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Original Articles

Phenotypic Spectrum of 3 Pseudohypoparathyroidism type 1a, and 2 Pseudopseudohypoparathyroidism Chinese Patients with Novel *GNAS* Mutations

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Abstract We present three cases (case 1-3) of Pseudohypoparathyroidism type 1a (PHP1a) with congenital hypothyroidism, Albright Hereditary Osteodystrophy (AHO) and mental retardation, and two cases (case 4-5) of Pseudopseudohypoparathyroidism (PPHP) with only features of AHO. A novel de novo heterozygous missense mutation c.152C>T in exon 2 was found in case 1, and a previously reported heterozygous missense mutation c.308T>C in exon 4 was found in case 2, a novel de novo heterozygous missense mutation c.719A>T in exon 10 was found in case 3, case 4 was the mother of case 2 and she also shared the heterozygous missense mutation c.308T>C in exon 4, and case 5 had a heterozygous nonsense mutation c.34C>T in exon 1; in the *GNAS* gene. The mother of case 2, who also carried the same missense mutation as her daughter, had only features of AHO; thus exhibiting the imprinting effect on *GNAS* gene, resulting in a PPHP phenotype. The authors discussed the phenotypic spectrum amongst the cases of PHP and PPHP; and emphasised the importance of early identification of female PPHP patients for genetic counselling.

Key words Albright Hereditary Osteodystrophy; Amenorrhoea; *GNAS* gene; Pseudohypoparathyroidism; Pseudopseudohypoparathyroidism

Introduction

Pseudohypoparathyroidism (PHP) is characterised by biochemical hypoparathyroidism with serum hypocalcaemia, increased production of parathyroid hormone (PTH) and PTH target tissue unresponsiveness. PHP type 2 is differentiated from type 1 by normal urinary cAMP excretion. Pseudohypoparathyroidism type 1a (PHP1a) differs from other type 1 subtypes by reduced Gsα (the α subunit of the heterotrimeric G protein) protein production. Pseudopseudohypoparathyroidism (PPHP) refers to normocalcemic PHP1a patients due to absence of hormone resistance, and shares with PHP1a, albright hereditary osteodystrophy.

Albright Hereditary Osteodystrophy (AHO) is a genetically heterogeneous phenotype that occurs in Pseudohypoparathyroidism (PHP) type 1a and type 1c, Pseudopseudohypoparathyroidism (PPHP), acrodysostosis and subtelomeric chromosomal deletion 2q37.3.[1] It is characterised by short stature, round face, brachydactyly, subcutaneous calcification (42% in PHP and 27% in normocalcemic AHO)[2] and obesity.

Guanine nucleotide-binding proteins (GNBPs) regulate many physiological processes, in particular cell growth and differentiation. The heterotrimeric G proteins that are coupled to G protein-coupled receptors (GPCRs) is a major class of GNBPs. The α subunit (Gsα) of the heterotrimeric G protein is encoded by the *GNAS* gene. The protein is the stimulatory component of the adenylyl cyclase complex that is required for transducing the extracellular activating hormonal signals to the intracellular second messenger, the cyclic AMP, to produce a physiological response. Both

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PHP1a and PPHP are caused by heterozygous loss of function mutations in the *GNAS* gene, and they are dominantly inherited with incomplete penetrance.

The *GNAS* gene consists of 13 coding exons that span 20 kb of genomic DNA, and was mapped to chromosome 20q13.2. It is biallelically expressed in most tissues, and is paternally imprinted and maternally expressed in specific hormone target sensitive tissues.[3] This explains the observed maternal inheritance of *GNAS* mutations causing PHP1a and paternal inheritance of *GNAS* mutations causing PPHP.

We report 3 cases (case 1 to 3) of PHP1a, with 2 presented with congenital hypothyroidism detected by the neonatal screening program of the Clinical Genetic Service (CGS), Department of Health, and subsequently developed AHO phenotype and hormonal resistance. Molecular analysis confirmed diagnosis of PHP1a and revealed two previously unreported mutations in the *GNAS* gene. Mutations in Case 1 and 3 were sporadic in their family. Case 2's mutation was inherited from her mother who had PPHP phenotype. We also report two cases (case 4 and 5) of PPHP and compare their phenotypic differences and discuss its diagnostic significance.

Subject and Methods

Case 1 (Figure 1) was an eighteen-year-old girl who was born full term to healthy non-consanguineous parents with birth weight 3.6 kg. Her cord blood Thyroid Stimulating Hormone (TSH) level was elevated at 45 mIU/l (Normal <15) detected by the neonatal screening program of CGS. She had transient neonatal hypothyroidism. No treatment was given while her thyroid function gradually returned to normal by the end of first month of life. She was diagnosed in her early childhood with hypothyroidism and was put on thyroxine replacement since then. Calcium supplement was added at the age of 3 after a hypocalcaemia associated epileptic episode, and has been seizure free. She was mildly retarded and studied at special school. She was referred at 17 years by Gynaecologist for primary amenorrhoea.

On examination, her height was 129.5 cm, 18 cm less than third percentile for her age. Coarse facial appearance was noted with round face, brachydactyly and relative obesity. Radiological examination showed thickened skull with intracranial calcification, shortened long bones, shortening of all metacarpals, distal phalanges and shortening of fourth and fifth proximal phalanges, bony exostosis in proximal right tibia and subcutaneous calcification in right lower leg. Her karyotype was 46,XX.

Case 2 (Figure 2) was a ten-year-old girl who was born at full term to non-consanguineous parents with birth weight 2.8 kg. She was confirmed by the neonatal screening program of CGS to have congenital hypothyroidism with cord blood TSH level of 70.9 mIU/l (Normal <15). Thyroid scan on day 15 postnatal life showed normal anatomical structure with reduced tracer uptake. She was commenced on Thyroxine replacement on same day. She was mildly

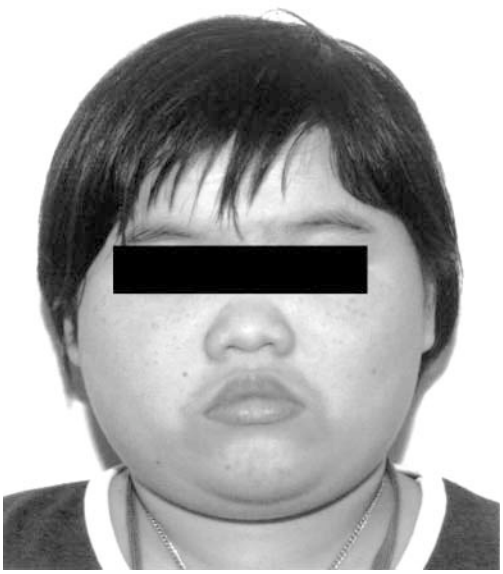

Figure 1 Case 1 with PHP1a.

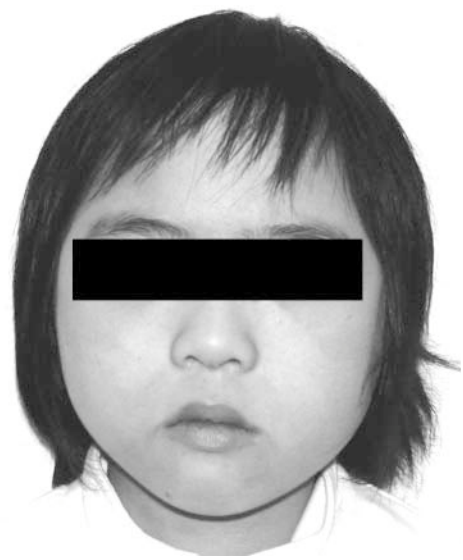

Figure 2 Case 2 with PHP1a.

delayed and was allocated placement in special school. She never had seizure; and was referred at 9 years of age by her attending paediatrician for suspected Turner syndrome.

On examination at 9.5 years old, her head circumference was 50.5 cm (10-25%), body weight was 29.8 kg (50-75%), height was 124 cm (3-10%). She had round face, relative obesity, brachydactyly of fingers and toes; in particular bilateral fourth and fifth toes. Biochemical investigation revealed hypocalcaemia and hyperphosphataemia. Her mother was mentally normal with short stature, slightly rounded face, brachydactyly of fingers and toes; in which her right third, fourth and fifth toes were asymmetrically involved, and was not obese. Proband's two elder siblings have normal phenotypes. Proband's mother had normal serum calcium and phosphate levels. Radiological examinations of Proband and her mother showed similar findings of thickened skull, shortening of metacarpals, metatarsals and distal phalanges.

Case 3 (Figure 3) was a 13-year-old girl who was referred to CGS by paediatrician at 5 years old for suspecting Prader Willi syndrome. She was born to non-consanguineous parents at term with birth weight 2.95 kg. She had relative obesity, short stature and developmental delay. She sat at one year, said "ma ma" at two years old, and walked at three years old. She was assessed by child assessment centre at 4.5 years old to be mildly delayed. Her delay gradually improved after appropriate training at special school; and currently, she is studying in Form 1 at a normal school but with very poor school performance. She never had seizure; and was pre-pubertal at 13 years old with primary amenorrhea. At aged 12, she was diagnosed Hypothyroidism and was put on daily thyroxine replacement since. Her cord blood TSH level was 12.5 mIU/l (Normal <15). She had one elder brother who performed extremely well at school and had normal stature. Her mother had normal intelligence and a height of 150 cm. Her father was normal in intelligence and height.

On examination at 5 years old, her head circumference was 50 cm (50%), body weight was 18 kg (75%), height was 98 cm (3%). She had a round and coarse face, short 4th and 5th metacarpals and metatarsals. Fingers and toes were short and stubby. No abnormal subcutaneous masses were palpable. At 12.5 years old, her body weight was 30.1 kg (3%), height was 127.5 cm (10.5 cm <3%). Her biochemical profile and brain imaging were normal. Her karyotype was 46,XX.

Case 4 (Figure 4) was a 39-year-old woman who had mild terminal phalanges shortening, and marked short and stubby toes. She did not have round face or endocrine abnormality. Her height was 156 cm (25%). She had tall parents and 5 tall siblings. She had 3 children; two of which aged 15 and 10 were very tall for their age and performed well at school. Case 2 was her youngest child.

Case 5 (Figure 5) was a 19-year-old girl who was referred to CGS at 14 years of age for suspected Turner Syndrome. Apart from scoliosis that required bracing, she was healthy all along. She had spontaneous menarche at 11 years old and her periods have been regular; and she

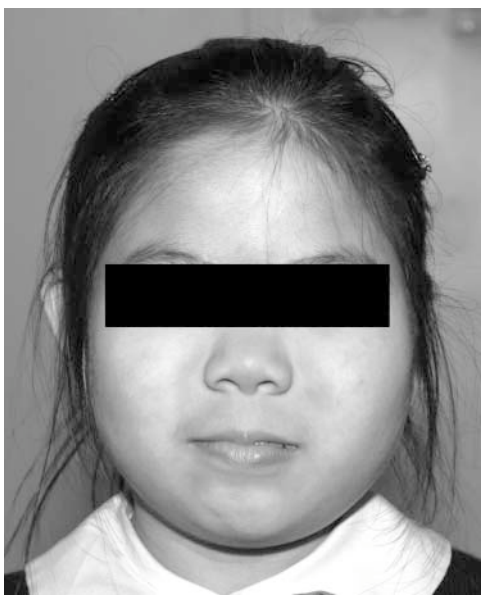

Figure 3 Case 3 with PHP1a.

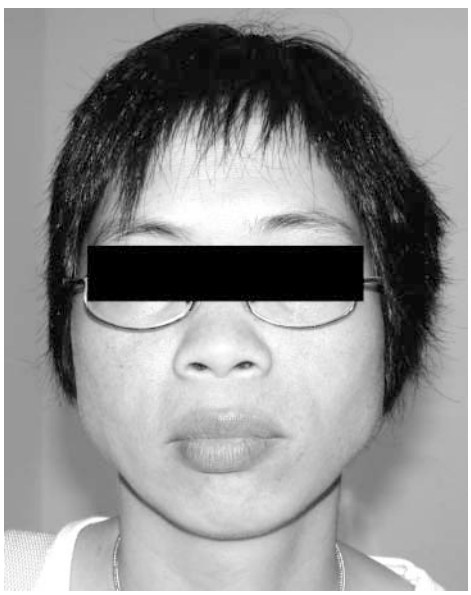

Figure 4 Case 4 with PPHP.

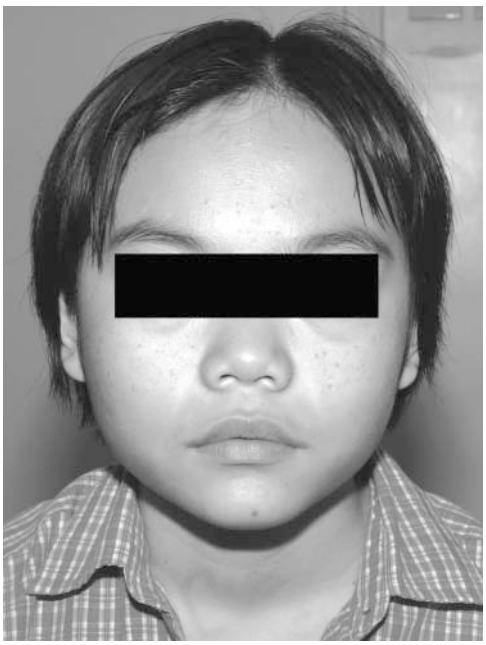

Figure 5 Case 5 with PPHP.

had no other endocrine abnormalities. Her biochemical and hormonal profiles were normal and brain imaging showed calcification at the falx cerebri near the vertex. No abnormal basal ganglia calcification was noted. She had normal intelligence. Her parents had normal stature, and the rest of her family history was unremarkable.

On examination at 14 years old, her head circumference was 56 cm (90-97%), body weight was 41.8 kg (25-50%), height was 141.5 cm (3cm <3%). She had a round face, short 4th and 5th metacarpals and metatarsals. Fingers and toes were short and stubby. No abnormal subcutaneous masses were palpable. Her karyotype was 46,XX.

Genomic DNA was extracted from peripheral blood using QIAamp DNA blood mini kit (Qiagen). DNA was amplified in a final reaction volume of 50 ul by using 100 ng genomic DNA, 1x FX buffer, 0.2 mM of each dNTPs, 20 pmole primers and 1 unit AmpliTaq Gold polymerase. Primers and the annealing temperatures were described previously by Mantovani et al, 2000.[4] PCR products were purified using QIAquick PCR purification kit (Qiagen) and then subjected to cycle sequencing in a 20 ul reaction with the ABI BigDye Terminator V1.1 cycle sequencing kit. Each reaction contained 3.2 pmole forward or reverse primer, 8 ng template DNA, 2 ul BigDye Terminator ready reaction mix, 3 ul of BigDye sequencing buffer and using water to make up to 20 ul. Unincorporated dye and other contaminants were removed using the CentriSep columns (Applied Biosystems). The purified extension products were then sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems).

Parental consents for publication that included photos and radiographs were obtained in all cases.

Results

Sequence analysis of the *GNAS* gene found a novel heterozygous missense mutation c.152C>T in exon 2 for case 1. This mutation is expected to cause substitution of phenylalanine for serine at codon 51 (S51F). The same mutation was not found in 100 normal healthy control subjects nor either of her parents.

A previously reported[5] heterozygous mutation c.308T>C in exon 4 of the *GNAS* gene was found in case 2. This mutation is expected to cause substitution of threonine for isoleucine at codon 103 (I103T). The same mutation was found to be inherited from her mother (case 4). Proband's two elder healthy siblings and mother's healthy elder sister with normal phenotype were available for c.308T>C mutation testing. The results were all normal.

A novel heterozygous missense mutation c.719A>T in exon 10 of the *GNAS* gene was found in case 3. The mutation changed the amino acid code of the 240th codon from aspartic acid to valine. The missense mutation was not found in 100 normal healthy control subjects, or in her mother. Her father was not available for testing.

Molecular analysis of *GNAS* gene for case 5 revealed a heterozygous c.34C>T mutation in exon 1. This nonsense mutation changed the amino acid code of the 12th codon from glutamine to a STOP codon. The same mutation was not found in either of her parents.

Discussion

The *GNAS* gene has several functional domains. Exon 1 and 2 encode the GTPase activity domain.[6] The GTPase activity domain acts as molecular switches; regulating the extracellular signal transduction to downstream signaling components by switching between the bounded inactive guanosine diphosphate (GDP) and the active guanosine triphosphate (GTP). The missense mutation in case 1 is a novel mutation in exon 2 which is expected to inactivate the GTPase activity of the gene. Search on NCBI website revealed mutation occurred at highly conserved nucleotide position.

Exon 4 and 5 are known to encode the Adenylyl Cyclase activity domain of the Gsα protein.[6] Following ligand binding to GPCR and GDP-GTP exchange, dissociation of the G protein occurs. The α subunit will bind to and activate adenylyl cyclase to initiate a signaling cascade with the resultant generation of second messengers and effectors to produce a physiological response. The missense mutation in case 2 was a previously reported[5] pathogenic missense mutation in exon 4 that is expected to abolish this function.

Phenotypic differences in the three cases of PHP were evident. Case 1 and 3 had a much coarser facial appearance than case 2 with case 1 undoubtedly had the most "coarse" face while case 2 was rather normal looking. Apart from that, all had round face, relative obesity, short stature and mild developmental delay. Interestingly, case 2's height was within normal limit (so was her mother's height) while case 1 and 3 were well below third centile. As for multi-hormone resistance, case 1 and 2 presented at birth with congenital hypothyroidism; and later, gonadal failure in case 1. Case 1 also had multiple subcutaneous calcifications, a manifestation of Gsα deficiency,[7] presented as palpable hard lumps on her right leg that was confirmed on radiograph; whereas subcutaneous calcification was absent in case 2 and case 3 when last examined at age 10 and 13 respectively. Intelligence wise, case 1 was studying at special school when last seen, while case 2 and 3 managed poorly at normal school.

The two PPHP patients (case 4 and 5) also had phenotypic differences. Case 4 (mother of case 2) had only minimal features of AHO. She had normal stature, no rounded face and mild shortening (mainly distal phalanges) of fingers and markedly short and stubby toes. However she did not have obesity (in fact she looked thin) or subcutaneous calcification. She was otherwise healthy. Maternal grandfather passed away already and was said to be tall, and there was no family history of short stature. Case 5, in the contrary, had more features of AHO, with a rounded face, short stature, brachydactyly with marked short 4th and 5th metacarpals and metatarsals. Interestingly, mutation in case 5 was a reported mutation previously causing AHO and Progressive osseous heteroplasia.[7] However case 5 had no ectopic calcification when last examined at 19 years old.

Case 2 has two different phenotypes co-segregating in same family, both sharing identical *GNAS* mutation. This demonstrated the imprinting effect on the dominant inheritance of the same mutation on clinical phenotypes. The *GNAS* gene is normally biallelically expressed[8,9] and is paternally imprinted in certain tissues; proximal renal tubules, thyroid tissues, pituitary gland[10] and gonadal tissues.[11] Therefore inheritance of mutations on maternal alleles would lead to PHP1a and hypothyroidism as in case 2. Case 1 and 3, although their mothers do not carry the same mutation; knowing the mechanism of causing AHO with hormonal resistance, it is highly likely that they are mutated on their maternal allele. Both case 1 and 3 novel missense mutations were not found in 100 normal healthy control subjects, and a search on NCBI website revealed mutation occurred at highly conserved nucleotide positions. Case 4 (mother of case 2) and case 5 were presumed to carry their heterozygous mutation on their paternal allele (case 4's parents were not available) as they only had PPHP without multi-hormone resistance. The significance of identifying PPHP patients is apparent because there is 50% chance of inheriting the mutated allele to her children, therefore giving rise to PHP1a. This is particularly important to case 5 as she is approaching reproductive age, and that prenatal diagnostic procedures are made available to her after adequate genetic counselling.

Mantovani et al, 2003[10] showed that PHP1a patients, in addition to other hormone resistance, had variable degree of Growth Hormone releasing hormone (GHRH) resistance in pituitary gland, consistent with *GNAS* imprinting in human pituitary. The AHO phenotype of all our patients may be attributed to *GNAS* haploinsufficiency in many tissues, but exact mechanism that led to AHO phenotype is unknown. Rickard and Wilson, 2003[12] reported that there was no obvious parental origin effect of *GNAS* mutation on AHO phenotype. The degree of GH deficiency, if any, contributed to final height in PHP1a is also unknown. In this series, it is possible that other factors such as environmental or other genetic factors may be in place to explain why case 2 and 4 have exceptional height in their respective groups.

PHP1a females with clinical hypogonadism commonly present as delayed puberty, incomplete sexual maturation or amenorrhoea.[13] Case 1 was referred for primary amenorrhoea and delayed puberty suspecting chromosomal abnormality. The additional congenital hypothyroidism and mental retardation should raise the possibilities of other alternative causes such as PHP.

Congenital hypothyroidism is genetically heterogeneous. Apart from structural abnormalities, thyroid scan performed at birth in neonates with elevated TSH often could not give rise to definitive diagnosis. Almost all PHP1a presents with elevated TSH at birth,[14] and that the TSH levels may normalise for several months before becoming elevated again.[14] Case 1 had transient elevation of TSH at birth with

gradual improvement over the subsequent weeks to normal level. The girl presented again in early childhood with frank hypothyroidism, and thus illustrated the importance of paediatric follow-up, of all initial significantly abnormal cases at birth, especially in the first few years of life. One would have to be vigilant in examining a girl with hypothyroidism, short stature, delayed puberty and mental retardation; as not to miss other important differential diagnosis such as PHP. Case 2 on the other hand had a thyroid scan on Day 15 which showed decreased tracer uptake and commenced thyroxine replacement since then, and treated as congenital hypothyroidism. Despite adequate thyroxine replacement, there was poor growth in height but still managed to fall within normal centile range. Developmental delay and learning difficulties became obvious with age. The subsequent development should alert the possibility of other differential diagnoses by examining closely the skeletal changes for AHO.[15] Case 3 did not present with Congenital Hypothyroidism, and her phenotypic severity was intermediate amongst these three patients.

Karyotyping is important to exclude Turner syndrome and the so called AHO-like syndrome; the Deletion 2q37.3 phenotype, in which responsible genes have been known to produce phenotype that simulate AHO.[1] Molecular analysis is a valuable tool[16] and should be performed in suspected AHO cases for definitive diagnosis.[17]

In conclusion, we emphasised the importance of molecular analysis in suspected PPHP and PHP1a cases when PTH infusion test, Gsα activity, PTH tissue resistance or other highly technical hormonal analysis are not readily available. The gene testing is relatively easy to perform. The beneficial impact of such result on genetic counselling with potential view on prenatal diagnosis and family planning of involved families is great.

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