/day}$) and calcium carbonate (2,400 mg/day) was therefore initiated.

The female patient 149/II-1 was born at term. Her growth and development had been normal, but menarche did not occur until 15 years. At that age, she presented with an enlarged thyroid gland and was found to have an elevated TSH (21.9 μ U/ml; normal range: 0.5-5.0) and low FT4 (0.28 ng/dl; normal range: 0.7-1.48). A thyroid ultrasonography revealed no abnormality and she showed no evidence for an abnormal regulation of calcium homeostasis. Her thyroglobulin antibodies (titer: 1:25,600; normal: <1:25) and thyroid peroxidase antibodies (76 IU/ml; normal: <50) were elevated. L-thyroxine treatment was therefore started with 100 μ g per day. Her older sister had a goiter, but normal thyroid function tests; her maternal grandmother and several relatives of her maternal grandfather had hyperthyroidism. At the age of 28, she was investigated further because of cramping pains in the upper gastrointestinal and esophageal region; an endoscopic evaluation revealed no macroscopic or microscopic abnormalities. Although showing no clinical evidence for hypocalcemia, she was found to have a low total serum calcium (6.7 mg/dl) and an elevated alkaline phosphatase (134 U/L; normal range: 35-105). Treatment with cholecalciferol (20 μ g) and 1000 mg calcium carbonate was started, which resulted in resolution of the abdominal symptoms. Ten days later, serum PTH was found to be elevated (1186 pg/ml) and serum phosphate was at the upper end of normal (4.3 mg/dl); total serum calcium was again significantly below the normal range and the ionized calcium was only 0.76 mmol/L. Her 1.25(OH)₂ vitamin D level was normal (166 pmol/L). She was referred to an endocrine unit, where gastroscopy, neck ultrasound, abdominal ultrasound, and radiographic examination of long bones revealed no abnormalities. On treatment with cholecalciferol (50 μ g/day) and calcium carbonate (3000 mg/day), the patient remained asymptomatic. When referred to P.A. and O.M., her PTH levels had improved (501 pg/ml), but total serum calcium had not reached the normal range (8.44 mg/dl). Alfacalcidol 0.25 μ g twice per day was therefore started and 3,000 mg/day of calcium carbonate was continued. Two months after starting alfacalcidol serum total calcium was normal (8.92 mg/dl).

The male patient 147/II-1 was jaundiced during the perinatal period, but had a normal TSH on the neonatal screen. At 3 years of age, he showed increasing lethargy compared to his non-identical twin brother. Upon referral to W.R., he was found to have a normal total T4 (8.3 μ g/dl; normal range: 4.6-12.0) and a mildly elevated TSH level (5.9 μ U/mL; normal range: 0.2-4.0 μ U/ml) and treatment with L-thyroxine (25 μ g/day) was therefore started. Anti-thyroglobulin and anti-thyroperoxidase antibodies as well as islet cell antibodies and anti-nuclear antibodies (ANA) were negative. The family history was positive for multiple autoimmune disorders. His mother, maternal grandmother, and a maternal great aunt were diagnosed with hypothyroidism. The maternal great-grandmother had scleroderma and lupus, and the mother was positive for ANA. The father's first cousin had type 1 diabetes. Clinical examination revealed a normally developed child without AHO features; his thyroid gland was normal to palpation. Over the subsequent years, the dosage of L-thyroxine was adjusted in order to maintain TSH values in the normal range. At the age of 22 years, concurrent with mononucleosis, he presented with muscle cramps, tetany, and tingling of the face and hands, and was found to be hypocalcemic (calcium 5.6 mg/dL). Further investigations revealed an elevated PTH (795 pg/mL), reduced 25OH vitamin D (17 ng/mL), and low magnesium (1.2 mg/dL) and potassium levels (3 mmol/L). Serum phosphorus was always within normal limits. After treating 147/II-1 with cholecalciferol his serum 25OH

vitamin D level normalized, but his PTH remained elevated and he was therefore started on calcitriol (0.5 µg/day) and calcium carbonate (3,000 mg/day). When last investigated at the age of 25.5 years, while on treatment with L-thyroxine his FT4 and TSH were normal (1.4 ng/dl and 4.0 µU/ml, respectively); he had no detectable anti-thyroperoxidase antibodies, and his thyroid was normal in size and echogenicity as determined by ultrasonography.

Pediatric and adult patients with sporadic subclinical hypothyroidism

Because of the delayed onset of PTH-resistance in the above patients, we also studied 62 patients (23 pediatric and 39 adult cases) with sporadic subclinical hypothyroidism, who were referred from different regions of Italy to M.T. and A.M.

Pediatric patients—15 males and 8 females with subclinical hypothyroidism (defined as an elevation in TSH without a reduction in FT4; data are presented as mean±SD) had mildly elevated TSH (5.78±1.74 µU/ml; normal range: 0.5-3.5) and normal FT4 levels (1.13±0.25 ng/dl; normal range: 0.7-1.7). Two of these patients were identified at the neonatal screening for congenital hypothyroidism, and both had thyroid glands that were normal in size and location. All other individuals had been negative in the neonatal screening test, but TSH levels were found to be elevated at 6.3±4.2 years when measured for non-specific complaints.

Adult patients—11 males and 28 females were diagnosed with subclinical hypothyroidism (levels of TSH: 8.08±2.21 µU/ml; FT4: 0.96±0.2 ng/dl) at 30.7±9.9 years of age.

Additional biochemical and clinical investigations allowed in all the above pediatric and adult patients exclusion of autoimmune thyroiditis, since testing for anti-thyroperoxidase, anti-thyroglobulin, and anti-TSH receptor antibodies was negative. For all cases, serial serum dilutions excluded assay interference with heterophilic antibodies and all TSH measurements run in parallel to the standard curve (data not shown). Imaging by ultrasound showed properly located thyroid glands with normal echogenicity. Nucleotide sequence analysis of all exons encoding the TSH receptor revealed no pathogenetic mutation except for a well-known polymorphisms, a C to A transversion in nucleotide 154 (CCC/ACC) affecting the proline residue at position 52 which is replaced by threonine. The elevated TSH levels therefore remained unexplained in these patients, who were followed for 4.4±3.4 years (range: 0.5-13) and did not develop alterations in PTH, calcium, and phosphate concentration during that time.

The study was approved by the local ethical committees and informed consent was obtained from all subjects or their guardians.

Results

We studied four individuals, who were diagnosed initially with subclinical or overt hypothyroidism, yet developed PTH-resistant hypocalcemia and hyperphosphatemia 3-20 years later (see Table 1). These changes in the regulation of mineral ion homeostasis were associated with abnormal *GNAS* methylation in genomic DNA from blood leukocytes, thus

little or no Gs α protein is made in this portion of the kidney thus leading to hormonal resistance¹⁻³. The responsible mechanisms that reduce Gs α expression from the paternal allele appear to have variable efficacy, since PTH-resistance does not become apparent in PHP-1b patients until the ages of 2-3 years, and clinically symptomatic hypocalcemia usually does not develop until the beginning of the second decade of life^{5,11}. Furthermore, even in families in which the frequently encountered 3-kb deletion in *STX16* has been documented on the maternal allele, laboratory evidence for PTH-resistance can be highly variable with some patients showing little or no elevation of PTH levels^{5,11,22,23,31} although *GNAS* methylation changes are established shortly after fertilization and thus affect all tissues. Consistent with these findings in patients, mice show biallelic Gs α expression in the proximal renal tubules during early post-natal development. Subsequently, however, a major reduction of Gs α expression occurs at the paternal *Gnas* locus, thus leading, at least, in rodents with maternal ablation of *Gnas* exon 1 to PTH-resistance by the third week of life²¹.

The silencing mechanisms that reduce the amount of Gs α derived from the paternal allele appear to be present not only in the proximal renal tubules²¹, but also in other tissues, including pituitary, brown fat, and thyroid^{21,32,33}. In the latter tissue, substantially reduced paternal Gs α signalling is thus expected to lead in the presence of maternal *GNAS* mutations to transient or persistent TSH-resistance, possibly influenced further by genetic factors, environmental influences (i.e. iodine intake), or physiological changes during puberty and pregnancy. It is therefore conceivable that silencing of paternal Gs α transcription, combined with maternal *GNAS* methylation changes can cause TSH-resistance and consequently elevated TSH levels and even reduced FT4 levels. In three of our patients, these abnormalities occurred in the absence of anti-thyroid antibodies and a thyroid gland that appeared normal in size and texture as determined by ultrasonography.

However, patient 149/II-1 presented with autoimmune thyroiditis as well as TSH-resistance due to *GNAS* methylation changes. Her hypothyroidism could thus be explained by two distinct, possibly additive mechanisms, just like in a previously described patient with a homozygous TSH-receptor mutation, who later developed autoimmune thyroiditis³⁴. Since autoimmune thyroid disease occurs relatively frequently in the general population, our findings in patient 149/II-1 emphasize that *GNAS* methylation changes need to be considered even if anti-TPO antibodies have been detected.

By using MS-MLPA, we have now demonstrated broad *GNAS* methylation changes in three of our patients and an isolated loss of exon A/B methylation in one patient. The initial laboratory and clinical findings in one patient (149/II-1) were consistent with autoimmune hypothyroidism, but the epigenetic *GNAS* changes were undoubtedly present before and may have contributed to the TSH elevation and the reduced FT4 levels. The remaining three individuals revealed no evidence for anti-thyroid antibodies, thus their subclinical or overt hypothyroidism had developed well before PTH-resistant hypocalcemia. These findings suggest that silencing of Gs α transcription from the paternal allele and thus predominantly maternal expression of this ubiquitously expressed signaling protein can be as efficient in the thyroid as in the proximal renal tubules. The protein(s) contributing to the silencing of the Gs α promoter thus appear to be expressed at variable levels in different tissues with

predominantly monoallelic *Gsa* expression. In fact, its expression levels may vary throughout life thus explaining the lack of PTH-resistance in infants, who later develop PHP-Ib, and the major delay in disease onset in some adult patients^{5,11}, including those presented in this report.

We failed to demonstrate *GNAS* methylation changes in a cohort of pediatric and adult patients with idiopathic subclinical hypothyroidism. However, more such patients need to be investigated to exclude epigenetic *GNAS* changes and thus diminished *Gsa* expression as a cause of this disorder. The observation that one of our PHP-Ib patients, namely patient 147/II-1, did not develop symptomatic hypocalcemia until 20 years after establishing the diagnosis of hypothyroidism, raises the question whether hypothyroid patients need to be screened for extended periods of time for changes in serum calcium and phosphate. While the genetic and/or epigenetic analysis of the *GNAS* locus is unlikely to be cost-effective, the periodic measurement of serum PTH, calcium, and phosphate levels should be considered. Because PTH levels are highly variable in PHP-Ib patients, even when caused by the same genetic mutation^{4,11}, it would be reasonable to search for *GNAS* methylation changes in all those hypothyroid patients, who present with any PTH elevation, unless 25OH vitamin D levels are reduced as evidence for vitamin D deficiency. Measuring only calcium, without PTH and phosphate, is probably insufficient for establishing the diagnosis of PHP-Ib in patients with idiopathic hypothyroidism since normal calcium blood levels can be maintained for extended periods of time because of PTH-stimulated bone resorption.

In conclusion, TSH elevation as evidence for subclinical or overt hypothyroidism can occur in some PHP-Ib patients with methylation change at the *GNAS* locus before PTH-resistant hypocalcemia becomes apparent. Our current data and previously reported findings indicate that significant silencing of *Gsa* expression from the paternal *GNAS* allele may occur in tissues other than the proximal renal tubules, including the thyroid. In fact, it is conceivable that other disorders, such as isolated obesity, intrauterine growth retardation, or short stature can be caused in some patients by *GNAS* methylation changes without obvious changes in PTH and/or TSH levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PHP	Pseudohypoparathyroidism
LOM	loss of methylation
AHO	Albrights Hereditary Osteodystrophy
PTH	parathyroid hormone

TSH	thyroid-stimulating hormone
GHRH	growth hormone-releasing hormone
CT	calcitonin
ANA	anti-nuclear antibodies
SD	standard deviation

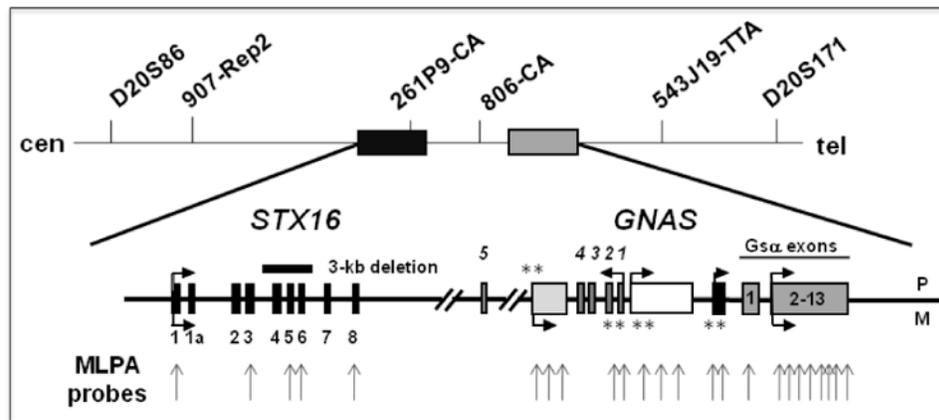


Figure 1. Schematic presentation of the *GNAS-STX16* region on chromosome 20q13.3. The approximate locations of different microsatellites markers used in this study are shown, as are the multiple probes used MLPA and MS-MLPA (grey vertical arrows). Horizontal arrows depict direction of transcription; **, indicate the locations of differentially methylated regions and whether methylation occurs on either the paternal (P) or the maternal (M) allele. The location of the 3-kb deletion involving *STX16* exons 4-6 is indicated by a horizontal black bar.

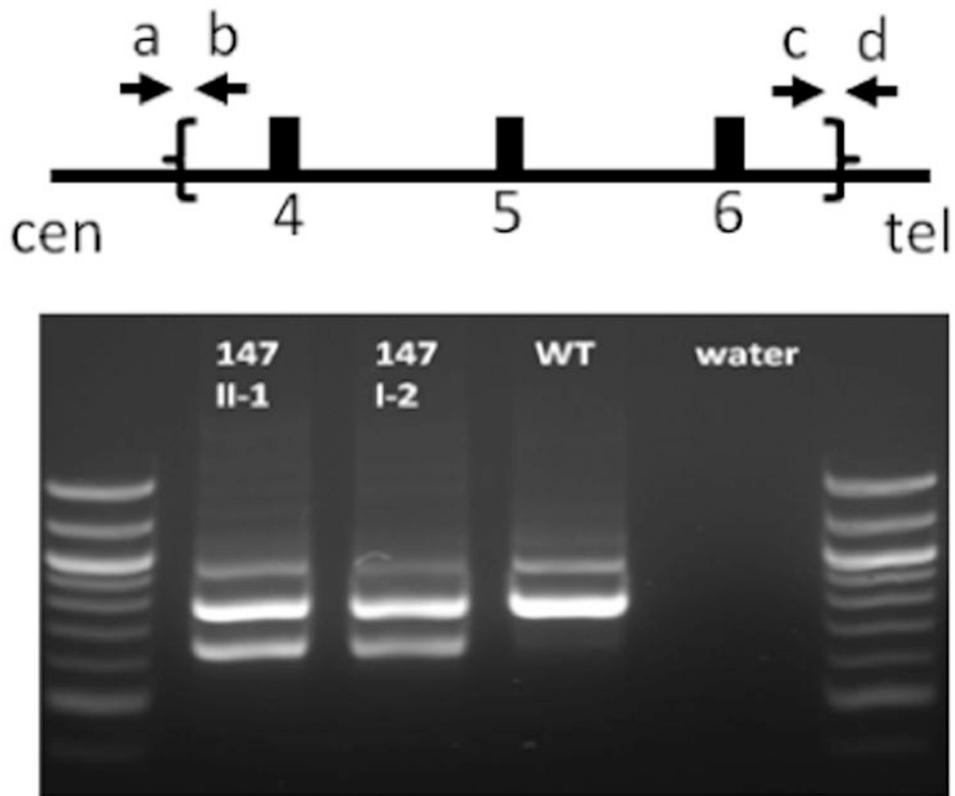


Figure 2.

Schematic presentation of *STX16* exons 4-6 (open boxes) and location of the primers (indicated by arrows a-d) that were used for multiplex PCR to assess the presence of the previously described 3-kb *STX16* deletion²⁷. Lane #1, the patient 147/II-1; lane #2, the patient's healthy carrier mother; lane #3, healthy individual; lane #4, negative control. The 663 bp band is only observed when using primers a and d that are outside of the deletion borders and visible only in subjects with the 3-kb *STX16* deletion.

Expected bands: Wild-type DNA: primers a and b, 966 bp; primers c and d, 793 bp; primers a and d, 3641 bp (too long to be amplified with the PCR system used). DNA from patient with 3-kb *STX16* deletion: additional band of 663 bp with primers a and d. The 100 bp ladders are shown on both sides of the gel.

Table 1

Patient characteristics, laboratory, and epigenetic findings in four patients initially diagnosed with hypothyroidism before establishing the diagnosis of PHP-Ib; note that patient 149/II-1 has autoimmune thyroid disease and that PHP-Ib most likely developed independently. The normal ranges for PTH, calcium, and phosphate (adult) were similar for the laboratories at the different institutions; for the other parameters, institution-specific reference ranges are provided; * = only total T4 was measured at the time (normal range: 4.6–12.0 µg/dl). To convert FT4 from ng/dl to pmol/L multiply by 12.87. Analysis of *GNAS* methylation at exons A/B, XL, AS, and NESP55: -non-methylated; + methylated. 3-kb *STX16* deletion: yes, present; no, absent.

	130/II-1		148/II-1		149/II-1		147/II-1	
	0,2	3,5	0,5	3,0	15,0	28,0	3,0	23,0
Age (yrs)								
TSH (µU/ml)	10,6 (0,5-6,0)	1,9 (0,5-5,0)	13,2 (0,4-5,5)	8,0 (0,4-4,2)	21,9 (0,5-5,0)	0,1 (0,5-5,0)	5,9 (0,2-4,0)	4,0 (0,5-4,5)
FT4 (ng/dl)	0,67	1,90 (0,95-2,10)	1,25	1,49 (0,85-1,46)	0,28	1,19 (0,70-1,48)	8,3*	1,39 (0,9-1,80)
PTH (pg/ml) (10-65)	n.d.	291	n.d.	668	n.d.	1186	n.d.	795
Calcium (mg/dl) (8,5-10,5)	10,0	7,0	10,5	7,9	n.d.	6,7	n.d.	5,6
Phosphate (mg/dl) (adult: 3,0-4,5)	5,4	6,4	7,5	8,5	n.d.	4,3	n.d.	3,5
25OHD (ng/ml)	n.d.	31 (>30)	n.d.	19 (30-100)	n.d.	34 (>30)	n.d.	17 (30-100)
<i>GNAS</i> methylation								
A/B	-	-	-	-	-	-	-	-
XL	-	-	-	-	-	-	+	-
AS	-	-	-	-	-	-	+	-
NESP55	+	+	+	+	+	+	+	-
3-kb <i>STX16</i> deletion		no		no		no		Yes