Quantitative analysis of methylation defects and correlation with clinical characteristics in patients with Pseudohypoparathyroidism type I and GNAS epigenetic alterations.

Original Citation:
Quantitative analysis of methylation defects and correlation with clinical characteristics in patients with Pseudohypoparathyroidism type I and GNAS epigenetic alterations. / Elli FM; de Sanctis L; Bollati V; Tarantini L; Filopanti M; Barbieri AM; Peverelli E; Beck-Peccoz P; Spada A; Mantovani G. - In: THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM. - ISSN 0021-972X. - ELETTRONICO. - 99:3(2014), pp. E508-E517.

Availability:
This version is available http://hdl.handle.net/2318/154086 since 2016-11-30T11:49:49Z

Published version:
DOI:10.1210/jc.2013-3086

Terms of use:
Open Access
Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)
This is the author's final version of the contribution published as:

Elli FM; de Sanctis L; Bollati V; Tarantini L; Filopanti M; Barbieri AM; Peverelli E; Beck-Peccoz P; Spada A; Mantovani G. Quantitative analysis of methylation defects and correlation with clinical characteristics in patients with Pseudohypoparathyroidism type I and GNAS epigenetic alterations. THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM. 99 (3) pp: E508-E517.
DOI: 10.1210/jc.2013-3086

The publisher's version is available at: http://press.endocrine.org/doi/10.1210/jc.2013-3086

When citing, please refer to the published version.

Link to this full text: http://hdl.handle.net/2318/154086
Quantitative analysis of methylation defects and correlation with clinical characteristics in patients with Pseudohypoparathyroidism type I and GNAS epigenetic alterations

Francesca M Elli, Luisa de Sanctis, Valentina Bollati, Letizia Tarantini, Marcello Filopanti, Anna Maria Barbieri, Erika Peverelli, Paolo Beck-Peccoz, Anna Spada, Giovanna Mantovani

Department of Clinical Sciences and Community Health, University of Milan, Endocrinology and Diabetology Unit, Fondazione IRCCS Ca’ Granda Policlinico, Milan (F.M.E., A.M.B., M.F., E.P., P.B-P, A.S., G.M.); Department of Public Health and Pediatrics, University of Turin, Regina Margherita Children’s Hospital, Turin, (L.d.S.); XX (V.B., L.T.); Italy

Short title: Phenotype/Epigenotype correlation in Pseudohypoparathyroidism type I

Word count: Text: XX, Abstract: 249, Tables: 1, Figure: 5

DISCLOSURE STATEMENT: The authors have nothing to disclose.

Our findings further demonstrate that similar GNAS molecular alterations may lead to a broad spectrum of diseases, from isolated PTH resistance to multi-hormone resistance and Albright hereditary osteodystrophy

Corresponding address:
Giovanna Mantovani, MD, PhD
Unità di Endocrinologia- Pad. Granelli
Fondazione IRCCS Ca’ Granda Policlinico
Via Francesco Sforza, 35
20122 Milano- Italy
Phone: +39 02 50320613
FAX: +39 02 50320605
Email: giovanna.mantovani@unimi.it
Abstract

Context: Pseudohypoparathyroidism type I (PHP-I) includes two main subtypes, PHP-Ia and –Ib. About 70% of PHP-Ia patients, who show Albright hereditary osteodystrophy (AHO) associated with resistance toward multiple hormones (PTH/TSH/GHRH/gonadotropins), carry heterozygous mutations in \( GNAS \) Gs\( \alpha \)-coding exons 1-13, whereas about 60% of PHP-Ib patients, who classically display hormone resistance limited to PTH and TSH with no AHO sign, have methylation defects in the imprinted \( GNAS \) cluster. Recently, methylation defects have been detected also in patients with PHP and different degrees of AHO, indicating a molecular overlap between the two forms.

Objectives: To collect patients with the following characteristics: clinical PHP-I (with or without AHO), no mutation in Gs\( \alpha \)-coding sequence but presence of \( GNAS \) methylation alterations; to investigate the existence of correlations between the degree of the epigenetic defect and the severity of the disease.

Patients and methods: We quantified \( GNAS \) methylation alterations by both PCR-pyro-sequencing and MS-MLPA in genomic DNA from 54 patients with PHP-I and correlated these findings with clinical parameters (age at diagnosis, calcium, phosphorus, PTH, TSH levels; presence or absence of each AHO sign).

Results: By both approaches, the degree of the imprinting defect did not correlate with the onset of the disease, the severity of endocrine resistances, nor with the presence/absence of specific AHO signs.

Conclusions: Similar molecular alterations may lead to a broad spectrum of diseases, from isolated PTH resistance to complete PHP-Ia.
Introduction

Pseudohypoparathyroidism (PHP) and Albright hereditary osteodystrophy (AHO) (short stature, rounded face, brachydactyly, ectopic ossifications and mental retardation) are rare, related, highly heterogeneous and deeply impairing disorders with proven genetic component. PHP is historically the first hormone resistance syndrome, and the term now encompasses a heterogeneous group of metabolic diseases, all characterized by end-organ resistance to the action of PTH (reviewed in refs 1-3).

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, about 70% of PHP-Ia patients, who show Albright hereditary osteodystrophy (AHO) associated with resistance toward multiple hormones (PTH/TSH/GHRH/gonadotropins), are affected by heterozygous, maternally-derived mutations in GNAS exons 1-13 (the gene encoding Gsα i.e. the alpha-subunit of heterotrimeric stimulatory G protein). The same mutations inherited from the father lead to pseudo-PHP (PPHP), 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 (4), due to a reduction in its α-subunit mRNA and protein.

On the other hand, the majority of PHP-Ib patients, who classically display hormone resistance limited to PTH and TSH with no AHO sign and normal Gsα activity in erythrocytes and fibroblast, display methylation defects in the imprinted GNAS cluster (5,6). In fact, beside Gsα, GNAS drives the transcription of other genes, including XLαs, A/B, NESP55 and AS, each promoter being characterized by a parent-specific methylation pattern (Figure 1) (reviewed in refs. 7&8). The most consistent defect common to all PHP-Ib patients is the loss of imprinting at the exon A/B differentially methylated region (DMR), and the percent of methylation at this site seems to correlate with PTH concentration at the time of diagnosis (6,9). The biallelic and thus abnormal expression of this paternally derived transcript located upstream of the gene encoding Gsα, presumably leads to a decreased Gsα expression in renal proximal tubules and few additional cells.
The familial form of the disease (AD-PHP-Ib) is typically caused either by an isolated loss of imprinting at the exon A/B DMR due to microdeletions disrupting the upstream STX16 gene or, less frequently, by loss of all the maternal GNAS imprints associated with deletions removing the NESP55 DMR. Conversely, sporadic PHP-Ib cases show broad GNAS imprinting abnormalities involving multiple DMRs, with no known underlying genetic lesion (reviewed in refs. 10&11).

During the last decade, incoming data on both clinical and molecular aspects of these complex disorders have challenged the distinction of different GNAS-related diseases. In particular, in a subset of patients with PHP and variable degrees of AHO, epigenetic defects of GNAS similar to those classically found in PHP–Ib patients have been detected by independent groups, suggesting a molecular overlap between PHP-Ia and Ib (12-15), but the correlation between these molecular findings and the severity of the disease has not been elucidated. In the present study we therefore investigated the presence of correlations between the degree of epigenetic defects and the severity of the disease in a large series of patients with PHP type I.

Patients and Methods

Patients

The inclusion criteria in the present study were the presence of PHP together with methylation defects at the GNAS locus in the absence of mutations in GNAS Gsα-coding exons. Following these criteria, we included 54 patients (23 females and 31 males), all born from healthy, non-consanguineous parents. In all of them the clinical diagnosis of PHP was based upon the presence of PTH resistance, i.e. hypocalcemia, hyperphosphatemia and raised serum PTH levels in the absence of vitamin D deficiency. Twelve patients presented with additional hormone resistances and 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 -2SDS at the metacarpophalangeal profile pattern in at least one metacarpal bone or distal phalanx) (12,16), short stature (height below the 3th percentile for chronological age), obesity (BMI >30 kg/m² in
adults and >97th centile in children), round face, ectopic ossifications (either clinically evident or at X-ray), and mental retardation, defined in case of history of delayed motor and/or speech milestones or need of extra help in pre-school or mainstream school. Since many of these signs are highly non-specific, the presence of at least brachydactyly and/or heterotopic ossifications was required for the definition of AHO. In this case, despite the absence of mutations in \textit{GNAS} G\alpha-coding exons, a clinical diagnosis of PHP-Ia was made.

In the remaining 42 patients PTH resistance presented either alone or, as for more than half of these patients (N=17), together with TSH resistance. In addition to the absence of distinguishing AHO signs (brachydactyly and ectopic ossifications), no other hormone resistances were observed in this series. These patients were thus classified as having PHP-Ib.

The age at which diagnosis of PHP-I was made ranged between 3 and 51 year-old (mean age: 14 for PHP-Ia patients and 36 for PHP-Ib patients). TSH resistance was documented by raised serum TSH levels (TSH>3.9 mU/L), in the absence of anti-thyroid antibodies and in the presence of a normal thyroid scan (data not shown). Evident resistance to gonadotropins was present in 1 PHP-Ia patient only (elevated LH and FSH levels, together with low oestradiol levels and secondary amenorrhea), while in 2 of 5 PHP-Ia female patients the onset of puberty was regular but thereafter oligomenorrhoic menstrual cycles occurred. No case of male hypogonadism was demonstrated.

Clinical and biochemical details are shown in Table 1. Informed consent for genetic and epigenetic studies was obtained from all subjects involved in the study.

\textit{GNAS Methylation analysis and quantification}

Differential methylation of \textit{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 (15). In brief, 360 ng DNA were treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the manufacturer’s protocol, and were amplified by PCR using
ZymoTaq DNA Polymerase (Zymo Research, Orange, CA). Primers for the amplification of the four different GNAS exons were specifically designed to amplify bisulfite-modified DNA (primer sequences available upon request). Either the forward or the reverse primer was biotin-labeled, depending on the proximity to CG sites, in order 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 0.2M NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Westborough, MA), as recommended by the manufacturer. A 0.3 µM pyrosequencing primer was then annealed to the purified single-stranded PCR product, and pyrosequencing was performed using the PyroMark MD (Pyrosequencing) (Figure 2). Every sample was tested three times for each marker to confirm the reproducibility of our results. Methylation quantification was performed using the provided software. The degree of methylation was expressed for each considered DNA DMR as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines. The degree of the imprinting defect was compared to the percentage of methylation at each DMR in respect with a pool of 20 age- and gender-related normal subjects. These pyrosequencing assays allowed to analyze 3 CGs at chromosome position 57415673-57415972 (NESP DMR), 3 CGs at chromosome position 57426653-57426945 (AS DMR), 4 CGs at chromosome position 57429173-57429469 (XL DMR) and 5 CGs at chromosome position 57463553-57463848 (A/B DMR) (according to GRCh37/hg19 build reference sequence) (Figure 1). Mean methylation levels of NESP, AS, XL and A/B DMRs in healthy individuals were 43.1 % ± 4.8, 37.9 % ± 3.2, 40.5 % ± 3.8 and 46.5 % ± 5.1, respectively. These data are consistent with expected methylation levels for imprinted loci in physiological conditions. (Figure 2)

*Methylation Specific - Multiplex Ligation-dependent Probe Amplification assay (MS-MLPA) of STX16 and GNAS loci*
Differential methylation of GNAS DMRs (A/B, AS, XL, and NESP) was then confirmed by Methylation Specific Multiplex Ligation-specific Probe Amplification (MS-MLPA) using a commercially available probe mix (ME031 MLPA probemix; MRC-Holland, Amsterdam, The Netherlands). Probes used to detect methylation contain a HhaI digestion site (methylation sensitive restriction enzyme; recognition site GCGC). In brief, according to the manufacturer’s instructions, 400 ng of genomic DNA in a final volume of 5 µl was hybridized to MLPA probes overnight at 60°C. Subsequently, half of the sample was ligated, whereas for the remaining ligation was combined with an HhaI digestion resulting in ligation of the methylated sequences only. PCR amplification of ligated probes was carried out for 40 cycles in a final volume of 25 µl. PCR products were separated by capillary electrophoresis using a 3100 Genetic Analyzer (Perkin-Elmer Corp.) with an internal size standard GeneScan 500LIZ (Applied Biosystems, Foster City, CA). Data analysis was performed using PeakScanner v1.0 (Applied Biosystems, Foster City, CA) and Coffalyser v9.4 (MRC-Holland, Amsterdam, The Netherlands). The methylation status was calculated by dividing the normalization constant of each MS-MLPA probe obtained in the digested sample by the normalization constant of each MS-MLPA probe obtained in the same undigested sample (Supplementary Figure 1).

Statistical analysis

Continuous variables, i.e. percentage of methylation, hormonal and biochemical parameters were reported as mean ± standard deviation when normally distributed or median and interquartile range (IQR) otherwise, whereas the presence of PHP signs (dichotomous variables) were expressed as proportions. Differences between means and proportions were checked by Student’s t and Chi-squared tests, respectively. Bivariate correlation between methylation level at A/B DMR and continuous variables were checked by Pearson’s r correlation coefficient computation, whereas correlation with ordinal variables (i.e. number of AHO signs) were calculated by Spearman’s test. Hierarchical classification analysis was carried out by Ward method on Euclidean
distances calculation, considering the percentage of methylation at all \textit{GNAS} DMRs at the same time. Number of clusters were limited to five.

A P less than 0.05 was considered significant. Statistical computations were performed by SPSS 18.0 software (IBM Inc. Armonk, NY, USA).

**Results**

According to the apparent sporadic form of the disease, in addition to methylation defects at exon A/B, 45 out of 54 patients showed multiple additional methylation changes (LoM at XL and AS DMRs and gain of methylation at NESP DMR) (classified from the molecular point of view as having spor-PHP-Ib), while 9 patients showed deletion of the imprinting control region (ICR) at the \textit{STX16} gene associated with an isolated LoM at the A/B DMR (classified from the molecular point of view as having AD-PHP-Ib). In particular, as for the A/B DMR, a condition considered sufficient to determine PHP manifestations, percentages of methylation at the five investigated CpG sites were highly homogeneous, with a variation coefficient < 2%. Moreover, the 5 x 5 correlation matrix showed high Pearson’s r values (between 0.939 and 0.986, P < 0.001), indicating that the mean value of methylation at these five CpG sites can be considered as an overall index of methylation at the entire DMR.

Pyrosequencing analysis did not show correlation between the percentage of methylation at the A/B exon DMR and the precocity of the disease (defined as the age at diagnosis) or the severity of hormonal resistances (defined as PTH, calcium, phosphorus, and TSH levels at diagnosis) was observed (Figure 3A for PTH; supplementary figure 2 for TSH, calcium, phosphorus). All these results turned out similar either considering the series taken as a whole, or dividing patients into PHP-Ia vs Ib groups (Figure 3B&C).

Similarly, no correlation was observed in our study between the degree of methylation defects and the severity of AHO, defined as the number of AHO signs ($r=-0.01041$, $P=0.9404$, 0.9404, 0.9404).
Spearman’s correlation test) or the presence or absence of each sign in patients affected with clinically defined PHP-Ia (Table 1 and data not shown).

Because of the wide scattering of A/B methylation percentages in the investigated cohort, a cluster analysis was carried out in order to unveil possible homogeneous subgroups (Figure 4, left panel). As expected, hierarchical classification showed the presence of 3 major clusters, labelled as cluster 1 (healthy controls and PHP-Ib patients without imprinting defects), cluster 2 (isolated LoM at the A/B DMR) and cluster 3 (methylation defects at all 4 GNAS DMRs). Furthermore, the same analysis indicated that cluster 3 could be subdivided in group 3.1, defined as “severe” methylation defect (mean methylation % ± SD: NESP, 81.5% ± 6.3; AS, 9% ± 5.8; XL, 10.3% ± 5.8 and A/B, 5.6% ± 4.5) and 3.2, defined as “mild” methylation defect (mean methylation % ± SD: NESP, 67% ± 12.8; AS, 16.3% ± 6.5; XL, 19.7% ± 7.1 and A/B, 22.3% ± 9.4) (Figure 4, right panel). As shown in figure 5, no differences were observed in terms of age at diagnosis and hormonal/biochemical parameters among clusters 2, 3.1 and 3.2.

By definition, all patients showed at least LoM at exon A/B, a condition sufficient to determine PHP manifestations. We therefore next considered the methylation degree at the A/B DMR as marker of the imprinting status of GNAS locus. Correlation analysis taking into consideration methylation at the A/B DMR only and clinical/biochemical variables was repeated without finding statistically significant results (data not shown).

Finally, the absence of correlation between the severity of methylation defects and clinical parameters was confirmed by repeating statistical analysis considering MS-MLPA results.

Discussion

During the last decade, incoming data on both clinical and molecular aspects of PHP have challenged the distinction of different GNAS-related diseases. We report a clinical/molecular correlation analysis in a large series of patients with either PHP-Ia or PHP-Ib, all showing GNAS methylation alterations in the absence of mutations in Gsα-coding exons, and we demonstrate that
similar molecular alterations may equally lead to a broad spectrum of diseases, from isolated PTH resistance to complete PHP-Ia.

The distinction between PHP-Ia and PHP-Ib is usually performed according to the presence (PHP-Ia) or absence (PHP-Ib) of AHO and, as for the underlying molecular mechanism, to the presence of mutations in \textit{GNAS} Gsα-coding exons (PHP-Ia) or \textit{GNAS} imprinting dysregulation (PHP-Ib). Until recently, this clinical and molecular classification was in reciprocal agreement. Nevertheless, although \textit{GNAS} mutations are identified in the majority of patients with PHP–Ia as well as in relatives affected with PPHP, in about 30% of patients no \textit{GNAS} mutation is found. In the past few years, in a subset of patients with PHP and variable degrees of AHO phenotype but absence of mutations in Gsα–coding exons, \textit{GNAS} epigenetic defects similar to those classically found in PHP–Ib patients have been detected, suggesting a molecular overlap between PHP-Ia and Ib (12-15). This would propose that features of AHO may result from either mutations in Gsα–coding exons or epigenetic defects causing disruption of imprinting control elements in \textit{GNAS} locus, both defects resulting in silencing or reduction of Gsα transcription in selected tissues, where it is postulated that the protein is mainly derived from the maternal allele (17-20). According to this line of evidence, the present study further expands the number of patients with PHP and AHO carrying \textit{GNAS} methylation abnormalities, thus confirming all recently published reports by us and others.

To date, although we previously reported that the presence or absence of imprinting defects in clinically classified PHP-Ia patients was not associated with the severity or number of signs of AHO, as well as number or type of hormone resistances (15), no study has investigated the existence of an epigenotype/phenotype correlation in PHP-I patients. In this study we therefore aimed to investigate the presence of a correlation between the degree of epigenetic defects and the severity of the disease in a very large series of these patients. The data presented here confirm the absence of correlation between the phenotype of 54 PHP-I patients and their epigenotype,
established by a quantitative analysis (pyrosequencing) and subsequently confirmed by a semi-quantitative method (MS-MLPA).

Our results are apparently in contrast with a work by Maupetit-Méhouas and colleagues indicating that the percent of methylation at the exon A/B DMR correlates with PTH concentration at the time of diagnosis in a series of 19 patients with PHP-Ib investigated by real-time PCR (9). Moreover, the authors also found a sub-cluster of patients who did not show any abnormality at the XL DMR, despite having alterations at all the other DMRs (A/B, NESP and AS). Noteworthy, in our series a small subset of patients (pts 5, 9, 38 and 43) may belong to this entity as they display higher levels of methylation at the XL DMR than those detected at the other investigated DMRs. However, statistical analysis did not identify this cluster of patients and, anyhow, no phenotypic difference was observed in these cases if compared with the others. In our study, pyrosequencing analysis was chosen to quantify methylation, this being a quantitative technique able to analyze a stretch of cytosines at the same time, in contrast with Southern Blot or cytosine specific real-time PCR that allow the investigation of one cytosine at a time. The different approach is the most likely explanation for the discrepancy between our results and the previously reported ones, also taking into account that cytosines investigated by the French group are not included in the stretch of cytosines analyzed by pyrosequencing in the present series. In order to exclude that our negative results might be due to the concurrent investigation of many cytosines and that methylation of single residues may be crucial in the determination of the severity of single clinical manifestations, statistical analysis was performed considering not only the percent of methylation at the cytosine stretch taken as a whole, but also each cytosine at a time. In our view, this methodological approach together with the size of the investigated series, should give strength and reliability to the results presented here.

Nevertheless, quantitative measurement of methylation at the 4 GNAS DMRs did allow the identification of different subgroups of patients based on the presence of a severe or milder imprinting defect. Again, the comparison of these two clusters in terms of clinical phenotype did
not lead to significant differences. By definition, all patients showed at least LoM at exon A/B, a condition sufficient to determine PHP manifestations. We therefore proceeded considering the methylation degree at the A/B DMR as marker of the GNAS locus imprinting status and thus performed the correlation analysis taking into consideration the A/B DMR alone, with similar negative results.

Overall, the finding of different degrees of methylation defects is in accordance with previously published data on smaller series (9) and could suggest the presence of somatic mosaicism leading to the contribution of both normal cells and cells with an imprinting defect in a given tissue. This phenomenon has been described in the context of several diseases involving loci submitted to parental imprinting such as Angelman, Silver–Russell and Beckwith–Wiedemann syndromes in which a post-zygotic error of the imprinting has been proposed (21-25). On the other hand, profound and broad epigenetic changes (sporadic cases in sub-cluster 1) could also underlie the presence of uniparental disomy (hetero- or isodisomy), as already shown by different authors in a small number of sporPHP-Ib patients (26-29). Nevertheless, the latter hypothesis is unlikely in this case series as most of our patients were previously found to be heterozygous for at least one polymorphic site within GNAS (15).

In the present study, all patients displaying both hormone resistance and AHO were clinically classified as having PHP-Ia. Therefore, we did not make a distinction between PHP-Ia and PHP-Ic, as a large subset of these patients were not evaluated for Gsα activity. Measurement of Gsα activity in erythrocyte membranes is considered crucial to distinguish PHP-Ia subjects from PHP-Ic ones, being reduced in the former group and normal in the latter. Similarly to PHP-Ic, this activity is classically normal in PHP-Ib patients. Indeed, a very recent study demonstrated a reduction of Gsα activity in erythrocyte membranes of patients with PHP due to epigenetic alterations at the GNAS locus, the maximum decrease being observed in patients with AHO signs (30). The absence of GNAS mutations is the second hallmark of PHP-Ic. Nevertheless, mutations in GNAS carboxyl-terminus with conserved adenylyl cyclase receptor-independent activation but
disrupted receptor-mediated activation have been recently detected in few PHP-Ic patients (31,32). Taken together, these findings further underscore the overlap among PHP-I subtypes and suggest that PHP-Ia and –Ic are indeed clinically, biochemically and genetically indistinguishable disorders.

Finally, the severity of hormone resistance in PHP-Ia and Ib has not been deeply investigated to date, with the exception of a single paper reporting the absence of clinical differences between the sporadic and the familial form of PHP-Ib (33). Taken advantage of the large number of patients included in this study, we also compared patients clinically classified as having PHP-Ia in respect with those affected with PHP-Ib and no difference was found in terms of calcium and PTH levels at the time of diagnosis, as well as TSH and phosphorus levels.

In conclusion, we describe a large, clinically and molecularly characterized series of patients affected with PHP type I, in whom we performed a phenotype/epigenotype correlation analysis. Our findings demonstrate that similar GNAS molecular alterations may equally lead to a broad spectrum of diseases, from isolated PTH resistance to multi-hormone resistance associated with Albright hereditary osteodystrophy. Despite we strongly believe that each PHP patient should be screened for both GNAS genetic and epigenetic alterations in order to have a proper genetic counselling, the data presented here do not suggest that the molecular characterization may help clinicians to anticipate the severity and the type of different PHP/AHO manifestations.

Acknowledgements

This work was supported by a grant from the Italian Ministry of Health to G.M. (GR-2009-1608394).
References


10. **Juppner H, Bastepe M** 2006 Different mutations within or upstream of the GNAS locus cause distinct forms of pseudohypoparathyroidism. J Pediatr Endocrinol Metab 19 Suppl 2:641-646


large cohort of 11p15-related foetal growth disorders (Russell Silver and Beckwith Wiedemann syndromes) reveals simultaneous loss of methylation at paternal and maternal imprinted loci. Hum Mol Genet 18:4724–33.


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

<table>
<thead>
<tr>
<th>ID</th>
<th>clinical diagnosis</th>
<th>gender</th>
<th>age</th>
<th>diag. age</th>
<th>PTH</th>
<th>TSH</th>
<th>SS</th>
<th>Ob</th>
<th>RF</th>
<th>EO</th>
<th>MR</th>
<th>Br</th>
<th># AHO signs</th>
<th>Ca</th>
<th>P</th>
<th>% methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n.</td>
<td>n.a.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
<td>n.</td>
</tr>
</tbody>
</table>

Elli et al., 19
| ID | PHP-ib  | M  | 22 | 454 | 4.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8.3 | 4.3 | 76.8 | 8.0 | 7.6 | 2.8 |
|----|---------|----|----|-----|-----|----|---|---|---|---|---|---|---|---|-----|-----|-----|-----|-----|-----|
| 49 | PHP-ib  | M  | 36 | 22  | 454 | 4.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8.3 | 4.3 | 76.8 | 8.0 | 7.6 | 2.8 |
| 50 | PHP-ib* | M  | 12 | 11  | 164 | 2.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.1 | 9   | 41.1 | 34.3 | 39.5 | 5.5 |
| 51 | PHP-ib  | F  | 10 | 9   | 40  | 2.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.1 | 2   | 81.4 | 6.9 | 7.5 | 1.5 |
| 52 | PHP-ib  | M  | 3  | 2   | 110 | 3.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.2 | 1.8 | 82.8 | 6.3 | 6.9 | 1.2 |
| 53 | PHP-ib  | F  | 18 | 17  | 333 | 3.3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 7.6 | 4.8 | 81.5 | 7.0 | 7.8 | 14.3|
| 54 | PHP-ib  | F  | 9  | 4   | 890 | 5.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1   | 0   | 7.2  | 8.3 | 82.5 | 6.7 |

Legend: ID column contains patient’s identificative number. PHP column indicates the clinical diagnosis (※: presence of STX16 gene deletion). M, male. F, female. Age, actual age. Diag age, age at diagnosis. PTH, normal range: 10-65 pg/mL. TSH, normal range: 0.4-3.9 mU/L. Calcaemia, normal range: 9-10.5 mg/dL. Phosphataemia, normal range: 2.8-4.5 mg/dL. SS, short stature. Ob, obesity. RF, round face. EO, ectopic ossification. MR, mental retardation. Br, brachydactyly. Brachydactyly and ectopic ossifications were considered the most specific AHO signs and thus highlighted in bold were present.

NESP/AS/XL/AB columns show the percentage of measured methylation at each DMR. n.a., data not available.
Legend to figures:

**Figure 1:** The upper panel shows a schematic graph of the GNAS locus and the STX16 gene. Exons are indicated as black rectangles, STX16 and NESP-AS deletions as brackets, and allelic origin of transcription as broken arrows on the paternal (Pat) or maternal (Mat) allele. The four differentially methylated regions (DMRs) are represented beneath the genomic line as black boxes or + (methylated) or white boxes or – (unmethylated) on the paternal (Pat) or maternal (Mat) allele. The lower horizontal line shows CpG islands identified by the Genome Browser software.

The lower panel describes the analysis of GNAS DMRs methylation status performed by pyrosequencing. Representative pyrograms for each analyzed DMR (NESP, AS, XL, A/B) are shown. The x-axis represents the DMR sequence, whereas the y-axis shows the percentage of incorporated nucleotide for each given position. Upper pyrograms show the normal CpG pattern of methylation in a wild-type subject, whereas lower pyrograms demonstrate methylation alterations (indicated by arrows) at the same position in a representative patient.

**Figure 2:** The graphs show the distribution of methylation percentages (mean ± SD) detected by pyrosequencing at each GNAS DMR in subjects without imprinting defects, defined as controls (ctrls) (A), and PHP-I patients with imprinting defects (B). Mean methylation levels in individuals with a wild-type methylation pattern are consistent with expected methylation levels for imprinted loci in physiological conditions (NESP, 43.1 ± 4.8; AS, 37.9 ± 3.2; XL, 40.5 ± 3.8 and A/B, 46.5 ± 5.1). As expected, PHP-I cases exhibit methylation levels in accordance with the presence of LoM at GNAS DMRs (NESP, 71 ± 16.7; AS, 15.8 ± 12.5; XL, 17.9 ± 12.7 and A/B, 10.2 ± 10.8).

**Figure 3:** Correlation between the degree of the epigenetic defect and PTH concentration at the time of diagnosis. A) Percentage of methylation at A/B DMR vs PTH. B) Percentage of methylation at A/B DMR in patients with clinical diagnosis of PHP-Ia vs PTH. C) Percentage of methylation at
A/B DMR in patients with clinical diagnosis of PHP-Ib vs PTH. All graphs show the absence of correlation between the percentage of methylation at A/B DMR and PTH concentration.

**Figure 4:** *Left panel:* The cluster dendrogram, obtained after methylation quantification at GNAS DMRs, allows the identification of different subtypes of methylation defects. In particular, 3 main clusters were identified: cluster 1, which includes healthy controls and PHP-Ib patients without imprinting defects, cluster 2, which includes 9 AD-PHP-Ib patients, and cluster 3, which includes 45 patients affected with spor-PHP-Ib. The latter can be further divided in 2 sub-clusters: 3.1 and 3.2. *Right panel:* For cluster 2 and sub-clusters 3.1 and 3.2, obtained by hierarchical clusterization, values and means ± SD of detected percentage of methylation at each DMR are shown. Patients belonging to cluster 2 have severe imprinting defects limited to A/B DMR (5.4 ± 7.6). Patients included in cluster 3 show variable degrees of epigenetic defects at all GNAS DMRs. In particular, in sub-cluster 3.1 methylation defects are more severe than in sub-cluster 3.2 (NESP: 81.5 ± 6.3 vs 67 ± 12.8; AS: 9 ± 5.8 vs 16.3 ± 6.5; XL: 10.3 ± 5.8 vs 19.7 ± 7.1; A/B: 5.6 ± 4.5 vs 22.3 ± 9.4; p=XXX).

**Figure 5:** Absence of significant differences in terms of PTH levels (A), TSH levels (B), calcaemia (C), phosphataemia (D), number of AHO signs (E) and age at diagnosis (F) among clusters 2, 3.1 and 3.2.

**Supplementary figure 1:** Analysis of GNAS DMRs methylation status by MS-MLPA. The figure shows representative chromatograms of undigested and digested samples obtained from a negative control and from a patient with methylation defects at each analyzed DMR. Digested probes cannot be amplified and do not produce signal during capillary electrophoresis. In contrast, in case of sample DNA methylation, the ligated probes are protected against HhaI digestion generating a peak. The table summarizes data analysis by Coffalyser software in a wild-type sample and in samples
with methylation defects (spor-Ib and AD-Ib). The expected ratio for imprinted targets is 0.5 (range 0.25-0.75), for LoM is < 0.25 and for GoM is > 0.75.

**Supplementary figure 2:** Absence of correlation between the degree of the epigenetic defect at the A/B DMR and patient’s TSH (A.) calcium (B.) phosphorus (C.) at the time of diagnosis.
### Table

<table>
<thead>
<tr>
<th>Probe</th>
<th>Chg pos</th>
<th>Gene</th>
<th>WT</th>
<th>sper-Lb</th>
<th>AS-Lb</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA33 probe  1032-104208</td>
<td>20x102</td>
<td>NTM</td>
<td>0.32</td>
<td>0.33</td>
<td>0.35</td>
</tr>
<tr>
<td>ST1 probe  2039-209306</td>
<td>20x102</td>
<td>STOM-n119</td>
<td>0.35</td>
<td>0.35</td>
<td>0.36</td>
</tr>
<tr>
<td>ST1 probe  2014-212200</td>
<td>20x102</td>
<td>STOM-n118</td>
<td>0.35</td>
<td>0.35</td>
<td>0.36</td>
</tr>
<tr>
<td>ST1 probe  7520-176856</td>
<td>20x102</td>
<td>STOM-n116</td>
<td>0.33</td>
<td>0.33</td>
<td>0.36</td>
</tr>
<tr>
<td>ST1 probe  7520-163123</td>
<td>20x102</td>
<td>STOM-n115</td>
<td>0.33</td>
<td>0.33</td>
<td>0.36</td>
</tr>
<tr>
<td>ST1 probe  7520-151223</td>
<td>20x102</td>
<td>STOM-n114</td>
<td>0.33</td>
<td>0.33</td>
<td>0.36</td>
</tr>
<tr>
<td>ST1 probe  7520-132423</td>
<td>20x102</td>
<td>STOM-n113</td>
<td>0.33</td>
<td>0.33</td>
<td>0.36</td>
</tr>
</tbody>
</table>

### Diagram

- **WT uncut**
- **WT cut**
- **sper-Lb uncut**
- **sper-Lb cut**

### Additional Information

- The table lists various probes targeted to specific genes with the corresponding expression levels for WT and sper-Lb conditions.
- Diagrams illustrate the expression profiles for WT and sper-Lb conditions, showing differences in gene expression patterns.