Pseudohypoparathyroidism Type Ib Associated with Novel Duplications in the GNAS Locus

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Abstract

Context

Pseudohypoparathyroidism type 1b (PHP-Ib) is characterized by renal resistance to PTH (and, sometimes, a mild resistance to TSH) and absence of any features of Albright's hereditary osteodystrophy. Patients with PHP-Ib suffer of defects in the methylation pattern of the complex GNAS locus. PHP-Ib can be either sporadic or inherited in an autosomal dominant pattern. Whereas familial PHP-Ib is well characterized at the molecular level, the genetic cause of sporadic PHP-Ib cases remains elusive, although some molecular mechanisms have been associated with this subtype.

Objective

The aim of the study was to investigate the molecular and imprinting defects in the GNAS locus in two unrelated patients with PHP-Ib.

Design

We have analyzed the GNAS locus by direct sequencing, Methylation-Specific Multiplex Ligation-dependent Probe Amplification, microsatellites, Quantitative Multiplex PCR of Short Fluorescent fragments and array-Comparative Genomic Hybridization studies in order to characterize two unrelated families with clinical features of PHP-Ib.

Results

We identified two duplications in the GNAS region in two patients with PHP-Ib: one of them, comprising ~320 kb, occurred "de novo" in the patient, whereas the other one, of ~179 kb in length, was inherited from the maternal allele. In both cases, no other known genetic cause was observed.
Conclusion
In this article, we describe the to-our-knowledge biggest duplications reported so far in the GNAS region. Both are associated to PHP-Ib, one of them occurring ’de novo’ and the other one being maternally inherited.

Introduction
Pseudohypoparathyroidism (PHP) is a group of rare endocrine diseases characterized by hypocalcemia, hyperphosphatemia and an elevation of PTH values due to a variable resistance to this hormone in its target organs, mainly the proximal renal tubule [1]. Its exact prevalence in diverse populations is currently unknown, although it has been estimated to be 0.79/100.000 (according to Orphanet Report Series (http://www.orpha.net/consor/cgi-bin/index.php) and [2]).

Depending on the associated phenotype and biochemical features, PHP is divided in different subtypes: PHP-Ia (OMIM#103580), PHP-Ib (OMIM#603233), PHP-Ic (OMIM#612462), PHP-II (OMIM#203330) [2]. PHP type I is caused by defects at the GNAS locus (20q13.2–13.3), a complex cluster which generates five transcripts using alternative first exons. Between them, the alpha subunit of the heterotrimeric stimulatory G protein (Gsα) [3], which is a signaling protein essential for the actions of PTH and many other hormones (TSH, glucagon, gonadotropins, ...) through the adenylyl cyclase pathway [4].

As mentioned above, GNAS is a highly complex imprinted locus that generates multiple gene products through the use of multiple promoters and first exons that splice onto a common set of downstream exons. Moreover, these transcripts can be expressed by the maternal, the paternal or both alleles as the locus is regulated by imprinting mechanisms [5] (Fig. 1A).

Patients with PHP-Ia present a characteristic phenotype called Albright’s hereditary osteodystrophy (AHO), which comprises short stature, rounded face, brachydactyly, ectopic ossifications and/or mental retardation. There is a minor response in cAMP production after exogenous PTH administration and a variable resistance to multiple hormones that act through the G protein (TSH, glucagon, gonadotropins, ACTH, GHRH...). At a molecular level, most of PHP-Ia cases are caused by haploinsufficiency because of maternally-inherited heterozygous inactivating mutations in Gsα [6]. On the contrary, when the germline mutation is inherited from the paternal allele, patients suffer of pseudo-pseudohypoparathyrodism (P-PHP, OMIM#612463). These patients present with AHO features but do not have biochemical alterations. This occurs because Gsα is predominantly transcribed from the maternal allele in the tissues where PTH, TSH, LH, FSH act (proximal renal tubule, thyroid, ovary, pituitary), so they exert their actions normally if the mutation is transmitted by the paternal allele [7].

PHP-Ib is characterized by renal resistance to PTH and absence of AHO phenotype and resistance to other hormones (although there is sometimes a mild resistance to TSH) [8]. There is also a minor cAMP production after PTH administration. PHP-Ib is due to changes in the methylation pattern of the GNAS locus [9]. There is a loss of methylation in the maternal exon A/B, sometimes combined with other epigenetic defects in other regions in the locus. It can be inherited in an autosomal dominant pattern (AD-PHP-Ib), although most of the cases are sporadic [2]. AD-PHP-Ib is typically characterized by an isolated loss of methylation at exon A/B, associated with microdeletions in the STX16 gene, encoding syntaxin 16, 220 kb upstream of GNAS [10–12]. Less frequently, AD-PHP-Ib is caused by other microdeletions affecting NESP55 and NESPAS differentially methylated regions (DMRs) [13–15], causing broad loss of
Fig 1. Graphic representation of the GNAS complex locus. (A) Alternative exons and transcripts in the region. Each first alternative exon is depicted in a different format (the scratched rectangle corresponds to NESP55, the dark grey rectangle to XLα, the black rectangle to exon A/B and the dotted rectangle to GSα exon 1). NESPAS exons 1–5 are shown as soft grey rectangles. White rectangles represent common exons 2–13 and the continuous line represents PHP-Ib and GNAS Locus Duplications.
imprinting at the \( \text{GNAS} \) locus. Sporadic cases also show broad imprinting abnormalities at the \( \text{NESPAS}, \text{XL} \alpha \text{s and A/B DMRs} \), with no other changes in \textit{cis-} or \textit{trans-}acting elements explaining these phenomena up to now. Some PHP-Ib cases present paternal 20q disomies as the cause of the disease\[16\]). Moreover, an autosomal recessive form has also been hypothesized to explain the molecular mechanism of sporadic PHP-Ib in some families\[17\].

Recent articles have described methylation defects in some patients with PHP and AHO phenotype\[18–21\], an overlapping that has also been referred about the activity of the \( Gs \alpha \) protein\[22\], indicating a possible molecular and clinical superposition between PHP-Ia and PHP-Ib\[23\].

Few genomic rearrangements have been described at the \( \text{GNAS} \) locus, such as terminal deletions\[24\] or an inversion\[25\], both of them related to PHP-Ia patients. To our knowledge, there is only a report describing an intralocus duplication in \( \text{GNAS} \) in an AD-PHP-Ib kindred\[13\]. In this article, we report the biggest duplications detected so far in the \( \text{GNAS} \) region, both associated to a PHP-Ib phenotype and arising in the maternal allele. One of them (which occurred ‘\textit{de novo}’ in the patient) was associated with a complete loss of methylation at the \( \text{NESPAS}, \text{XL} \alpha \)s and A/B DMRs, whereas the other one (present both in the index case and in her mother) was concomitant with an isolated loss of methylation in exon A/B. We did not detect any other change in the locus in either patient that could explain the differences in the methylation status, although the existence of a still unknown agent co-responsible for the (epi)phenotype cannot be discarded.

**Patients**

**Case 1**

The first case (GS0131) is a 16-year-old male, the only child of healthy unrelated parents, born after a normal pregnancy and a normal delivery (weight 3200 g, length: 51 cm). The mother was 26 and the father 27 years of age at the time of the pregnancy (mother’s height 157cm, father’s height 175cm). No familial history of PHP was reported. He has been properly vaccinated, with a good psychomotor development, with weight and height in the range of standard values. He has passed through an ordinary and complete puberty, and is phenotypically normal, with no symptoms of Albright’s hereditary osteodystrophy.

At age 14 years, he presented with generalized convulsions. After an altered electrocardiography was observed in the Emergency Department, he was referred to the Pediatric Cardiology Unit. All analyses were normal except for a long Q-T segment. Referred to Neuropediatrics, he was treated with different anticonvulsants, presenting a normal Magnetic Resonance Imaging (MRI). The electroencephalography showed normal but he still suffered of convulsive crisis. A scanner revealed calcifications and a biochemical evaluation showed the following values: Ca, 6.5mg/dl; P, 7.1mg/dl; PTH, 621.9pg/dl. One month later, hypocalcemia, hyperphosphatemia and PTH high values persisted (Ca, 6.1mg/dl; P, 7.8mg/dl; PTH, 884pg/dl), the patient being diagnosed of pseudohypoparathyroidism. He started oral treatment with 500 mg of calcium carbonate and 0.5 \( \mu \)g of calcitriol each 24 hours. Calcium and phosphate values as well as electrocardiographic alterations normalized in the first month of treatment. He is properly following the treatment and all biochemical controls have turned out to be normal, with no need of changes in the doses. He has not presented any intercurrent disease. The thyroid function analysis showed a slight hyperthyrotropinemia in the beginning, which settled down later.
The last physical evaluation (16y 5m) showed a height of 169.9cm (p25–50) and a weight of 61kg (p25-p50) (BMI: 21.1).

Case 2

The second case (GS0155) is a 27-year-old female. She had had some episodes of right hemiparesis and a bowel volvulus in pediatric age. She was studied in the Neurology Unit after an episode of right hemiparesis, paresthesia and a visual field deficit. Imaging studies did not show any significant alteration. Biochemical analysis showed calcium values in the lower limit of normality (8.5–9.3mg/dl) and elevated PTH (200–300pg/ml), so she was diagnosed of pseudohypoparathyroidism. She did not present any symptom of Albright’s hereditary osteodystrophy (height 1.65m; weight 68.5kg; BMI: 25.1). No other hormone deficit was detected (TSH, 3μU/ml). She referred regular periods. Calcium and vitamin D supplements (both cholecalciferol and calcifediol) were prescribed, although she is not properly following the treatment.

In the last visit, she referred paresthesia in upper extremities with no calcium supplementation for a month. She brought a report with analytical levels: Ca, 8.7mg/dl; P, 3.9mg/dl; Mg, 2.4mg/dl, TSH, 4.15μU/ml; PTH, 349pg/dl; 25(OH)D, 37.2 ng/ml; urine Ca, 76mg/day; CLcr, 177ml/min; Dual-energy X-ray absorptiometry (DXA) with a lumbar T score of -0.7 SD and a femoral T score of -0.3 SD. She is clinically stable, with no symptoms of latent tetany. Biochemical analysis were performed: Ca, 8.9mg/dl; P, 3.7mg/dl; HbA1c 5.4%, PTH, 417pg/dl; 25(OH) D, 44 ng/ml.

Her mother was diagnosed at age 48 years with a multinodular toxic goitre. She was treated with T318 that caused iatrogenic hypothyroidism, so she was prescribed levothyroxine 125μg/day. Her past clinical history showed symptoms of generalized anxiety, being followed in the Mental Health Unit. She does not present any characteristic phenotype, with a normal physic exploration (height 1.64m; weight 70kg; BMI: 26).

Methods

Ethics Statement

The study was approved by the Comité Ético de Investigación Clínica (CEIC) from the Hospital Universitario Cruces. The patients and their relatives provided their written informed consent to participate in this study. In case of the minor included in the cohort, we obtained written informed consent from his guardians prior to enrolment in the study.

Structural analysis of the GNAS locus

Peripheral blood samples from the probands and their parents were collected on EDTA tubes. DNA was extracted from whole blood using QIAamp DNA Blood Minikit (Qiagen) according to the manufacturer’s specifications. The structural analysis of the 13 Gsα exons and GNAS exon A/B was performed by polymerase chain reaction (PCR) amplification, ExoSap (USB) purification and sequencing of both strands on an ABI3130xl Genetic Analyzer (Life Technologies). Sequences were analyzed with Sequencing Analysis v.5.2 software (Life Technologies) and compared with the reference sequence (Ensembl identifier: GNAS locus ENSG00000087460) using SeqScape v.2.5 software (Life Technologies). Primer sequences and PCR conditions are available on request.

Methylation analysis of the GNAS cluster by MS-MLPA

Dosage and methylation analyses of the GNAS locus were studied by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) using the ME031-A2 kit (MRC-
Holland) [26]. This SALSA MS-MLPA probemix is intended to provide information on deletions, duplications and abnormal methylation of sequences in the 20q13.32 GNAS region. The ME031-A2 GNAS probemix contains 27 probes specific for the complex GNAS locus and one for the TH1L gene (located 90 kb q-telomeric of GNAS) (Table 1, Fig. 1B). Of these, 17 are MS-MLPA probes containing a HhaI recognition site specific to the differentially methylated regions of the locus GNAS: XLα, NESP55, NESPAS and GNAS exon A/B and one in STX16 (located 160 kb p-telomeric of the GNAS locus) and in TH1L. Furthermore, 6 regular MLPA probes (no HhaI site) specific for GNAS and 4 probes for the STX16 gene are included.

The protocol was implemented following the manufacturer’s recommendations. Analysis of the MS-MLPA PCR products was performed on an ABI3130xl genetic analyzer using GeneMapper v4.0 software (Life Technologies). Each MS-MLPA reaction generates two samples. For copy number analysis, data generated by the undigested sample was intra-normalized by dividing the peak area of each amplification product by the total area of the reference probes only. The ratios were then obtained by dividing the intra-normalized probe ratio in a sample by the average intra-normalized probe ratio of all reference runs. For methylation analysis (digested sample), the intra-normalized peak area of each MS-MLPA probe from the digested sample was divided by the value obtained for the undigested sample.

Analysis of polymorphic markers

To characterize chromosome 20q and rule out any uniparental disomy (UPD) at this locus, twenty three polymorphic markers (D20S99, D20S857, D20S1044, D20S913, D20S468, D20S832, D20S100, D20S746, D20S88, D20S102, D20S149, D20S158, D20S430, D20S451, D20S1063, D20S496, AFMB332XDS5, D20S459, D20S443, D20S171, AFMa202yb9, D20S173 and exon A/B pentanucleotide repeat) were typed by fluorescent PCR and electrophoresis on an ABI3130xl Genetic Analyzer using GeneMapper v.4.0 software (Life Technologies) (primers and conditions are available on request). Further, four single nucleotide variations (SNV) were also typed and used as markers during the structural analysis of Gsα (c.140–53, rs7121, rs3730173 and rs234628). The chromosomal location of all these markers is indicated in Table 1 and Fig. 1B.

Whole genome and 20q13-ter Comparative Genomic Hybridization (CGH) arrays

Detection of gene copy number was performed by array-Comparative Genomic Hybridization (a-CGH) experiments. Whole genome analysis was conducted using a human whole genome 400k array (SurePrint G3 Human CGH microarray 2x400, AMADID 021850, Agilent Technologies, Santa Clara, CA), following standard and manufacturer’s protocol [27]. For the analysis of the region chr20:47897125–62435965, the array was designed using Agilent’s e-array tool (Agilent Technologies) following the manufacturer’s instruction (https://earray.chem.agilent.com/). In both cases, healthy male and female donor samples were used as hybridization controls.

Microarray data were extracted and visualized using Feature Extraction software v10.7 and Agilent Genomic Workbench v5.0 (Agilent Technologies). Copy number altered regions were analyzed using ADAM-2 (set as 6) statistic provided by DNA Analytics, with a minimum number of 5 consecutive probes (the analysis resolution, thus, is approximately 25 kb for most of the regions for the whole genome array and 400 bp for the custom array). Genomic build hg19 was used for the experiments.
| Probe number (MS-MLPA) | Marker/Probe | Genomic location GRCh37/hg19 coordinates |
|------------------------|-------------|----------------------------------------|
| D20S99                 |             | Chr20:39613650–39613812                |
| D20S857                |             | Chr20:50108271–50108486                |
| D20S1044               |             | Chr20:52501053–52501308                |
| D20S913                |             | Chr20:53459303–53459503                |
| D20S468                |             | Chr20:53895474–53895673                |
| D20S832                |             | Chr20:54314065–54314276                |
| D20S100                |             | Chr20:54991350–54991700                |
| D20S746                |             | Chr20:55514140–55514427                |
| D20S88                 |             | Chr20:57226476–57226533                |
| D20S149                |             | Chr20:57236177–57236381                |
| P1                     |             | Chr20:57242563–57242626                |
| STX16 Ex1              |             | Chr20:57244321–57244372                |
| STX16 Ex2              |             | Chr20:57245568–57245646                |
| STX16 Ex3              |             | Chr20:57252459–57252516                |
| STX16 Ex5              |             | Chr20:57262647–57262633                |
| STX16 Ex6              |             | Chr20:57429229–57429286                |
| STX16 Ex7              |             | Chr20:57429288–57429336                |
| STX16 Ex8              |             | Chr20:57430117–57430171                |
| STX16 Ex9              |             | Chr20:57430201–57430258                |
| NESP55                 |             | Chr20:57464100–57464159                |
| NESP55                 |             | Chr20:57464278–57464282                |
| NESP55                 |             | Chr20:57464367–57464424                |
| NESP55                 |             | Chr20:57466769–57466826                |
| NESP55                 |             | Chr20:57468829–57468892                |
| NESP55                 |             | Chr20:57470713–57470779                |
| NESP55                 |             | Chr20:57474005–57474058                |
| NESP55                 |             | Chr20:57478577–57478646                |
| NESP55                 |             | Chr20:57480450–57480516                |
| NESP55                 |             | Chr20:57484181–57484241                |
| NESP55                 |             | Chr20:57484583–57484643                |

(Continued)
**STX16 deletions**

All samples from both families were screened for the 3-kb and 4.4-kb STX16 deletions by PCR and agarose gel electrophoresis followed by ethidium bromide staining, as previously described [10, 11], with slight modifications in the PCR conditions.

**Determination of the parental origin of the duplication in family GS0131**

D20S443, D20S459 and D20S1063 microsatellites, located inside the duplicated region (chr20:57381447–57701869), were genotyped by fluorescent PCR and capillary electrophoresis in order to study the inheritance of the alleles. Only D20S459 turned out to be informative (see Table 2). Quantitative multiplex PCR of short fluorescent fragments (QMPSF) was subsequently carried out for this marker together with amplicons from AIRE (21q22), HNF1A (12q24) and/or HNF1B (17q12) genes in a FAM-labeled multiplex PCR under semiquantitative conditions. Resulting PCR products were analyzed on an ABI3130xl Genetic Analyzer using GeneMapper v.4.0 software (Life Technologies). Data analysis was performed in Excel (Microsoft Office 2007).

**Results**

**Structural analysis of the GNAS locus**

As expected, neither patient GS0131 nor GS0155 present any point mutation along the GNAS gene.

**MS-MLPA**

Dosage analysis of the GNAS locus by MS-MLPA revealed that GS0131 carried a duplication which included the 23 probes corresponding to the region from NESP55 to TH1L (Fig. 2A, black bars). Methylation analysis with the same technique revealed a complete alteration of the maternal methylation pattern (loss of methylation at exon A/B, XLαs and NESPAS and a gain of methylation at exon NESP55) (Fig. 2B, black bars).

The MS-MLPA study of his parents revealed that the duplication occurs ‘de novo’ in GS0131, as we could not see any alteration occurring in either parent. Both their gene dosage and their methylation pattern were normal (Fig. 2C and 2D, upper panels).
In the second family, dosage analysis of the GNAS locus by MS-MLPA showed that GS0155 presented with a duplication affecting 10 probes from NESP55 to XLα, and not affecting exon A/B or the GSkα promoter (Fig. 2A, grey bars). Regarding methylation, she suffered a complete

Fluorescent PCR and genotyping results of the 27 polymorphic markers in chromosome 20q, situated in the table according to their chromosomal location.

Figures indicate the obtained size of the amplicons, or the number of pentanucleotide repeats, in the case of the A/B pentanucleotide marker. In all cases, first columns indicate paternally inherited alleles; the second columns indicate maternally inherited alleles (in the cases in which alleles are not directly informative, this inheritance has been assigned assuming both paternal alleles are present; see text for details). It was not possible to determine the inheritance of the single nucleotide variation (SNV) c.140–53. Bold figures indicate informative markers.

| Marker/Probe | Index | Mother | Father | Result                  | Index | Mother | Father | Result                  |
|--------------|-------|--------|--------|-------------------------|-------|--------|--------|-------------------------|
| D20S99       | 161   | 159    | 159    | 161 161                  | Excludes isodisomy               | GS0131 | | |                          |
| D20S857      | 208   | 208    | 208    | 216 208 214              | Excludes heterodisomy            | 198    | 216    | 216    | 198 216 216              | Informative               |
| D20S1044     | 212   | 212    | 212    | 212 212                  | Uninformative                    | 255    | 255    | 257    | 255 257 255              | Excludes heterodisomy     |
| D20S913      | 255   | 257    | 257    | 255 255                  | Informativne                      | 208    | 192    | 192    | 208 208 202              | Excludes isodisomy        |
| D20S468      | 207   | 207    | 207    | 207 207 201              | Excludes paternal heterodisomy    | 207    | 207    | 207    | 207 207 201              | Excludes paternal heterodisomy |
| D20S832      | 210   | 210    | 210    | 210 210 212              | Excludes paternal heterodisomy    | 207    | 210    | 210    | 210 210 212              | Excludes isodisomy        |
| D20S100      | 203   | 207    | 203    | 203 203                  | Excludes isodisomy               | 197    | 205    | 207    | 197 207 207              | Informative               |
| D20S746      | 253   | 253    | 253    | 253 253                  | Uninformative                    | 253    | 253    | 253    | 253 253 253              | Uninformative               |
| D20S88       | 445   | 431    | 431    | 443 445                  | Informative                      | 441    | 437    | 437    | 441 437 432              | Informative               |
| D20S102      | 180   | 182    | 182    | 180 180                  | Informative                      | 180    | 180    | 180    | 180 180 180              | Excludes paternal heterodisomy |
| D20S149      | 296   | 288    | 288    | 296 296 286              | Informative                      | 288    | 296    | 284    | 288 286 288              | Informative               |
| D20S158      | 388   | 392    | 392    | 388 388                  | Informative                      | 378    | 382    | 386    | 378 382 382              | Excludes isodisomy        |
| D20S430      | 204   | 224    | 224    | 230 204 204              | Informative                      | 204    | 204    | 216    | 204 216 208              | Excludes heterodisomy     |
| D20S451      | 314   | 304    | 304    | 322 314 318              | Informative                      | 304    | 314    | 296    | 304 296 304              | Informative               |
| D20S1063     | 202   | 202    | 202    | 202 203                  | Excludes paternal heterodisomy    | 202    | 202    | 202    | 202 202 202              | Uninformative               |
| D20S496      | 205   | 205    | 205    | 205 205                  | Uninformative                    | 205    | 205    | 205    | 205 205 205              | Uninformative               |
| AFMB332XD5   | 75    | 75     | 75     | 75 75 75                 | Uninformative                    | 75     | 75     | 75     | 75 75 75                 | Uninformative               |
| D20S459      | 237   | 231    | 231    | 235 237 231              | Excludes isodisomy               | 231    | 231    | 231    | 231 231 231              | Uninformative               |
| A/B Pentan   | 3     | 3      | 3      | 3 3 3                    | Excludes heterodisomy            | 2      | 3      | 3      | 3 3 3                    | Excludes isodisomy        |
| SNV (c.140–53) | C T C T C T                        | Excludes isodisomy               | C T T C C C                        | Excludes isodisomy               |
| rs7121       | C     | C      | C      | C C                       | Excludes maternal heterodisomy   | T      | T      | T      | T C C T C                | Excludes heterodisomy     |
| rs3730173    | C     | C      | C      | C C                       | Uninformative                    | T      | C      | C      | C C T C                   | Excludes isodisomy        |
| rs234628     | T     | T      | T      | C T                       | Excludes heterodisomy            | C      | T      | C      | C T C C                    | Excludes isodisomy        |
| D20S443      | 139   | 139    | 139    | 139 139                  | Excludes maternal heterodisomy   | 139    | 139    | 135    | 139 135 139              | Excludes heterodisomy     |
| D20S171      | 132   | 128    | 128    | 134 132 132              | Informative                      | 132    | 138    | 138    | 132 138 132              | Excludes isodisomy        |
| AFMa202yb9   | 243   | 243    | 243    | 243 243                  | Uninformative                    | 243    | 243    | 243    | 243 243 243              | Excludes paternal heterodisomy |
| D20S173      | 182   | 184    | 184    | 182 182                  | Excludes isodisomy               | 184    | 182    | 182    | 184 182 182              | Excludes isodisomy        |

Table 2. Genotyping results of the 27 polymorphic markers at 20q in both families.

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loss of methylation at exon A/B, a partial gain of methylation at exons XLα and NESPAS and a partial loss of methylation at NESP55 (Fig. 2B, grey bars).

In this case, the study of her parents revealed that GS0155 inherits the duplication from her mother, who also had 10 affected probes (Fig. 2C, lower panel, grey bars). GS0155’s mother...
also presents an altered methylation pattern (Fig. 2D, lower panel, grey bars), with a partial loss of methylation at exons XLαs and NESPAS (probes P9 to P15), and a partial gain at NESP55 (probes P6-P8). In this case, however, she does not have any epigenetic alteration in exon A/B (probes P16 and P17). GS0155’s father has normal dosage and methylation patterns (Fig. 2C and 2D, lower panels, black bars). As suggested by Mantovani et al [2], methylation status was validated by methylation-specific PCR (data not shown). MS-PCR confirmed the observed results in GS0131, and the complete loss of methylation at exon A/B in GS0155, while partial methylation results in the family, although apparent, were a little bit more difficult to interpret. We intend to perform pyrosequencing analysis of the methylation status in both families in the future, in order to triple check the obtained results.

Analysis of polymorphic markers

We then characterized twenty seven polymorphic markers in chromosome 20q to rule out the possibility of a uniparental disomy occurring simultaneously with any of both duplications, as it has been described for PHP-Ib [15]. Although several markers are not directly informative about their inheritance, some can individually exclude either parental isodisomies or heterodisomies, whereas the combination of all the results let us assume both paternal alleles where present in the index cases (Table 2).

Whole genome and 20q13-ter Comparative Genomic Hybridization (CGH) arrays

In order to check whether there was any big rearrangement in the genome of these families, we performed a whole genome aCGH analysis, which showed there was no other genomic alteration in either patient or their parents, apart from the 20q duplication. Besides, analysis of the parents confirmed that the duplication occurred ‘de novo’ in GS0131 and was inherited from the mother in GS0155.

We then focused on the chromosomal region 20q13, using a high-density array specific only for the chromosomal coordinates chr20:47897125–62435965. Using this customized array, we were able to determine the limits of both duplications: according to hg19, GS0131 presented a ~320 kb duplication, affecting chr20:57381447–57701869 (Fig. 3A), whereas GS0155 presented a ~179 kb duplication, affecting chr20:57281908–57461486 (Fig. 3B, upper panel). The presence of the same duplication was confirmed in GS0155’s mother (Fig. 3B, lower panel).

STX16 deletions

We discarded the presence in both families of the 3-kb and 4.4-kb STX16 deletions associated with A/B isolated loss of methylation [10, 11] (data not shown).

Determination of the parental origin of the duplication in family GS0131

In order to study the parental origin of the allele in which the duplication occurred in the GS0131 family, we carried out a QMPSF analysis of microsatellites inside the duplicated region. Only D20S459 turned out to be informative, revealing the duplication arose in the maternal allele (Fig. 4).
Fig 3. High density aCGH assay results for the 20q13 region of both families. In both cases, affected genes and regions mentioned in the text are highlighted in red on the X-axis. On the Y-axis, binary logarithm of the sample/control ratio. miR296 location is indicated in bold (corresponds to chr20:57392669–57392748). Red arrows indicate the location of the probes at each breakpoint. (A) Results for GS0131 index case. Won probes correspond to coordinates chr20:57381447–57381506. (B) Results of family GS0155 (upper panel, index case; lower panel, mother). Won probes correspond to coordinates chr20:57461436–57461486.

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Search of specific sequences

Trying to clarify the origin of both duplications, we searched within the sequences at breakpoints for different elements susceptible to being responsible for hypothetical non-allelic homologous recombination (NAHR) or non-homologous end joining (NHEJ), such as LINE or SINE elements, using RepeatMasker (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker); homologous sequences at the two ends of each duplication, using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or DNA palindrome Finder (http://www.alagu-molbio.net/palin.html) [28]. We were not able to localize any significant element in any of the patients.

Discussion

In this article we report the biggest duplications detected so far in the GNAS region, both present in two PHP-Ib patients and arising in the maternal allele.

We did not find any significant common element susceptible to be responsible for both rearrangements (i.e. specific sequences at the breakpoints, such as palindromes or SINE)
elements), so we cannot conclude whether this is a specific region prone to suffer these recombinations or both duplications are due to stochastic mechanisms.

The duplication in family GS0131 occurred ‘de novo’ in the patient. It was associated with a complete loss of methylation at the NESPAS, XLαs and A/B promoters. No other change was detected in the locus, either in the patient or in his parents. Paternal 20q disomies were also ruled out by polymorphic markers’ analysis.

This 320 kb duplication (chr20:57381447–57701869) also affected four genes located 3’ to the GNAS locus: TH1L (encoding NELFC and NELFD negative elongation factors), CTSZ (cathepsin Z), TUBB1 (tubulin beta-1 chain), and ATP5E (the epsilon subunit of the mitochondrial ATPase F1 complex). Mutations in these genes have been associated with macrothrombocytopenia (TUBB1, [29]), mitochondrial complex V (ATP synthase) deficiency nuclear type (ATP5E, [30]) or even tumour metastasis (CTSZ, [31]). As far as we know, there is not any report in the literature of any duplication in this region. Furthermore, at this point, the patient does not show any sign of these disorders, so the duplication he presents only seems to clinically affect the GNAS locus and its phenotypic manifestations, and the clinical significance of the overdose of the rest of the genes still needs to be elucidated.

In the same way, the duplication also affects miR-296 and miR-298, two microRNAs located in the GNAS region (Fig. 3A). These microRNAs have been described to target IKBKE and other genes regulating signaling pathways [32]. Moreover, they have been shown to have an imprinted expression, as they are paternally expressed and maternally repressed. In the case of GS0131, only the “silenced” maternal allele is duplicated, so the function of both miRNAs should not be affected, although further research should be performed to check this issue.

The second duplication, present in family GS0155, appeared both in the index case and her mother. It comprised 179 kilobases (chr20:57281908–57461486) and did not affect any other gene downstream GNAS, although both miR-296 and miR-298 and the NPEPL1 gene (located outside the GNAS locus on chr20:57264187–57294294) were also included in the affected region. In the case of the miRNAs, again, the duplicated “silenced” maternal allele is predicted not to affect their function, but this should be tested. On the other hand, NPEPL1 codifies for the protein known as “probable aminopeptidase NPEPL1”, whose exact function has not been reported so far, but it probably catalyzes the removal of unsubstituted N-terminal amino acids from various peptides. NPEPL1 proteins might function as tumor suppressors in tumorigenesis under the control of the miR-19a microRNA, although this has also not been consistently proved [33], so the effect of the overdose of this gene also remains elusive.

Duplications and deletions affecting other imprinted loci have also been reported. For instance, ICR1 and ICR2 in 11p15, associated with Silver-Russell and Beckwith-Wiedemann syndromes [34]. The interpretation of the copy number variation in this region is complicated, because the clinical features in carriers of these alterations depend on the size, breakpoint positions and the parental inheritance of the aberrations. Some interstitial microduplications have also been reported in the Prader-Willi and Angelman critical region, in 15q11-q13, also subject to imprinting. These duplications are the most common alteration in autism. In this case, maternal duplications are associated with autism, while paternal duplications usually have a normal phenotype [35].

Regarding the GNAS locus, as mentioned, few genomic rearrangements have been reported, and most of them are related to PHP-Ia. In 2005, Bastepe et al described the up-to-date only mentioned duplication in the GNAS locus, present in an AD-PHP-Ib kindred [13]. In that case, the duplicated region encompassed 15kb from the region between XLαs and A/B. Furthermore, the duplication was located in place of a 4kb deletion removing NESP55 DMR and NESPAS exons 3 and 4. The authors stated that, when inherited maternally, this deletion caused the loss of methylations of the DMRs normally methylated in the maternal allele.
Inherited paternally, no epigenetic alteration was observed. The phenotype observed in this patient was thus due to the disappearance of the NESP55 DMR rather than to the simultaneous duplication.

In our study, there is a maternal inheritance of the duplication in the index case in family GS0155, whereas methylation status of NESPAS, NESP55 and XLos invites to hypothesize that there is a paternal inheritance of the duplication in her mother (the partial loss of methylation at exons XLos and NESPAS, and partial gain at NESP55 agrees with a doubled paternal allele together with a unique maternal allele). Besides, the duplicated region should have undergone proper methylation. If this is the case, it could be speculated that only the maternal inheritance of the duplication would lead to the phenotypic manifestation of PHP-Ib, as the index’s mother is asymptomatic. Unfortunately, we did not have access to the grandparents’ samples in order to confirm this latter point. Besides, GS0155 index case also suffered a complete loss of methylation at the A/B promoter, which was not present in her mother (Fig. 2B and 2D). Therefore, we hypothesize that two different mutational events could have coincided in the index case: the first one, a duplication of a region in the locus that provoked the methylation level change, inherited from her mother. The presence of two maternal alleles would lead to the observed proportional increase in methylation levels at NESPAS and XLos and the decrease in NESP55. The second mutation, if present, would be responsible for the isolated total loss of methylation of the A/B promoter in the patient. For this reason, we tested for the presence of the already described 3-kb and 4.4-kb STX16 deletions associated with A/B isolated loss of methylation [10, 11] in this region, not affected by the duplication, but the analysis turned out to be negative (data not shown). We then performed a high-density cGH array analysis in the 20q region in order to be able to find any small deletion inside the duplication capable of causing an isolated loss of A/B methylation, as previously described [15] but, again, we were unable to detect any second alteration. Thus, this apparent isolated loss of A/B methylation remains unexplained. It is possible that the structure of the locus itself, together with the duplication of the maternal allele make it difficult to detect this deletion, if it exists. Or, maybe, there is another cis-acting element (or elements), still not described, controlling the methylation status of the different promoters, located in the region comprising chr20:57381447–57461486 (the common elements to both duplications) whose duplication in the maternal allele may cause a change in the methylation pattern of the region, either in a broad way, as it occurs in GS0131, or just in the A/B promoter, as it happens in GS0155.

Anyway, further studies are required in order to characterize the GNAS locus and the 20q region, as its imprinting pattern can be modified either by small deletions, small duplications or, as in our cases, by big duplications, which indicates that maybe there are several still unknown modifier agents which take part in the establishment or maintenance of the methylation of the different promoters in the region.

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Author Contributions
Conceived and designed the experiments: GPN LC. Performed the experiments: GPN TV. Analyzed the data: GPN TV LC. Contributed reagents/materials/analysis tools: AV MMT LC. Wrote the paper: GPN TV AV MMT LC. Clinically studied the patients: AV MMT.
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