CASE REPORT

Early manifestation of calcinosis cutis in pseudohypoparathyroidism type Ia associated with a novel mutation in the GNAS gene

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Abstract

Objective: To clarify the molecular defect for the clinical finding of congenital hypothyroidism combined with the manifestation of calcinosis cutis in infancy.

Case report: The male patient presented with moderately elevated blood thyrotropin levels at neonatal screening combined with slightly decreased plasma thyroxine and tri-iodothyronine concentrations, necessitating thyroid hormone substitution 2 weeks after birth. At the age of 7 months calcinosis cutis was seen and the patient underwent further investigation. Typical features of Albright's hereditary osteodystrophy (AHO), including round face, obesity and delayed psychomotor development, were found.

Methods and results: Laboratory investigation revealed a resistance to parathyroid hormone (PTH) with highly elevated PTH levels and a reduction in adenylyl cyclase-stimulating protein (Gs α) activity leading to the diagnosis of pseudohypoparathyroidism type Ia (PHP Ia). A novel heterozygous mutation (c364T > G in exon 5, leading to the amino acid substitution Ile-106 $\rightarrow$ Ser) was detected in the GNAS gene of the patient. This mutation was not found in the patient's parents, both of whom showed normal Gs α protein activity in erythrocytes and no features of AHO. A *de novo* mutation is therefore likely.

Conclusions: Subcutaneous calcifications in infancy should prompt the clinician to a thorough search for an underlying disease. The possibility of AHO and PHP Ia should be considered in children with hypothyroidism and calcinosis cutis. Systematic reviews regarding the frequency of calcinosis in AHO are warranted.

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Introduction

Subcutaneous calcification is a rare clinical symptom in infancy. Calcifications are most frequently reported after subcutaneous fat necrosis following extravasation of calcium gluconate or following hypothermia in neonates. Progressive calcifications of the subcutis in early infancy and later are often associated with progressive osseous heteroplasia (OMIM 166350) or pseudohypoparathyroidism (PHP; OMIM 103580).

PHP is characterized by target-organ resistance to parathyroid hormone (PTH) (1). Depending on the differences in pathogenesis and phenotype, PHP can be classified as types Ia–Ic and II (2). Type Ia is biochemically characterized by reduced activity of the Gs α protein. Other endocrinopathies, such as hypothyroidism

and hypogonadism, are often associated with PHP Ia. Patients with PHP Ia show a distinct physical phenotype characterized by short stature, obesity, brachydactyly, subcutaneous calcifications and mental retardation known as Albright's hereditary osteodystrophy (AHO) (1). The underlying cause for the reduced activity of the Gs α protein is the inactivation of the protein due to heterozygous mutations within the coding GNAS gene (3). The human GNAS gene is located on chromosome 20q13, with a coding region consisting of 13 exons (4). Various inactivating mutations have been identified in the GNAS gene of patients with AHO and PHP Ia (a list of mutations is available at <http://mammary.nih.gov.aho/>). Except a 4-bp deletion in exon 7 no mutational hotspot is apparent (2). A clear genotype-to-phenotype correlation is not possible, as PHP Ia and AHO show

high clinical and genetic diversity. PHP Ia shows an autosomal-dominant mode of inheritance. In the case of maternal transmission children develop PHP Ia; in the case of paternal transmission they develop a condition called pseudopseudohypoparathyroidism, characterized by signs of AHO and $Gs\alpha$ protein deficiency but without biochemical evidence of hormone resistance. This parental origin effect provides evidence that imprinting is a regulatory mechanism for $Gs\alpha$ transcription (2).

This report describes a boy with PHP Ia and AHO. He presented with mild congenital primary hypothyroidism and showed the remarkable feature of an early onset of disseminated calcinosis cutis at the age of 7 months. The diagnosis of PHP Ia was confirmed by a decrease in $Gs\alpha$ subunit protein activity in erythrocyte membranes and the identification of a novel heterozygous mutation in the *GNAS* gene.

Patient and methods

The baby boy was the first child of unrelated parents of German descent. He was born at term by Caesarean section because of fetal distress. Pregnancy was otherwise uneventful. His birth weight was 3144 g (-1.1 SDS), his length 47 cm (-2.5 SDS). The neonatal period was complicated by delayed pulmonary adaptation, hypoglycemia and prolonged jaundice. In addition, a small ventricular septum defect and a marked dysplasia of the hip were found. Neonatal screening revealed a slightly elevated thyrotropin level (20 mU/l; normal, <15 mU/l) with normal free

thyroxine and free tri-iodothyronine levels. Thyroid autoantibodies were negative. In sonography the thyroid gland appeared normal. At the age of 12 days plasma thyrotropin further increased to 50.6 μ IU/ml (normal range for this age, 1.4–8.8 μ IU/ml), whereas free thyroxine and free tri-iodothyronine were below normal (free thyroxine, 0.7 ng/dl; normal range for this age, 1.08–2.03 ng/dl; free tri-iodothyronine, 2.0 pg/ml; normal range for age, 2.9–6.8 pg/ml). Treatment with levothyroxine (50 μ g/day) was therefore initiated. Hip dysplasia required surgical revision at the age of 5 months. Although thyroid hormone substitution was sufficient, as established at various time points, the psychomotor development was mildly retarded. At the age of 7 months, he presented with multiple subcutaneous lesions distributed ubiquitously on trunk and extremities. The widespread lesions appeared as firm reddish plaques (Fig. 1A). Skin biopsy and histopathologic examination revealed deposits of calcium within the dermis and the adjacent subcutaneous tissue with a surrounding foreign-body giant-cell reaction (Fig. 1B). Further laboratory tests were carried out, looking for evidence of AHO and PHP. At 10 months of age, serum calcium was 2.24 mM (normal range, 2.1–2.65 mM), phosphate 1.91 mM (normal range, 1.32–2.31 mM), creatinine 0.3 mg/dl (normal range, 0.24–0.4 mg/dl), and alkaline phosphatase activity 403 U/l (normal range, 200–600 U/l). Tubular phosphate reabsorption and transport maximum was 96.9% (normal range, 85–97%) and 7.5 mg/dl (normal range, 4–8 mg/dl), respectively. Intact serum PTH was highly elevated with 202.0 pg/ml (normal range, 15.0–55.0 pg/ml).

Figure 1 (A) Photograph of a typical subcutaneous calcification appearing as livid, rigid plaque. (B) Histopathologic examination of a representative skin biopsy revealing deposits of calcium in the dermis and the subcutaneous tissue with a surrounding foreign body giant cell reaction (van Kossa stain; original magnification, $\times 50$).

Serum 25-hydroxyvitamin D and serum 1,25-dihydroxyvitamin D were 37.5 ng/ml (normal range, 10–50 ng/ml) and 102 pg/ml (normal range, 30–90 pg/ml), respectively. The urinary calcium/urinary creatinine ratio was 9.23 µg/mg (normal range, 30–810 µg/mg). The urinary cAMP/urinary creatinine ratio was within the low normal range (5.2 nmol/mg; normal range, 4–11 nmol/mg). A radiograph of the left hand at age 24 months revealed diffuse osteopenia and shortened metacarpal bones, with a diminished metacarpal index (5) of 3.76 (–4.8 SDS; normal range for age, 5.84 ± 0.43) (Fig. 2).

The Gs α protein activity in erythrocyte membranes was analyzed from heparinized blood samples *in vitro* as previously described (6, 7). In brief, the generation of cAMP in the presence of ATP using adenylyl cyclase from turkey red cell membranes after solubilization and activation of the Gs protein with GTP γ S (guanosine 5'-[γ -thio]triphosphate) was measured by RIA (Immuno Biological Laboratories, Hamburg, Germany). Results obtained in triplicate are expressed as percentages of the mean from healthy controls. The normal range was 85–115%.

Genomic DNA was isolated from peripheral leukocytes by standard procedures. Exons 2–13 of the GNAS gene including intron/exon boundaries were individually amplified in 10 fragments by PCR using the oligonucleotide primers reported previously (8). 100 ng DNA in a final volume of 50 µl using 20 pmol of each primer, 200 µmol or 2 mmol dNTP, 0.5 or 1 U Taq polymerase (AmpliTag; Perkin-Elmer Corp., Norwalk, CT, USA), 20 mmol Tris (pH 8.4),

1.0–2.5 mmol MgCl₂ were applied to an initial denaturation at 94 °C for 5 min, followed by 34 cycles of annealing at 52–62 °C for 90 s, elongation at 72 °C for 2 min, and denaturation at 94 °C for 75 s, and a final elongation at 72 °C for 5 min. The PCR products were between 141 and 381 bp long. The fragment containing exons 4 and 5 was digested with the restriction enzyme HaeII to yield two fragments of 150 and 230 bp used for further analysis. A non-isotopic, single-strand conformation analysis (SSCA) on 5–0% polyacrylamide gels was used for mutational screening as described previously (9). Electrophoretic band shifts were visualized by silver staining, and DNA samples with an aberrant migration pattern were reamplified from genomic DNA and sequenced directly. Direct sequencing of DNA was performed with CY5-labeled primers in the sense and antisense directions, analyzed in an automatic sequencer (ALF express II; Amersham Biosciences, Freiburg, Germany) using the Biozym sequencing kit (Biozym, Hessisch Oldendorf, Germany) as recommended by the manufacturer.

Results

The patient's Gs α protein activity was clearly diminished, at 47.8% (normal range, 85–115%). The mother's and father's Gs α protein activities were normal with 92.8 and 100.5%, respectively.

Direct sequencing of exons 2–13 of the GNAS gene of the patient using genomic DNA revealed a heterozygous mutation at nucleotide 364 in exon 5 (according to the sequence NM000516 published in GenBank) from normal thymidine to mutant guanine (c364T > G; Fig. 3). This mutation results in an amino acid replacement in codon 106 from isoleucine to serine (Ile-106 → Ser). The mutation was verified in two separately generated PCR fragments from two different blood samples by sequencing in the sense and antisense directions. Genotyping in the parents revealed no sequence variation at nucleotide position 364. Therefore, a *de novo* mutation has to be postulated. Patient and parental RNA samples or an informative DNA polymorphism in the vicinity of the mutation were not available to test the allelic origin. The nucleotide variant c364T > G in the GNAS gene was not found in over 100 analyzed alleles from healthy controls of German descent. Based on the combination of elevated PTH, diminished Gs α protein activity and a heterozygous mutation in the GNAS gene in association with features of AHO, the disease was classified as PHP Ia.

Discussion

In this report we present a patient with primary hypothyroidism in the neonatal period, mild retardation of psychomotor development and the unusual and remarkable feature of calcinosis cutis in early infancy. As an underlying defect, a novel missense

Figure 2 Radiograph of the left hand at age 24 months showing diffuse osteopenia and shortened metacarpals (metacarpal index, 3.76 (–4.8 SDS)) (5).

Figure 3 Mutational analysis of *GNAS* showing the heterozygous substitution of serine (AGT) for isoleucine (ATT) at codon 106 in the index patient.

mutation within the *GNAS* gene was identified leading to AHO and PHP Ia. Progressive subcutaneous calcifications in early infancy are rare. In addition to AHO, it can be seen in hyperparathyroidism, sarcoidosis, pseudoxanthoma elasticum, Ehlers–Danlos syndrome, dermatomyositis and scleroderma, or as an idiopathic condition. The calcifications in AHO are not secondary to hypocalcemia or hyperphosphatemia, as calcifications are not found in patients with primary hypoparathyroidism. Subcutaneous calcifications or calcinosis cutis is reported to be a typical feature of AHO. As with the additional features of AHO (short stature, brachydactyly, centripetal obesity, depressed nasal bridge, hypertelorism, mental deficits, developmental delay), there seems to be a considerable variation in the severity of these calcifications. The phenotype in AHO patients with proven inactivating G_{α} mutations varies from many to complete absence of clinical abnormalities (10). There are no systematic reviews regarding the frequency of calcinosis in AHO. From the literature it can be estimated that about 25–50% of patients with AHO and PHP Ia suffer from subcutaneous calcifications (8, 10–15). However, the time of appearance of calcinosis in AHO and PHP Ia within a larger cohort is not reported but it seems to be an earlier symptom of AHO. From our personal experience we presume that there is a wide individual variation of occurrence. Congenital hypothyroidism is another notable feature in our patient, as most individuals with AHO and PHP Ia show signs of hypothyroidism only in later life (8, 11, 15). A typical finding in patients suffering from the combination of AHO and congenital hypothyroidism is disturbed psychomotor

development in spite of sufficient thyroid hormone-substitution therapy (16). As all diseases associated with calcinosis cutis in childhood are very uncommon, the combination of primary hypothyroidism and subcutaneous calcification is highly indicative of AHO and PHP Ia.

Multiple heterozygous inactivating mutations within the G_{α} coding exons have been identified in PHP Ia (17). A 4 bp deletion in exon 7 has been identified in several unrelated families (18). Most of the other mutations have been identified only in individual kindred. An investigation of the complete *GNAS* gene is therefore always warranted in cases of AHO and PHP Ia. G proteins are integral components of various signalling pathways. Each G protein consists of a specific α -subunit associated with a β - and γ -subunit. The α -subunit consists of two domains; a GTPase or Ras-like domain and a helical domain (19). The helical domain is unique to heterotrimeric G-proteins. It is a regulator and activator of the G_{α} -subunit function although it is not directly involved with the nucleotide-binding site or the interdomain interface (20). The part within the helical domain that extends from residue 70 to 140 is functionally important for adenylyl cyclase activation, although the exact steric sequence is unclear (21). Our patient's mutation in residue Ile-106 is located within this important part of the helical domain. Since the G_{α} protein activity was severely reduced in erythrocyte membranes, the Ile-106 $\rightarrow$ Ser mutant protein in the reported patient is assumed to be defective. We speculate that the exchange of the hydrophobic amino acid isoleucine to the polar amino acid serine leads to the clinical phenotype reported, as a result of conformational changes within the helical domain of the G_{α} protein, affecting adenylyl cyclase activation for example. However, from our study we cannot predict the precise mechanism causing loss of function of the Ile-106 $\rightarrow$ Ser mutant protein.

It is generally accepted that hormone resistance in PHP Ia develops only after maternal transmission of *GNAS* mutations (2). This imprinting model assumes that G_{α} protein in specific hormone target tissues (e.g. renal proximal tubules) is primarily translated from the maternal allele, which carries the *GNAS* mutation in the case of PHP Ia. Thus, urinary cAMP response to PTH should be markedly reduced. Consistent with this, baseline cAMP excretion was low in relation to the elevated PTH levels in our patient. Unfortunately, PTH was not available for stimulative testing in our patient. Verification of the allelic origin of the mutation was not possible in our case as patient RNA was not available and an informative DNA polymorphism in the vicinity of the mutation was not found. As our patient showed typical signs of hormone resistance we can therefore only deduce from the imprinting model that the Ile-106 $\rightarrow$ Ser mutation occurred *de novo* on the maternal *GNAS* allele.

In conclusion, the combination of hypothyroidism and subcutaneous calcifications during early infancy in the patient reported allowed us to diagnose AHO and PHP Ia due to a novel missense mutation in the GNAS gene at an early age. The diagnosis of AHO is often delayed due to the high variability of the age of the patient at first appearance of clinical signs typical for the disease. Calcinosis cutis in an infant or child should always give rise to the suspicion of AHO and PHP Ia and should prompt an appropriate diagnostic work-up.

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