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Similar frequency of paternal uniparental disomy involving chromosome 20q (patUPD20q) in Japanese and Caucasian patients affected by sporadic pseudohypoparathyroidism type Ib (sporPHP1B)

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INTRODUCTION

Pseudohypoparathyroidism (PHP) is characterized by resistance to parathyroid hormone (PTH) in proximal renal tubules that leads to hypocalcemia and hyperphosphatemia (1). Different PHP variants have been recognized, which are all associated with loss or severely reduced expression of the alpha-subunit of the stimulatory G protein (Gsα) in this portion of the kidney thus causing impaired signal transduction of PTH and other hormones via the cAMP/PKA signaling pathway (2, 3). Gsα is encoded by *GNAS* located on the long arm of

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chromosome 20 (20q13.3), a complex imprinted locus that generates multiple sense and antisense transcripts. Through the utilization of alternative first exons and promoters, *GNAS* furthermore gives rise to several additional transcripts. These include the A/B transcript, which may encode an amino-terminally truncated form of Gsα (4) and non-coding antisense transcripts (AS) (5, 6), as well as transcripts encoding the extra-large Gαs variant (XLαs) and a 55-kDa neuroendocrine secretory protein (NESP55).

Patients affected by PHP type Ia (PHP1A) show resistance to several hormones that mediate their actions through G protein-coupled receptors and display various features of Albright's Hereditary Osteodystrophy (AHO), including short stature, round face, obesity, brachydactyly, ectopic ossifications, and/or various degrees of mental retardation (1). PHP1A is caused by inactivating heterozygous mutations involving one of the 13 *GNAS* exons or introns on the maternal allele. Subjects presenting with certain AHO features, but without hormonal resistance, obesity, and mental abnormalities, are classified as pseudopseudohypoparathyroidism (PPHP). This disorder is also caused by mutations affecting Gsα, but these are located on the paternal *GNAS* allele, rather than the maternal allele as in PHP1A (7).

Resistance toward PTH in the proximal renal tubules occurs also in PHP type Ib (PHPIB). These patients can furthermore show resistance towards other hormones, particularly towards TSH, and they may present with AHO features that can be indistinguishable from those observed in PHP1A (8–11). PHP1B is not caused by *GNAS* mutations involving the region encoding Gsα, but instead by loss-of-methylation (LOM) at *GNAS* exon A/B located within a differentially methylated region (DMR) (7). LOM can also be observed at additional *GNAS* exons, namely AS and XL, which is usually associated with a gain-of-methylation (GOM) at *GNAS* exon NESP.

The autosomal dominant (AD) form of PHP1B (AD-PHP1B) can be caused by maternal heterozygous deletions in *STX16* (3-kb, 4.4-kb, and 24.6-kb deletions), the gene encoding syntaxin-16 located approximately 220 kb upstream of *GNAS* exon A/B (12). These *STX16* deletions are associated with LOM affecting only *GNAS* exon A/B (12–14), which leads through unknown mechanisms to a reduction or loss of Gsα expression from the maternal allele. Indistinguishable LOM affecting only *GNAS* exon A/B occurs also with a maternal deletion comprising exon NESP and the upstream region (15).

AD-PHP1B can also be caused by maternally inherited deletions involving NESP and/or AS, which are associated with loss of all maternal *GNAS* methylation imprints (16, 17). Similarly broad methylation changes are also observed in most sporadic PHP1B (sporPHP1B) patients, but the molecular mechanism underlying these epigenetic changes remains unclear for the majority of these patients. In a few sporPHP1B patients the disease is caused by paternal uniparental isodisomy involving chromosome 20q (patUPD20q), which includes the *GNAS* locus (18–22). Such chromosomal rearrangements have not yet been described for the Japanese population. We therefore investigated 23 Japanese sporPHP1B patients, all of whom showed broad methylation changes involving all four DMRs of the *GNAS* locus, which led to the identification of two patients with patUPD20q. We furthermore mapped the known duplications and assessed the frequency of UPD20q

based on this and previous reports, which will help guiding the evaluation and genetic counseling of sporPHP1B patients.

MATERIALS AND METHODS

Patients and healthy family members

We investigated 23 Japanese sporPHP1B cases, who had presented with PTH-resistant hypocalcemia and hyperphosphatemia, and mild AHO features in some; clinical and laboratory information of most of these patients was previously reported (23). None of the available parents, siblings, and children showed abnormalities in calcium and phosphate homeostasis. Clinical features, biochemical results, and epigenetic findings for each patient are presented in Table 1.

Molecular studies

The study was approved by the Ethics Committee of Chiba University and the Massachusetts General Hospital. Genetic analyses were performed after obtaining informed consent from the patient or parents. Genomic DNA was extracted from peripheral blood leukocytes, as described (23).

Methylation analysis

Southern blot analysis and methylation specific-polymerase chain reaction (MS-PCR) were performed, as described (23). Multiplex ligation-dependent probe amplification (MLPA) and methylation specific-MLPA (MS-MLPA) were performed using the SALSA MLPA kit ME031 GNAS (MRC-Holland, Amsterdam, The Netherland) following the manufacturer's instructions. Analysis of the PCR products was performed on an ABI3130 genetic analyzer and using the GeneMapper Software (Applied Biosystems) at the DNA Core Facility of the Massachusetts General Hospital.

Analysis of SNPs and microsatellite markers

The PCR to search for single nucleotide polymorphism was performed with QIAGEN Taq DNA polymerase and the other reagents supplied with the same kit following the manufacture's protocols. PCR primers are listed in Supplemental Table 4. The PCR products were purified using ExoSap-IT (Affymetrix) and sequenced at the DNA Core Facility of the Massachusetts General Hospital. Analysis of microsatellite markers across the entire chromosome 20 was performed by the Center for Human Genetic Research of the Massachusetts General Hospital.

RESULTS

Laboratory and clinical findings in our cohort of sporPHP1B patients

We investigated a total of 23 Japanese subjects with sporPHP1B; sixteen of these patients were recently described (23), while the additional patients had previously not been reported (see Table 1). Parents and available siblings of our patients have/had no mineral ion abnormalities. All 23 patients showed, when first diagnosed, hypocalcemia and hyperphosphatemia associated with a significant increase in serum PTH levels. Seven

patients also had elevated TSH levels. Ten patients presented with mild AHO features. Taken together, these findings in our cohort of sporPHP1B cases were similar to those observed by us (23,24) (Table 2) and others (8,9,11,21).

GNAS methylation status and search for deletions in *GNAS* and *STX16*

MS-MLPA of genomic DNA of all patients revealed broad *GNAS* methylation changes (see Table 1) that are indistinguishable from those previously reported by us (23, 24) and others (8,9,11,21). MLPA provided no evidence for an allelic loss within *GNAS* or *STX16*; the 3-kb deletion in *STX16*, which is the most frequent cause of AD-PHP1B (24–26), was furthermore excluded in all 23 patients by PCR analysis, as previously described (12). The child of patient P24 is reportedly healthy and showed no epigenetic change at the *GNAS* locus.

Analysis of SNPs and microsatellite markers at the *GNAS* locus

Paternal uniparental disomy of chromosome 20q (patUPD20q) and the associated methylation changes at all four *GNAS* DMRs had provided a molecular explanation for some of the sporPHP1B patients (18–22). Most of these cases had revealed isodisomy rather than heterodisomy, and we therefore first analyzed three frequent SNPs (rs1800900, rs1800905, and rs138461295) and the pentanucleotide repeat polymorphism at *GNAS* exon A/B (309F20-GGCGC) (Fig. 1 and Suppl. Table 1) to explore the possibility of patUPD20q in our cohort.

Eight patients were heterozygous for two or more of these variants, making a duplication of the paternal chromosome 20q unlikely. Eleven patients were homozygous for the four SNPs within *GNAS* and four patients were heterozygous for one of these variants. The latter fifteen individuals were therefore analyzed further through the analysis of six polymorphic microsatellite markers surrounding the *GNAS* locus (see Fig. 1 and Suppl. Table 2). Two of these individuals revealed homozygosity for all six markers raising the possibility of patUPD20q.

Analysis of patients and parents through microsatellite markers across the entire chromosome 20

We furthermore analyzed numerous polymorphic markers across the entire chromosome 20 for patient P44 and both of her parents, as well as for patient P18 and her mother (her father is deceased). The studies revealed paternal isodisomy involving the entire long arm of chromosome 20, yet showed bi-parental inheritance for the short arm of chromosome 20. Furthermore, MLPA revealed no evidence for an allelic loss in the *STX16-GNAS* region leading us to the conclusion that both sporPHP1B cases are affected by patUPD20q (Fig. 2). The non-identical healthy twin brother of P44 showed no evidence for *GNAS* methylation abnormalities, as determined by MS-MLPA. Analysis of microsatellite markers 907-rep2, 261P9-CA, and D20S171 furthermore excluded patUPD20q; other markers were not informative (data not shown). Analysis of these and additional polymorphic markers provided no evidence for patUPD20q for the other investigated patients.

DISCUSSION

Our Japanese sporPHP1B cases affected both sexes equally, and their ages and laboratory abnormalities at diagnosis were not significantly different from those previously reported by us (see Table 2) and others (8,9,11) for Caucasian sporPHP1B cases. Furthermore, no significant differences in clinical and laboratory findings were observed for the two patUPD20q patients presented herein and other previously reported cases with duplications involving the long arm of chromosome 20 (see Table 3). Patients with patUPD comprising the long arm of chromosome 20, the entire chromosome, and only segments of chromosome 20q showed no significant differences with regards to age at disease onset as well as levels of PTH, calcium, and phosphate at presentation. This suggests that no another imprinted gene or functionally relevant polymorphisms on chromosome 20 contributes to mineral ion homeostasis.

Dixit *et al.* had proposed a novel phenotype related to PHP1B due to patUPD20q, namely a relatively high birth weight and obesity, which was noticed during infancy and persisted until later in life (22). Moreover, macrocephaly and tall stature had been observed as possible additional changes (20). However, at the ages of 7 and 12 years, respectively, our two patUPD20q patients were only slightly above average in height and neither was obese (Fig. 2). Besides *GNAS*, the long arm of chromosome comprises only one other imprinted gene, namely *NNAT* (20q11.2–q12) encoding neuronatin. Loss of this paternally expressed gene has been implicated in obesity (35;38–41), while biparental *NNAT* expression as in patUPD20q does not seem to be associated with changes in weight. However, six of seven previously reported patients with patUPD20q comprising the *NNAT* region were taller than average, while three patients whose UPD regions do not extend to this locus were below average for height (see Table 3). This could imply that *NNAT* contributes to growth.

UPD is the state in which a chromosomal region or segment is inherited only from a single parent. The duplicated region may vary from segmental (interstitial or telomeric) to an entire chromosome. Several mechanisms resulting in the formation of UPD have been proposed, including monosomy rescue, trisomy rescue, gamate complementation, and post-fertilization errors (27). Clinically relevant consequences resulting from UPD include, besides trisomic mosaicism, genomic imprinting disorders and homozygosity for a recessive mutation, or a combination of both latter conditions. For example, a homozygous mutation in the adenosine deaminase was recently shown to lead to severe combined immunodeficiency because of a paternal duplication of the entire chromosome 20, which most likely caused PHP1B besides ADA-SCID (28).

UPD is a very rare event with an estimated frequency in newborns of 0.029% (29). More frequently, UPD is observed as a cause of imprinting disorders. For example, Silver-Russel-Syndrome (SRS) is caused in 5–10% of the cases by matUPD7 (30, 31), while 3–5% of patients with Angelman syndrome (AS) showed patUPD15 (32) and 20% of patients with Prader-Willi syndrome (PWS) revealed matUPD15 (33). Furthermore, patUPD6 was detected in 41% of the patients with Transient Neonatal Diabetes Mellitus (TNDM) (34) and segmental patUPD11p accounted for 20% of the cases with Beckwith-Wiedemann syndrome (BWS) (35). In addition, it appears plausible that matUPD15 causes some forms

of central precocious puberty since mutations in the imprinted gene MKRN3 were found only in 15 out of 32 investigated patients (36).

Since the first description of patUPD20q as a cause of sporPHP1B (18), an additional 10 patients with this disorder have been reported (19–22), including those described in this manuscript. Most of these cases (6 out 11) revealed duplication of only one paternal long arm or the entire chromosome 20 (isodisomy), although the boundaries of the duplication were not always conclusively defined (see Fig. 1). The remaining sporPHP1B cases had smaller duplications involving chromosome 20q, including a duplication of only 7.6 Mb (20q13.31–q13.32) (22). Only one previous report had provided evidence for heterodisomy involving the short arm of chromosome 20 that was combined with interstitial isodisomy affecting chromosome 20q (20); this very infrequent cause of UPD could not be explored in the current study because parental DNA was not available for most patients.

The *GNAS* methylation changes for our patients with patUPD20q were indistinguishable from those of other reported cases with different extents of UPD. This indicates that a maternal segment that is no longer present within the duplicated 4.6 Mb region contains regulatory elements that allow establishment or maintenance of the normal methylation imprints at *GNAS.* Because of the telomeric boundaries of the duplicated region could not be conclusively defined in four cases, it remains uncertain whether the size of the genomic *GNAS* region that is critical for methylation could be smaller (see Fig.1).

We had previously reported 22 sporPHP1B patients, one of whom was later shown to have a duplication of the entire chromosome 20 (24, 19). Fernandez-Rebollo *et al.* identified patUPD20q in four out of twenty sporPHP1B patients (20), while Jin *et al.* observed patUPD20 in one out of seven Korean sporPHP1B cases (21). We now found two additional patUPD20q cases in our cohort of 23 Japanese sporPHP1B patients, suggesting that about 10% of all sporPHP1B cases may be caused by duplication of the paternal long arm of chromosome 20, and that all racial backgrounds are equally affected.

In comparison to the large duplicated regions identified in patUPD20 patients to date, about 20% of all patients affected by BWS revealed small duplicated segments in the 11p15.5 region that can be as small as 2.7 Mb (35, 42). Some of these patUPD11p patients were shown to be mosaic implying that postzygotic recombination events had occurred (35, 43, 44), i.e., a mechanism different from that for non-mosaic UPDs involving large chromosomal regions. It is conceivable that similarly small paternally duplicated regions comprising the *GNAS* locus can be a cause of some sporPHP1B cases or that mosaicism involving the chromosome 20q13 region could explain this disease variant.

In conclusion, two patients with patUPD20q were identified among 23 Japanese sporPHP1B cases. When combined with data from previous reports, these findings suggest that duplication of the paternal long arm of chromosome 20 may be a more frequent cause of sporPHP1B than initially thought and that patUPD20q should be considered in all sporPHP1B cases with broad *GNAS* methylation changes. Establishing patUPD20q would provide a molecular definition of their disease thus allowing appropriate genetic counseling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Pseudohypoparathyroidism type Ib (PHP1B) is caused by proximal tubular resistance to parathyroid hormone.
- In few sporPHP1B patients the disease is caused by paternal uniparental isodisomy involving chromosome 20q (patUPD20q).
- We investigated 23 Japanese sporadic PHP1B cases to determine whether patUPD20q can be their cause of PHP1B.
- PatUPD20q was confirmed for two patients.
- Paternal duplication of the chromosomal region comprising the *GNAS* locus appears to be a relatively common cause of sporPHP1B.

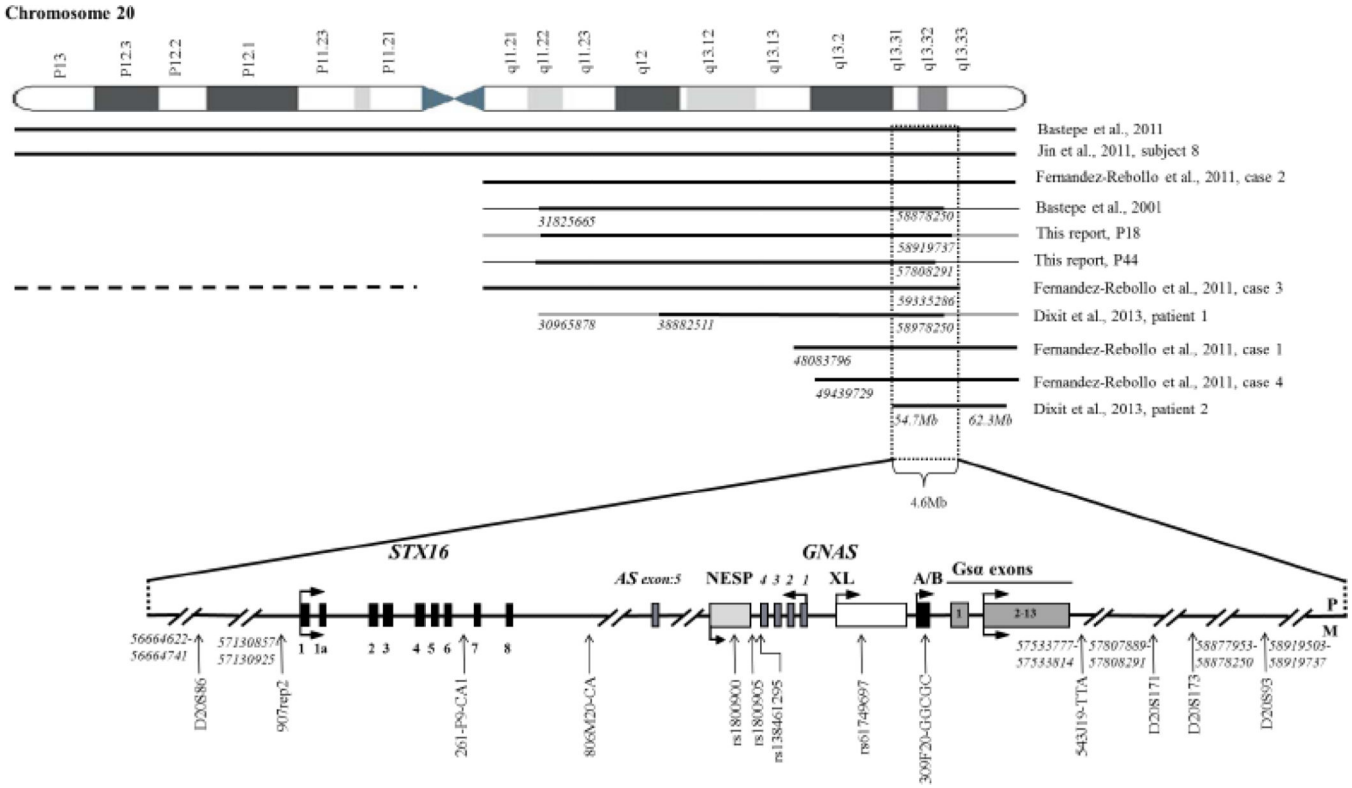
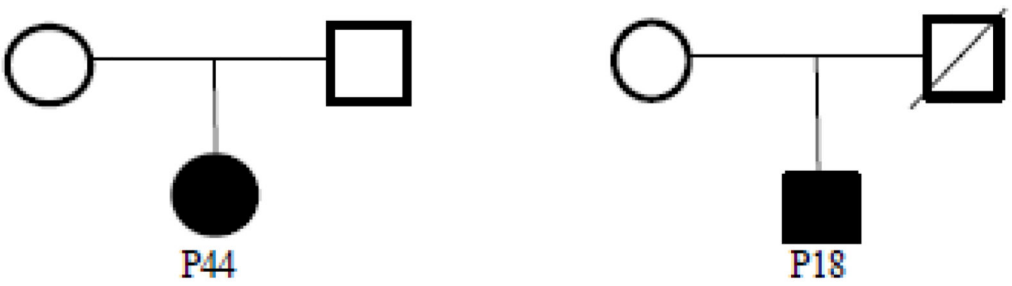


Fig. 1.

Schematic representation of the region on chromosome 20q extending from the *GNAS* locus to syntaxin 16 (*STX16*). Each horizontal thick line shows the extent of paternal uniparental isodisomy for the patients described in this report and previously described individuals. The region not conclusively defined as UPD is shown by a thin horizontal line. A horizontal broken line shows the position of the paternal heterodisomy of chromosome 20p. The two dotted vertical lines delineate the smallest region in which duplication of the paternal chromosome 20q may lead to sporadic PHP1B. Exons are indicated by boxes, introns by lines; arrows show direction of transcription. P, paternal; M, maternal. Numbers in *italic* were provided by hg19 (GRCh37) assembly. For the patient described in Bastepe et al., 2001, two additional microsatellite markers were analyzed (see Supplemental Table 3).



	P44						P18				
Age (years)			7						12		
Height (cm)			125.0 (+1.2 SDS)						160.7 (+1.5 SDS)		
Weight (kg)			27.3 (+1.2 SDS)						48.0 (+0.6 SDS)		
D20S117	180	184	184	178	178	172	182	180	180	151	20p
D20S115	238	238	238	238	238	238	238	238	238	238	
D20S186	122	138	138	128	128	126	126	132	132	124	
D20S112	224	226	226	220	220	222	220	224	224	222	
D20S195	146	146	**142**	**142**	**142**	146	140	146	**142**	**142**	20q
D20S107	212	214	**210**	**210**	**210**	216	216	216	**208**	**208**	
D20S119	117	117	**113**	**113**	**113**	113	109	111	109	109	
D20S178	183	189	**185**	**185**	**185**	189	183	187	183	183	
D20S196	284	286	**263**	**263**	**263**	263	284	286	286	286	
D20S86	315	327	315	315	315	315			327	327	
907-rep2	210	206	210	210	210	194	194	218	**214**	**214**	
261P9-CA	216	210	**212**	**212**	**212**	216	216	216	**210**	**210**	
806M20-CA	196	196	196	196	196	196	198	198	198	198	
543J19-TTA	156	149	149	149	149	149	149	156	149	149	
D20S171	140	144	**138**	**138**	**138**	140	140	146	**144**	**144**	
D20S93	324	328	328	328	328	328	328	340	336	336	

Fig. 2.

Microsatellite analysis of pedigrees P44 and P18: Affected individuals are represented by filled symbols; the parents are depicted by open symbols (circles=females; squares=male). Results for each microsatellite marker are shown below both pedigrees. **Bold** numbers indicate fully informative markers for the region with paternal UPD. Identical alleles have the same gray background colors.

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Table 1

Laboratory and epigenetic findings for the investigated PHP1B patients

				Serum biochemical values at diagnosis						MS-MLPA					
Code	**Sex**	**Age at diagnosis (years)**	**symptoms**	**Calcium (mg/dL) (8.5–10.2)**	**Phosphate (mg/dL) (2.4–4.3)***	**intact PTH (pg/mL) (10–65)**	**ALP (IU/L) (115–359)**	**1,25(OH)2D (pg/mL) (20–60)**	**TSH (μU/mL) (0.5–5)**	**AHO features**	**NESP**	**AS**	**XL**	**A/B**	**References**
P3	Male	14	none	7.8	4..8	129	493	N/D	1.5	−	+	−	±	−	23
P7	Male	6	loc, convulsion	4.6	10.3	347	N/D	45.6	5.9	RF	+	−	−	−	23
P8	Male	3	convulsion	5.2	6.8	720	681	22.0	4.8	Ob	+	−	±	−	23
P9	Female	9	loc	6.3	7.0	870**	258	N/D	2.4	Ob	+	−	±	−	23
P13	Female	6	convulsion	6.0	6.6	288	325	24.1	2.8	−	+	−	±	−	23
P18	Male	12	muscle cramp	4.8	9.1	139	479	23.6	1.4	−	+	−	−	−	23
P20	Male	11	convulsion	7.8	9.6	137	833	46.1	N/D	−	+	−	−	−	23
P21	Female	12	convulsion	5.7	9.1	1600**	451	31.0	2.3	Ob, RF, MR	+	−	−	−	23
P23	Female	8	convulsion	6.7	8.2	134	579	41.3	5.9	Ob, RF	+	−	−	−	23
P24	Female	35	tetany	5.3	5.2	96	237	24.5	4.4	Ob	+	−	±	−	23
P25	Male	8	convulsion	7.1	8.6	340	784	51.2	8.4	RF	+	−	−	−	23
P27	Female	12	convulsion	5.4	9.5	360	711	59.1	N/D	RF, SM	+	−	−	−	23
P29	Male	15	convulsion	4.8	7.2	190	1084	60.8	2.1	−	+	−	−	−	23
P32	Male	9	none	6.7	7.4	360	N/D	49.2	1.6	−	+	−	−	−	23
P40	Female	9	headache	8.3	5.9	480	727	47.3	2.7	−	+	−	−	−	23
P44	Female	7	convulsion	7.6	9.3	330	779	50.7	5.8	RF	+	−	−	−	
P46	Male	4	convulsion	7.0	7.5	300	1613	N/D	4.4	−	+	−	−	−	
P52	Male	6	convulsion	6.4	8.5	349	726	60.5	3.3	−	+	−	±	−	
P54	Male	13	convulsion	5.7	9.4	118	926	5.9	N/D	−	+	−	−	−	23
P55	Male	4	convulsion	low	high	high	N/D	N/D	7.2***	−	+	−	±	−	
P56	Male	10	N/D	5.2	6.3	354	N/D	N/D	N/D	N/D	+	−	−	−	
P57	Female	5	N/D	7.3	7.6	473	918	44.1	16.5	RF	+	−	−	−	
P58	Male	25	loc, tetany	7.2	5.2	457	263	13.0	1.5	−	+	−	−	−	

*adult normal range;

** elevated PTH was documented by a mid-PTH assay (normal range: 90–270 pg/mL), if the intact PTH assay was not available;

*** data at the age of 14 years during treatment with alfacalcidol; MS-MLPA, Methylation-Specific Multiplex Ligation-dependent Probe Amplification; N/D, not determined; loc, loss of consciousness; RF, round face; Ob, Obesity; MR, mental retardation; SM, short metacarpals

Table 2

Age at diagnosis and laboratory findings in our Japanese cohort of sporadic PHP1B patients in comparison to our previously reported data for Caucasian patients.

	Age at diagnosis (years)			Calcium (mg/dl)			Phosphate (mg/dl)			PTH (pg/ml)	
	sporPHP1B Linglart et al., 2007	sporPHP1B this report		sporPHP1B Linglart et al., 2007	sporPHP1B this report		sporPHP1B Linglart et al., 2007	sporPHP1B this report		sporPHP1B Linglart et al., 2007	sporPHP1B this report
Median	10.0	9.0	#NUM!	5.7	6.4	#NUM!	8.0	7.6	#NUM!	402	335
Mean	10.0	10.6	#DIV/0	6.0	6.3	#DIV/0	8.2	7.7	#DIV/0	634	305
SD	4.61	6.98	#DIV/0	1.17	1.08	#DIV/0	1.35	1.56	#DIV/0	742.9	154.3
SEM	0.98	1.45	#DIV/0	0.25	0.23	#DIV/0	0.29	0.33	#DIV/0	158.4	34.5
N	22	23	0	22	22	0	22	22	0	22	20

Table 3

Age at diagnosis, height, and laboratory findings in patients with sporPHP1B due to different forms of patUPD20

case	Type of patUPD20q	Sex	Age at diagnosis (years)	Serum biochemical values at diagnosis			
				Calcium (mg/dL) (8.5–10.2)	Phosphate (mg/dL) (2.4–4.3)	intact PTH (pg/mL) (10–65)	TSH (μU/mL) (0.5–5)
Bastepe et al., 2011	entire chromosome	Female	3.5	7.2	8.0	3685	N/D
Jin et al., 2011	entire chromosome	Male	8	5.6	5.6	677	7.2
Bastepe et al., 2001	entire q arm	Male	5	7.2	8.1	113	N/D
Fernandez-Rebollo et al., case 2	entire q arm	Male	9	8.8	6.5	940	2.0
this report, P18	entire q arm	Male	12	4.8	9.1	139	1.4
this report, P44	entire q arm	Female	7	7.6	9.3	330	5.8
Fernandez-Rebollo et al., case 3	segmental 20p heterodisomy + 20q interstitial isodisomy	Male	5	4.8	7.4	292	2.8
Fernandez-Rebollo et al., case 1	*Segmental 20q13.13-qter*	*Female*	*26*	*5.2*	*5.9*	*601*	*N/D*
Fernandez-Rebollo et al., case 4	*Segmental 20q13.13-qter*	*Male*	*46*	*6.4*	*4.6*	*127*	*1.2*
Dixit et al., patient 1	*Segmental 20q12–q13.33*	*Male*	*13*	*5.0*	*9.5*	*345*	*N/D*
Dixit et al., patient 2	*Segmental 20q13.31–q13.32*	*Male*	*5.5**	*9.0**	*6.1**	*422**	*35.0*

Patients without duplication of the region comprising NNAT are listed in italics.

* treatment with cholecalciferol commenced