CASE REPORT

A new heterozygous mutation (L338N) in the human $G\alpha$ (*GNAS1*) gene as a cause for congenital hypothyroidism in Albright's hereditary osteodystrophy

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Abstract

Objective: To identify the molecular defect by which psychomotor retardation is caused in two brothers with congenital hypothyroidism who received adequate treatment with L-thyroxine.

Case report: A six-year-old boy presented with psychomotor retardation and congenital primary hypothyroidism (CH). The patient had a normal blood thyrotrophin (TSH) level on neonatal screening, but low total serum thyroxine and triiodothyronine concentrations prompting thyroid hormone substitution shortly after birth. Nevertheless, psychomotor development was retarded and the patient underwent further investigation. Typical features of Albright's hereditary osteodystrophy (AHO) such as round face, obesity, and shortened 1st, 4th and 5th metacarpals were found.

Methods and results: Further investigation confirmed AHO with pseudohypoparathyroidism (PHP) type Ia. The boy had a mild resistance to parathyroid hormone and a reduced adenylyl cyclase stimulating protein ($G\alpha$) activity in erythrocytes. DNA analysis detected a new heterozygous mutation (L338N) in the $G\alpha$ protein (*GNAS1*) gene. This mutation was also present in the patient's brother who had similar features and was also treated with thyroid hormone because of CH, and in the phenotypically normal-looking mother who had a normal calcium metabolism but a reduced $G\alpha$ protein activity in erythrocytes suggestive of pseudopseudohypoparathyroidism.

Conclusion: In patients with CH, in whom the neurological outcome is poor even under adequate thyroid hormone substitution, PHP Ia may be suspected, especially when symptoms of AHO are present.

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Introduction

Congenital hypothyroidism (CH) occurs in 1 of 4000 births and is caused, in decreasing order of frequency, by thyroid dysgenesis, thyroid dysmorphogenesis, defects of thyrotrophin (TSH) or TSH-receptor, or hypothalamic–pituitary deficiency (1). Due to screening programs and early diagnosis, treatment of affected individuals is usually started within the first two weeks of life. With adequate therapy, growth and mental development proceed normally in infants with CH (1). However, in some patients with CH the neurological outcome is poor even under adequate hormone substitution. In such patients the retarded mental development may be related to other defects.

Pseudohypoparathyroidism (PHP) is biochemically characterized by hypocalcemia and hyperphosphatemia in association with an increased secretion of

parathyroid hormone (PTH) due to target tissue unresponsiveness to PTH (2). PHP type Ia represents one variant of this condition in which patients are not only resistant to PTH but also to other hormones that bind to receptors coupled to stimulatory G protein (G_s) to activate adenylyl cyclase (3). In addition, these patients have characteristic features of Albright's hereditary osteodystrophy (AHO) which are a round face, brachymetacarpus, short stature, obesity, mental retardation and subcutaneous calcifications (2). PHP Ia and AHO are caused by a reduced activity of the adenylyl cyclase stimulating protein ($G\alpha$). The gene encoding this protein (*GNAS1*) is located on chromosome 20q13.2-13.3. Heterozygous inactivating *GNAS1* mutations have been identified in patients with AHO and PHP (4), following an autosomal-dominant trait.

We studied a six-year-old boy and his eight-year-old brother with CH, who developed mental retardation

despite adequate treatment with L-thyroxine (L-T₄) started shortly after birth. Both boys displayed features of AHO. PHP Ia was suspected even though serum PTH was only slightly elevated in association with a normal serum calcium concentration. This diagnosis was confirmed in both boys by a decreased Gs α subunit protein activity in erythrocyte membranes and identification of a novel heterozygous mutation in codon 338 (L338N) of their *GNAS1* genes.

Subjects and methods

Patients

The proband was the second born to unrelated parents at term and after a normal pregnancy. His birth weight was 3325 g and his length was 49 cm. Because his brother was treated for congenital hypothyroidism, thyroid function tests were performed two days after birth. They revealed a normal serum TSH of 9.0 mU/l (normal range 3.0–18 mU/l) but low blood thyroxine (T₄) and triiodothyronine (T₃) concentrations. Therefore treatment with L-T₄ was initiated. Six weeks later respiratory problems became apparent. Tracheomalacia was diagnosed and the boy underwent surgery at the age of 3 months. The boy continued to receive L-T₄ in doses that were adjusted for his age and weight. He was tested repeatedly and always had thyroid function tests within the normal range. Development proceeded slowly and by the age of 2 years psychomotor retardation was evident. He was also found to have impaired conductive hearing. Further diagnostic tests, including electroencephalography and cranial computer tomography, failed to identify the underlying defect. Interestingly, the brother, who also received thyroid hormone replacement therapy, had a very similar phenotype so that an inherited disease was suspected. Review of his medical history revealed a normal neonatal screening result for CH with a TSH level below 15 mU/l. Prolonged jaundice prompted further evaluation at the age of 2 months. His serum TSH level was slightly elevated and his total T₃ and T₄ levels were below the lower limits of normal so that thyroid hormone substitution was started and adjusted subsequently for his age and weight.

At the age of 6 years the proband and his eight-year-old brother presented to our outpatient clinic. The children appeared physically and mentally retarded. Both were obese with round faces and had broad necks and brachydactyly. Their thyroid glands were normal in size, a finding confirmed by ultrasound. Laboratory tests revealed a suppressed serum TSH associated with high levels of total and free T₄, which can be explained by the high doses of L-T₄ given as replacement therapy (index patient (II₂) 150 μ g L-T₄, brother (II₁) 200 μ g L-T₄ daily). Thyroid antibodies were negative. Furthermore, analysis of the calcium metabolism showed normal phosphate and calcium but slightly elevated PTH levels in both

boys. The radiographs of the left hand of both boys showed shortened 1st, 4th and 5th metacarpals.

Following discontinuation of L-T₄ treatment serum TSH levels rose to 16 and 23.8 mU/l in the proband and in his older brother respectively, while serum free (f) T₄ and fT₃ remained well within the normal range. Values were 1.25 and 1.65 ng/dl for fT₄ and 3.31 and 2.67 pg/ml for fT₃ (for normal ranges see Fig. 1). Furthermore, administration of thyrotrophin-releasing hormone (TRH) increased their serum TSH at 30 min to peak values of 30.1 and 44.2 mU/l in the proband and in his brother respectively, with a further increase in the fT₃ values by 12–33% above baseline.

Laboratory tests carried out on all members of the family are shown in Fig. 1. Determination of Gs α protein activity in erythrocyte membranes was reduced in both boys (50.9% and 48.7%) and in the mother (45.5%) compared with healthy individuals suggesting a defect in the Gs α protein. To characterize the underlying molecular defect, the *GNAS1* gene was sequenced. All family members gave informed consent to participate in the study.

Clinical tests

Serum TSH, total T₄, total T₃, fT₄ and fT₃ concentrations were measured by chemiluminescent immunoassays (Chiron Diagnostics, Fernwald, Germany). For the measurement of PTH an Immulite-Assay (DPC-Biermann, Bad Nauheim, Germany) was used.

Gs α protein activity

The activity of the Gs α protein in erythrocyte membranes was analysed in heparinized blood samples as described previously (5). Briefly, after solubilization of the Gs protein from the patients' erythrocyte membranes and activation with GTP γ S, cAMP generation was measured by radioimmunoassay (Immuno Biological Laboratories, Hamburg, Germany) using adenylyl cyclase from turkey red cell membranes in the presence of ATP. Results obtained in triplicate were expressed as per cent of the mean of healthy controls (normal range 85–115%).

Molecular studies

Genomic deoxyribonucleic acid (DNA) was extracted from circulating white blood cells of all family members and was used for amplification of exons 2–13 of the *GNAS1* gene. The sequences of oligonucleotide primers and conditions for the polymerase chain reactions (PCR) have been described previously (6). Total ribonucleic acid (RNA) was extracted from cultured skin fibroblasts of the index patient using the phenol/guanidine isothiocyanate method (Trizol; Gibco BRL, Carlsruhe, Germany). The first complementary DNA (cDNA) strand that was used for PCR was synthesized

Figure 1 Pedigree of the family, thyroid function tests and results of DNA analyses. The AHO phenotype is indicated by hatched symbols. Black boxes show the presence of the heterozygous mutation, L338N. Figures in bold represent means outside the normal range. All data are aligned to the subjects' symbols on the pedigree. The mutation, L338N, removes a restriction site for *Rsa*I. Therefore, exon 12 was amplified from genomic DNA from all family members. The PCR products were then digested with *Rsa*I, electrophoresed on a 10% polyacrylamide gel and, after exposure to ethidium bromide, visualized under UV-light. In normal individuals the amplified PCR product of 252 bp is cut into a 130 bp and a 122 bp fragment. In the mutant allele which carries L338N the amplified band remains uncut.

with avian myeloblastosis virus reverse transcriptase (Promega, Mannheim, Germany) and an oligo-DT primer.

The PCR amplified products were purified and sequenced directly using an automated fluorescence-based sequencer (ABI 377, Perkin-Elmer, Weiterstadt, Germany).

The mutation L338N which is located in exon 12 of the *GNAS1* gene removes a restriction site for *Rsa*I. Genomic DNA was used to amplify exon 12 of all family members. The PCR products were then digested with *Rsa*I, electrophoresed on a 10% polyacrylamide gel and after exposure to ethidium bromide, visualized under UV-light. In normal individuals the amplified PCR product of 252 bp is cut into a 130 bp and a 122 bp fragment. In the mutant allele which carries L338N the amplified band remains uncut.

Results

Direct sequencing of exons 2–13 of the *GNAS1* gene of the proband using genomic DNA revealed a heterozygous transition of the normal guanine at nucleotide 1082 (according to the published sequence in Genbank Acc. No. NM000516) to a mutant cytosine (AAG → AAC). The same result was obtained by sequencing fibroblast cDNA. This mutation results in the replacement of the normal lysine with an asparagine in codon 338 (L338N) (Fig. 2).

The pedigree and genotyping analysis of all family members are shown in Fig. 1. The mutation removes a restriction site for *Rsa*I allowing verification and rapid screening for the mutation by digestion of the amplification product. The result shows that the proband is indeed heterozygous for L338N (Fig. 1)

Figure 2 Automated fluorescence based on direct sequencing of the patient's *GNAS1* gene from genomic DNA. Note the substitution of the normal guanine (G) with a mutant cytosine (C) in codon 338 (AAG → AAC) (arrow). This mutation results in the replacement of the normal lysine with an asparagine (L338N). The patient is heterozygous for the mutation since mutant C and normal G are present in position 1082.

since his DNA fragment is only partially digested while that of the unaffected father is fully digested. His affected brother and his mother, who also has a reduced $Gs\alpha$ protein activity (45.5%) but normal calcium metabolism and no brachymetacarpus or other obvious signs of AHO are heterozygous for the mutation.

Discussion

This study demonstrates that patients with CH and poor mental development despite sufficient thyroid hormone substitution therapy should be considered to have a deficiency of $Gs\alpha$ protein activity, especially when they display symptoms of AHO or PHP. In our patients the diagnosis of PHP Ia was delayed because neither boy developed hypocalcemia which could have led to the correct diagnosis earlier. It is known that patients with PHP Ia have resistance to other hormones that stimulate cAMP production in target cells via the $Gs\alpha$ pathway. This explains the elevated serum TSH levels in such patients (4). However, it is unclear whether the resistance to TSH results in true hypothyroidism and whether these patients benefit from treatment with thyroid hormone.

On neonatal screening both boys had normal TSH concentrations which is in contrast to the observation made by others who found elevated TSH levels in individuals with PHP Ia at birth (7–10). When thyroid hormone substitution was initiated in the older boy, he had low peripheral thyroid hormone levels in association with an elevated serum TSH concentration

indicating true primary hypothyroidism. The younger boy was treated with thyroid hormone because it was known that his older brother had hypothyroidism. He had allegedly a normal serum TSH but low peripheral thyroid hormone levels when thyroid hormone substitution was started. However, when thyroid hormone was stopped in both boys for a period of two weeks, serum thyroid hormone levels were still within the normal range whereas the TSH levels were elevated (see Results). The ability to further respond to TRH and for the thyroid gland to respond to TSH by an increase in serum T_3 , indicates that both brothers had partial resistance to TSH rather than primary thyroid gland failure.

This finding prompted us to compare our patients with others with PHP Ia and mutation in the *GNAS1* gene and resistance to TSH or who were considered to be hypothyroid (5, 6, 10–22). Of the 13 patients who were reported to have elevated serum TSH concentrations (10, 11, 13–15, 17–19, 22, 23) eight were treated with thyroid hormone (10, 13, 15, 17, 19). Four had low thyroid hormone levels (10, 13, 14, 17), whereas in four other individuals the levels were normal (11, 15, 22, 23). In the remaining five cases thyroid hormone levels were not mentioned (18, 19). Furthermore, in the vast majority of the published cases no detailed information regarding thyroid function tests and thyroid hormone treatment is given. Thus, it is uncertain how many patients with PHP Ia and known *GNAS1* mutation had hyperthyrotrophinemia. Consequently, a conclusion with regard to a beneficial effect from thyroid hormone substitution cannot be drawn.

It is not known why patients with PHP Ia are mentally retarded. The decreased $Gs\alpha$ protein activity which leads to a reduced cAMP concentration may explain why mental deficiency is associated with Gs protein defects (24). Because serum T_3 and T_4 concentrations were within the normal ranges in both boys and thyroid hormone substitution was initiated immediately, hypothyroidism is unlikely to be the cause of the delayed mental development. This observation would be supported if individuals with pseudopseudohypoparathyroidism (PPHP) who have AHO and $Gs\alpha$ protein deficiency but no hormonal resistance frequently developed psychomotor retardation. Unfortunately, the diagnosis of PHP versus PPHP is not clearly established in many hitherto reported cases and the description of the mental development of individuals with PPHP is limited.

Defects in the $Gs\alpha$ protein may result in either loss or gain of endocrine function. So far, 52 different loss-of-function mutations have been reported (4–6, 10–23, 25–35) in more than 70 affected individuals. A complete updated list of all known mutations is available online (<http://mammary.nih.gov.aho/>). In the present study a missense mutation in codon 338 of exon 12 of the *GNAS1* gene was identified which to our

knowledge has not previously been described. Since the Gs α protein activity was severely reduced in erythrocyte membranes of both boys we demonstrate that the mutant protein is defective. It is conceivable that substitution of a leucine which has a nonpolar side chain, by an asparagine with an uncharged polar side chain could result in a conformational change leading to a defective protein. However, based on our studies we cannot predict the precise mechanism by which loss-of-function is caused by L338N. Further studies are needed to exclude a targeting defect. Furthermore, we have no explanation for the absence of mental retardation in the mother despite a similar degree of impaired Gs α function.

In conclusion, Gs α protein defects can cause CH. This diagnosis should be considered in children with hypothyroidism that have a poor mental development despite adequate treatment with thyroid hormone and who display features of AHO. Whether these patients benefit from thyroid hormone substitution remains unclear although our patients did not. Elevated TSH levels simply reflect a defect in TSH signaling and do not necessarily indicate true hypothyroidism. Thyroid hormone levels should be monitored in these patients.

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