Association of GNAS imprinting defects and deletions of chromosome 2 in two patients: Clues explaining phenotypic heterogeneity in pseudohypoparathyroidism type 1B/iPPSD3

2q37 deletions in patients with Albright hereditary osteodystrophy phenotype and PTH resistance.

Elli FM[1], de Sanctis L[3], Madeo B[4], Maffini MA[1], Bordogna P[2], Pirelli A[1], Arosio M[1,2], and Mantovani G[1,2].

[1]University of Milan, Department of Clinical Sciences and Community Health, Milan, Italy;

[2]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Endocrinology Unit, Milan, Italy;

[3]Department of Public Health and Pediatric Sciences, University of Torino, Regina Margherita Children's Hospital-AOU Città della Salute e della Scienza, Torino, Italy.

[4]Unit of Endocrinology, Department of Internal Medicine, Endocrinology, Metabolism, and Geriatrics, Azienda Ospedaliero-Universitaria di Modena, Ospedale Civile di Baggiovara, Modena, Italy.

Corresponding author: Giovanna Mantovani, MD, PhD

Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Unità di Endocrinologia

Via Francesco Sforza, 35 - 20122 Milano, Italy.

Phone: +39 02 50320607; FAX: +39 02 50320605; Email: giovanna.mantovani@unimi.it

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Abstract

Pseudohypoparathyroidism (PHP) is a rare endocrine disorder deriving from a defective activation of the cAMP pathway by the parathyroid hormone (PTH) secondary to GNAS molecular defects. PHP subtypes were defined due to the presence/absence of specific clinical/biochemical features. PHP1A is characterized by resistance to multiple hormones with features of Albright hereditary osteodystrophy (AHO), while PPHP by AHO in the absence of PTH resistance. Small subsets of PHP and PPHP patients without known molecular defects have been re-diagnosed as affected by the brachydactyly-mental retardation syndrome (BDMR), also known as AHO-like syndrome.

This study aimed to analyze 24 PHP1A and 51 PPHP patients without molecular diagnosis for the presence of BDMR-associated 2q37 deletions to improve the differential diagnosis and to find features that might help avoiding a misdiagnosis.

Molecular investigations identified 4 deletions in 4 unrelated patients. The smallest region of overlap, containing gene/s potentially involved in the clinical presentation, removed about 3.5Mbp hosting 38 genes. Affected patients showed a combination of the most pathognomonic AHO features. Of note, 3 of them displayed also mild PTH resistance and none of them developed ectopic ossifications.

Our work confirmed the rarity of misdiagnosis of BDMR in PHP subjects by the identification of 4 patients bearing a 2q37 deletion in a cohort of 73 PHP patients (5.3%). Three deleted patients presented a PHP1A phenotype in the absence of any BDMR-specific findings. Further studies on larger case series are needed to elucidate the overlap between these clinical entities and to allow an early identification of patients.

Introduction

Pseudohypoparathyroidism (PHP) is a rare endocrine disorder deriving from a defective activation of the cAMP transduction pathway by the parathyroid hormone (PTH) secondary to molecular defects affecting the alpha subunit of the stimulatory G protein (Gsα), encoded by the *GNAS* gene [1]. Since the first description in 1942, several PHP subtypes (PHP type 1A/PHP1A - MIM#103580, Pseudopseudohypoparathyroidism/PPHP - MIM#612463, PHP type 1B/PHP1B - MIM#603233, PHP type 1C/PHP1C - MIM#612462, PHP type 2/PHP2 – MIM#203330 and POH - MIM# 166350) were defined due to the presence/absence of specific clinical/biochemical features and to the presence/absence of underlying genetic, further divided into maternal or paternal, or epigenetic molecular GNAS defects [2-4]. PHP1A is the clinical entity characterized by resistance to multiple hormones, mainly PTH and TSH, with features of Albright hereditary osteodystrophy (AHO) [3]. It is associated with *GNAS* genetic defects, both point mutations and deletions, on the maternal allele. PPHP is defined as AHO in the absence of PTH resistance and is associated with paternal GNAS genetic alterations [5].

Research studies on these diseases allowed the identification of both clinical and molecular overlap among subtypes and with closely related disorders deriving from alterations of elements involved in the cAMP transduction pathway different from the Gsα, making the classification obsolete [4, 6]. A novel nomenclature and classification including all disorders of the PTH/PTHrP receptor signaling pathway has been recently proposed and the new coined name is Inactivating PTH/PTH-rP signaling disorders (iPPSDs) [7]. One of the strengths of the new classification is to provide a category, iPPSDx, also for those patients lacking a known genetic/epigenetic molecular determinant [8].

In the past years, small subsets of clinically defined PHP and PPHP patients without known molecular defects have been re-diagnosed as affected by the brachydactyly-mental retardation

syndrome (BDMR, also known as AHO-like syndrome or 2q37 microdeletion syndrome, MIM#600430) as they carried deletions with breakpoint at or within chromosome 2 region q37 [9].

The BDMR syndrome is characterized by a spectrum of clinical features with different penetrance, including the AHO-like phenotype, with mild-moderate intellectual /developmental / behavioural abnormalities, short stature, obesity, characteristic facies and brachydactyly type E [10].

In the present study we screened 24 PHP1A and 51 PPHP patients without known genetic and epigenetic defects (hereafter, patients will be referred to as iPPSDx according with the novel proposed classification) for the presence of BDMR-associated 2q37 deletions to improve the differential diagnosis of iPPSDs and to find features that might help avoiding a misdiagnosis.

Methods

The study included 73 iPPSDx patients, defined as patients with a previous clinical diagnosis of PHP1A (n=24) and/or PPHP (n=49) without known molecular defects.

According to the recent classification of iPPSDs, clinical and biochemical major criteria for PHP and related disorders are PTH resistance, and/or subcutaneous ossifications, and/or brachydactyly type E. Minor criteria include TSH resistance, other hormonal resistances, motor or cognitive impairment, intrauterine and/or postnatal growth retardation, obesity/overweight and flat nasal bridge and/or maxillar hypoplasia and/or round face. Brachydactyly type E, being associated also with other diseases, requires to be combined with at least one more major or 2 minor criteria to establish the diagnosis of iPPSD [7, 8].

Among our whole series of iPPSDx patients, in the present study we selected patients presenting with either at least 2 major criteria or 1 major criterion plus at least 2 minor criteria. This means that we selected those patients with AHO major signs with or without hormone resistance. Clinical

details of the investigated cohort are resumed in Supplementary Table 1. All subjects involved in the study subscribed the informed consent for genetic and epigenetic studies.

Previous investigation of patient's genomic DNA samples, extracted from peripheral blood (Nucleon BACC2 genomic DNA purification kit, GE Healthcare, Piscataway, NJ, USA), by direct sequencing of *GNAS*, *PRKAR1A* and *PDE4D* coding exons and flanking intronic sequences (ENSEMBL reference sequence ENSG00000087460, ENSG00000108946 and ENSG00000113448, respectively) and *PDE3A* exon 4 (ENST00000359062), as well as methylation specific-multiplex ligand-dependent probe amplification (MS-MLPA) of the GNAS locus (ME031 GNAS probemix by MRC-Holland, Amsterdam, The Netherlands) did not reveal any (epi)genetic abnormality associated with iPPSDs [11-13].

The presence of 2q37 deletions was assessed by MLPA using the P264-Human Telomere-9 (MRC-Holland, Amsterdam, The Netherlands). MS-MLPA and MLPA data analysis was performed by the Coffalyser software (MRC-Holland, Amsterdam, The Netherlands).

The 2q37 variable number tandem repeats (VNTRs) genotyping was used to confirm deletions found by MLPA. Briefly, genetic markers were PCR-amplified and, after capillary electrophoresis on the ABI3130xl Genetic Analyzer, the peak pattern was evaluated with the Peak Scanner software (Applied Biosystems, Foster City, CA). We used a three primers approach based on the simultaneous use of a couple of sequence-specific primers associated with a fluorescently labelled universal forward M13 tailed FAM oligonucleotide. All primers and experimental conditions are available upon request.

Results

The MLPA analysis of the 2q37 region identified 4 deletions in 4 unrelated patients. The assay showed a 3Mb deletion from the *HDAC4* gene to the *PDCD1* gene in patient 2, a 3,5-4,5Mb

deletion from the *TRAF3IP1* gene to the *PDCD1* gene in patients 3 and 4 and a 5-8Mb deletion from the *COL6A3* gene to the *PDCD1* gene in patient 1 (Supp.Fig.1, Table 1).

Table 1. Table showing the extension of 2q37 deletions found in our patients according to analyzed variable number tandem repeats (VNTRs), MLPA probes (P264-Human Telomere-9, MRC Holland) and genes location (reference assembly GRCh37/hg19). Genes included in the smallest region of overlap (SRO) are in Italics.

gene/marker/probe ID	type	start	end	PT 1	PT 2	PT 3	PT 4
D2S206	VNTR	233.707.826	233.707.976	HET	HET	HET	HET
D2S2205	VNTR	234.400.231	234.400.401	HET	HET	HET	HET
D2S336	VNTR	235.777.094	235.777.202	HOMO	HET	HOMO	HET
D2S2202	VNTR	236.630.048	236.630.280	HOMO	HOMO	HOMO	HET
D2S338	VNTR	237.235.412	237.235.700	HOMO	HET	HET	HET
RDC1	gene	237.476.430	237.491.001				
D2S345	VNTR	237.802.011	237.802.265	HOMO	HET	HET	HOMO
D2S2968	VNTR	238.078.465	238.078.645	HOMO	HOMO	HOMO	HOMO
COL6A3/09038-L09292	gene/MLPA	238.296.676	238.296.745	DEL	NO DEL	NO DEL	NO DEL
LRRFIP1/09037-L09291	gene/MLPA	238.672.488	238.672.548	DEL	NO DEL	NO DEL	NO DEL
D2S1833	VNTR	238.690.825	238.690.951	HOMO	HOMO	HOMO	HOMO
RBM44	gene	238.707.032	238.751.451				
RAMP1	gene	238.767.536	238.820.756				
UBE2F	gene	238.875.469	238.951.236				
SCLY	gene	238.969.530	239.008.054				
ESPNL	gene	239.008.798	239.041.928				
KLHL30	gene	239.047.363	239.061.588				
FAM132B	gene	239.067.623	239.077.541				
D2S2338	VNTR	238.849.991	238.850.157	HOMO	HET	HET	HOMO
ILKAP	gene	239.079.042	239.112.370				
HES6	gene	239.146.908	239.149.303				
PER2	gene	239.152.679	239.198.743				
TRAF3IP1	gene	239.229.082	239.309.541				
TRAF3IP1/09036-L13880	gene/MLPA	239.306.132	239.306.198	DEL	NO DEL	DEL	DEL
ASB1	gene	239.335.383	239.360.891				
TWIST2	gene	239.756.673	239.795.893				
HDAC4/10036-L11449	gene/MLPA	240.274.425	240.274.494	DEL	DEL	DEL	DEL
NDUFA10/09034-L09288	gene/MLPA	240.960.582	240.960.651	DEL	DEL	DEL	DEL
OR6B2	gene	240.968.841	240.969.906				
PRR21	gene	240.981.230	240.982.399				
OR6B3	gene	240.984.494	240.985.489				
MYEOV2	gene	241.065.980	241.076.224				

OTOS	**gene**	**241.078.446**	**241.083.979**				
D2S125	**VNTR**	**241.168.126**	**241.168.221**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
GPC1	**gene**	**241.375.088**	**241.407.493**				
ANKMY1	**gene**	**241.418.839**	**241.508.626**				
DUSP28	**gene**	**241.499.471**	**241.503.431**				
RNPEPL1	**gene**	**241.505.221**	**241.520.789**				
***CAPN10*/15667-L17633**	**gene/MLPA**	**241.530.376**	**241.530.437**	**DEL**	**DEL**	**DEL**	**DEL**
GPR35	**gene**	**241.544.848**	**241.570.676**				
AQP12B	**gene**	**241.615.835**	**241.622.323**				
AQP12A	**gene**	**241.631.262**	**241.637.900**				
KIF1A	**gene**	**241.653.183**	**241.759.725**				
***AGXT*/10035-L11450**	**gene/MLPA**	**241.814.554**	**241.814.618**	**DEL**	**DEL**	**DEL**	**DEL**
SNED1	**gene**	**241.938.255**	**242.034.983**				
D2S2890	**VNTR**	**242.026.549**	**242.026.698**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
D2S1611E	**VNTR**	**242.026.692**	**242.026.776**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
MTERFD2	**gene**	**242.011.584**	**242.041.747**				
***PASK*/09031-L09285**	**gene/MLPA**	**242.080.099**	**242.080.171**	**DEL**	**DEL**	**DEL**	**DEL**
RH25358	**VNTR**	**242045824**	**242.045.969**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
PPP1R7	**gene**	**242.088.991**	**242.123.067**				
ANO7	**gene**	**242.127.924**	**242.164.792**				
HDLBP	**gene**	**242.166.679**	**242.256.476**				
D2S1516E	**VNTR**	**242.168.683**	**242.168.763**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
RH101	**VNTR**	**242.168.726**	**242.168.976**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
RH104464	**VNTR**	**242.177.515**	**242.177.649**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
NEDD5	**gene**	**242.254.515**	**242.293.442**				
FARP2	**gene**	**242.295.658**	**242.434.256**				
RH19982	**VNTR**	**242.384.394**	**242.384.560**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
SHGC-32276	**VNTR**	**242.418.120**	**242.418.223**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
***STK25*/10034-L10544**	**gene/MLPA**	**242.438.755**	**242.438.820**	**DEL**	**DEL**	**DEL**	**DEL**
BOK	**gene**	**242.498.136**	**242.513.546**				
THAP4	**gene**	**242.523.820**	**242.576.864**				
***ATG4B*/02782-L02224**	**gene/MLPA**	**242.606.067**	**242.606.128**	**DEL**	**DEL**	**DEL**	**DEL**
DTYMK	**gene**	**242.615.157**	**242.626.406**				
ING5	**gene**	**242.641.450**	**242.668.893**				
***D2HGDH*/15666-L17632**	**gene/MLPA**	**242.684.218**	**242.684.272**	**DEL**	**DEL**	**DEL**	**DEL**
GAL3ST2	**gene**	**242.716.240**	**242.743.623**				
***NEU4*/09029-L09283**	**gene/MLPA**	**242.758.251**	**242.758.314**	**DEL**	**DEL**	**DEL**	**DEL**
***PDCD1*/15664-L17629**	**gene/MLPA**	**242.793.165**	**242.793.217**	**DEL**	**DEL**	**DEL**	**DEL**
CXXC11	**gene**	**242.811.752**	**242.815.975**				
D2S2985	**VNTR**	**242.855.712**	**242.855.894**	**HOMO**	**HOMO**	**HET**	**HOMO**
D2S2988	**VNTR**	**242.856.375**	**242.856.543**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
D2S2986	**VNTR**	**242.866.489**	**242.866.638**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
D2S447	**VNTR**	**242.884.802**	**242.885.024**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
D2S2585	**VNTR**	**242.926.377**	**242.926.556**	**HOMO**	**HOMO**	**HOMO**	**HOMO**
CICP9	**gene**	**243.062.007**	**243.062.204**				
ABC6	**gene**	**243.160.372**	**243.160.825**				
chromosome end			**243.199.373**				

Although we were not able to identify the precise breakpoints location, analysis of VNTRs in the 2q37 region allowed to roughly delineate the extension of deletions, because heterozygous markers represented a biallelic condition. In particular, the deletion extension range and the number of deleted genes were: 2'924’948-3'893'175bps / 38-40 genes for patient 2, 3'549’580-4'005'555bps / 38-39 genes for patient 3, 3'893’241-4'526'825bps / 41-51 genes for patient 4 and 4'902’697-8'798’972bps / 53-54 genes for patient 1 (Supp.Fig.1, Table 1). Unfortunately, biological samples from parents were not available, thus we were not able to determine the inheritance pattern of defects (inherited vs *de novo*) nor the affected allele (maternal vs paternal).

The smallest region of overlap (SRO) containing the gene or genes potentially involved in the clinical presentation, that is the region ranging from nucleotides 239'306’199 and 242'855’711 where all four found deletions overlapped, removed about 3.5Mbp hosting 38 genes (Figure 1, Table 1).

Clinical details of deleted patients are summarized in Table 2. Of note, all deleted patients displayed brachydactyly and cognitive impairment, and 3 of them also mild resistance to PTH.

Table 2. Clinical and biochemical characteristics of deleted patients. Abbreviations: IUGR, intrauterine growth retardation; y, years; AGA, appropriate for gestational age. PTH normal range: 10-65 pg/ml

		patient 1	patient 2	patient 3	patient 4
Clinical and auxological data	**Pre- and perinatal growth retardation**	IUGR	AGA	AGA	AGA
	Postnatal growth retardation	NO	YES	NO	NO
	Obesity / overweight	YES (75°centile)	NO (<3°centile)	YES (>97°centile/BMI = 28.3 at 15y)	YES (BMI = 31)
	Brachydactyly	YES (brachymetacarpia)	YES (brachydactyly - brachymetacarpia - brachymetatarsia)	YES (brachymetacarpia - brachymetatarsia)	YES (brachymetacarpia)
	Mental retardation	YES	YES	YES	YES
	Facial dysmorphisms	NO	YES (prominent forehead, microphthalmia, rotated posteriorly auricles, small nose, short filter, wide mouth)	NO	YES
	Additional features	slightly increased bone age (13½-14y vs 12y of chronological age)	legs exostosis - 3 accidental fractures (ulna and wrist) - elbow dislocation due to joint laxity	febrile convulsions after 1y	phimosis, genu valgum, scoliosis, bilateral flat feet
Haematochemical	**PTH**	↑ (135 pg/mL)	↑ (130 pg/mL)	↑ (83 pg/mL)	↔ (49 pg/mL)
	Ca	↔	↔	↔	↔
	P	↔	↔	↔	↔
	TSH	↔	↔	↔	↔

Discussion

Pseudohypoparathyroidism demonstrated a bigger and bigger overlap from the clinical point of view with diseases in differential diagnosis, and nowadays it is often difficult to make a conclusive diagnosis without a molecular confirmation of the underlying genetic defect [1].

Despite the high detection rate of molecular defects, a percentage of patients with a clinical suspect of PHP still lack a confirming diagnosis, thus the need to screen for alterations associated to diseases sharing clinical similarity. Recently, deletions of chromosome 2q37.2 have been detected in a small subset of patients with PHP but no *GNAS* defects, further confirming a phenotypic overlap with the brachydactyly-mental retardation syndrome [1, 9].

The BDMR is a complex syndrome with a significantly variable presentation of characteristic dysmorphic features as patients show a range of severity of physical findings, mental retardation and behavioral characteristics. Despite dysmorphisms, malformations, additional late-onset abnormalities and neurodevelopmental/behavioral traits resumed in Table 3, the BDMR was initially named as the “AHO-like syndrome” being the hallmarks for diagnosis brachymetaphalangia (short fourth and fifth metacarpals and metatarsals), early developmental delay or frank mental retardation (from mild to severe), short stature (sometimes early overgrowth with subsequent premature closure of the epiphyses and short final height) and obesity [10].

Table 3. Table resuming features associated with the BDMR syndrome.

Additional features associated with the BDMR phenotype
Typical facial dysmorphisms (most cases)
• prominent forehead • arched eyebrows • upslanting palpebral fissures • midface hypoplasia • depressed nasal bridge • thin upper lip • anteverted lobules
Major malformations (about 30% of pts)
• congenital heart malformations (i.e. ventricular septal defects, aortic coarctation or hypoplasia) • gastrointestinal and renal anomalies (i.e. pyloric stenosis, duodenal or esophageal atresia) • genitourinary malformations (i.e. horseshoe kidney, hypospadias, hypoplastic gonads, bifid uterus and undescended testes) • central nervous system malformations (i.e. hydrocephaly, holoprosencephaly) • congenital skeletal malformations (i.e. hip dislocation, fused cervical vertebrae, fractures, arched or cleft palate)
Other features and late-onset abnormalities
• sparse or thin hair • eczema • recurrent otitis media • sinusitis and lower respiratory infections • joint laxity • umbilical and inguinal hernias • articulation dislocation
Neurodevelopmental and behavioral traits (frequently reported)
• hypotonia improving with time • seizure disorder unrelated to brain malformation • epilepsy • complex febrile seizures • autistic features (i.e. repetitive behaviors, deficit in communication and social interaction, stereotypic movements, intermittent aggression, hyperactivity, attention deficit, obsessive-compulsive disorder and sleep disturbances)

In the present study, we investigated for the presence of 2q37 deletions our cohort of 73 iPPSDx patients, in whom relevant differential diagnoses had been excluded. Molecular analysis identified 4 different overlapping 2q37 deletions, ranging from about 3Mbp to about 8Mbp extension, in 4 unrelated patients.

The detected deletions completely overlapped with previously reported 2q37 BDMR-associated abnormalities and the about 3.5Mbp smallest region of overlap hosted 38 genes, already proposed as related to the AHO-like phenotype (*GCP1*, *GPR35*, *HDAC4*, *CAPN10*, *HDLBP*, *PASK*, *FARP2* and *STK25*) [10, 15-19]. Our data further supported the hypothesis that one or more of these genes are important for skeletal morphogenesis and neurodevelopment, and that their gene products could interact with Gsα-mediated transduction pathways without affecting those activated by other mediators.

The common clinical features shared by our deleted patients were brachydactyly and mental retardation, in the absence of thyroid disfunction and ectopic ossifications.

It is interesting that none of these patients developed ectopic ossifications, so that extraskeletal bone formation should remain to be considered as a typical feature associated with iPPSDs only (and particularly with GNAS defects).

When we looked for additional BDMR-specific physical findings, we found that patient 2 presented congenital skeletal malformations including legs exostosis, frequent accidental fractures and joint laxity, patient 3 suffered from febrile convulsions after the first year, while patient 4 showed skeletal malformations including scoliosis and bilateral flat feet.

Comparing deleted patients with the whole cohort of iPPSDx patients, we did not find gene-specific clinical manifestations that helped defining patient-specific algorithms to properly address the genetic testing. As a matter of fact, deleted patients showed clinical signs and symptoms both common and uncommon to PHP and BDMR.

Three deleted patients showed a mild resistance to PTH, brachydactyly and mental and/or behavioural defects, so we further examined a cluster of 11 cases presenting those three features but we did not observe any noteworthy phenotypic difference.

To note that hormone resistance is typically absent in BDMR, while, in PHP, PTH levels are variable but frequently severely elevated. To our knowledge, only one patient with an AHO-like phenotype and raised PTH levels has been described, and after additional biochemical investigations the authors suggested a mild hormone resistance [14]. Possible explanations are that, in published studies, the endocrine function was not systematically evaluated or that patients had not developed an overt hormone resistance at the moment of the evaluation.

Being 2q37 deletions inheritable defects with a 50% recurrence risk in the offspring, for genetic counseling purpose patients and parents should be screened. Moreover, after the exclusion of defects affecting the Gsα-cAMP signaling pathway, screening for such defects should be considered in the evaluation of specific iPPSDx patients showing brachydactyly, mental and/or behavioural defects and, unexpectedly, elevated PTH serum levels.

In case of confirmation, patients should be re-evaluated and start a follow-up for BDMR: echocardiogram, renal ultrasound for the possible development of renal cysts, periodic hearing screen for the risk of middle ear disease/dysfunction, ophthalmologic evaluation. Obesity should be controlled, both in the child and the adult, and growth parameters monitored. In order to give an appropriate educational intervention, children should undergo an early developmental and behavioral assessment. Patients with joint laxity should practice low impact physical activities. The surveillance for Wilms' tumor should be performed in children with breakpoint at or proximal to band 2q37.1. For additional common features such as otitis, sinusitis, asthma and eczema, standard care should be provided [10].

In conclusion, with the present work we confirmed the rarity of misdiagnosis of iPPSD in BDMR subjects as we identified 4 deletions in 4 unrelated patients by the screening of 73 iPPSDx patients

(5.3%). All deleted patients presented brachydactyly and mental retardation, in the absence of thyroid disfunction and ectopic ossifications, while three of them also showed raised PTH levels. We did not find any additional BDMR-specific physical findings that might help in identifying patients deserving a molecular investigation for terminal 2q37 deletions. Further studies are needed to elucidate the clinical overlap between PHP and BDMR and to formulate new classification algorithms for an early identification of patients.

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Figure legends

Figure 1. Representative figure from the UCSC Genome Browser resuming the extension range of previously reported BDMR-associated deletions and genes included. The smallest region of overlap (SRO) among our deletions is highlighted in red.