Guanine Nucleotide-Binding Protein α Subunit Hypofunction in Children with Short Stature and Disproportionate Shortening of the 4th and 5th Metacarpals

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Key Words

Short stature · Metacarpal dysmorphology · Gsa protein · GNAS mutation · Epigenetic analysis

Abstract

Background: *GNAS* encodes the α subunit of the stimulatory G protein (Gsa). Maternal inherited Gsa mutations cause pseudohypoparathyroidism type Ia (PHP-Ia), associated with shortening of the 4th and 5th metacarpals. **Aims:** Here we investigated the Gsa pathway in short patients with distinct shortening of the 4th and 5th metacarpals. **Methods:** In 571 children with short stature and 4 patients with PHP-Ia metacarpal bone lengths were measured. In identified patients we analysed the Gsa protein function in platelets, performed *GNAS* sequencing, and epigenetic analysis of four significant differentially methylated regions. **Results:** In 51 patients (8.9%) shortening of the 4th and 5th metacarpals was more pronounced than their height deficit. No *GNAS* coding mutations were identified in 20 analysed patients, except in 2 PHP-Ia patients. Gsa activity was reduced in all PHP-Ia patients and in 25% of the analysed patients. No significant methylation changes were identified. **Conclusions:** Our find-

ings suggest that patients with short stature and distinct metacarpal bone shortening could be part of the wide variety of PHP/PPHP, therefore it was worthwhile analysing the Gsa protein function and *GNAS* gene in these patients in order to further elucidate the phenotype and genotype of Gsa dysfunction.

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Introduction

Infrequently, patients with short stature have shortening of the 4th and 5th metacarpals as is often found in Albright's hereditary osteodystrophy (AHO) [1]. This peculiar phenotype further includes classically short stature, heterotopic ossifications, mental retardation, and obesity with rounded face [1]. In some subjects it is associated with parathormone renal resistance and is known as pseudohypoparathyroidism (PHP) type Ia (OMIM:

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103580) [2–4]. The underlying cause is an inactivating mutation of *GNAS* [2]. The most well-characterized *GNAS* product is the G protein α subunit ($G\alpha$), which is encoded by exons 1–13 of *GNAS*. Although $G\alpha$ is expressed biallelically in most tissues, in the pituitary, thyroid, gonads, and the proximal renal tubule it is only maternally expressed and paternally silenced by imprinting [5, 6]. Thus, only maternal transmission of *GNAS* mutations leads to PHP-Ia with altered Gs-coupled pathway by the indicated hormones [7–10].

PHP-Ib (OMIM: 603233) is associated with a loss of methylation at the *GNAS* exon A/B (also called 1A). Apart from $G\alpha$, the *GNAS* locus gives rise to different products through the use of alternative promoters and first exons that splice onto a common set of downstream exons. These additional gene products are oppositely imprinted: extra-large stimulatory $G\alpha$ (XLas), NESPas, and A/B are paternally expressed and maternally silenced by methylation, whereas neuroendocrine secretory protein-55 (NESP55) is maternally expressed and paternally silenced [11–14]. Methylation changes in the NESP55, NESPas, and XLas promoter regions have been previously described in patients with a sporadic form of PHP-Ib [15], except for in a few families with additional NESP55 deletions [16].

Interestingly, recent studies reported PHP-Ia patients with no coding mutations in the *GNAS* gene, which is similar to methylation defects in PHP-Ib patients [17–19]. These data confirm the presence of an overlap between different forms of PHP at the clinical and molecular level.

Considering the complex regulated $G\alpha$ pathway, we hypothesized that this pathway is more frequently involved in the aetiology of short stature with disproportionate metacarpal shortening than currently appreciated. Furthermore, we investigated $G\alpha$ protein activity in freshly isolated platelets and analysed mutations within the $G\alpha$ coding exons and performed epigenetic studies of the *GNAS* locus.

Patients and Methods

Patients

We included 571 patients with short stature (height <-2 standard deviation score (SDS)) who presented consecutively between 2003 and 2005 in the paediatric endocrinology outpatient clinic of the University Children's Hospital Heidelberg, Germany, with the following diagnoses, confirmed as recommended by standard national guidelines: idiopathic short stature (ISS), small for gestational age (SGA), growth hormone deficiency (GHD), and Turner syndrome (TS) (fig. 1). Chronological ages, heights, height SDS, weights, and bone ages according to the method of Greulich and Pyle were documented [20–23]. Four patients with PHP-Ia were analysed, serving as positive

Fig. 1. Participant profile.

controls. Radiographs of the left hand previously made at routine controls to evaluate bone age were available for all 575 patients including the 4 patients with PHP-Ia. The length of metacarpals (1–5) and phalanges (distal, medial, proximal) were measured and expressed as SDS for bone age using references of healthy children [24].

Patients with significant shortening of the 4th and 5th metacarpals in relation to their height (mean 4th and 5th metacarpal length SDS <-2 , height SDS <-2 , and ratio of height SDS to mean 4th and 5th metacarpal length SDS ≤ 1) were selected. From these selected patients, 20 were presented randomly and consecutively in our outpatient department from June to December 2006 and agreed to further participation in this study. Blood samples were collected in order to analyse $G\alpha$ protein function, for *GNAS* sequencing, and epigenetic analysis (fig. 1). Additionally, 4 healthy children with normal stature and without disproportionate shortening of the 4th and 5th metacarpals were included, serving as negative controls analysing $G\alpha$ protein activity.

The study protocol was approved by the institutional review board, and $G\alpha$ protein activity, *GNAS* gene, and epigenetic analysis were performed after written consent was obtained.

Gα Protein Activity

In order to evaluate $G\alpha$ hypofunction, measurement of platelet $G\alpha$ function by collagen-induced platelet aggregation in the presence of $G\alpha$ -coupled receptor agonist (prostaglandin E_1) was performed in freshly isolated platelets as previously described [25, 26]. Additionally, $G\alpha$ protein activity was measured in 4 patients suffering from PHP-Ia, serving as positive controls.

GNAS Gene Sequencing

Genomic DNA was extracted from whole blood samples using a QIAamp Blood Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The *GNAS* gene (exons 1–13) was then amplified by PCR using specific primers as previously described [7, 27]. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). Sequencing of the PCR products using both sense and antisense primers was performed using an ABI PRISM Big Dye Terminator Kit following the protocol of the supplier (PE Applied Biosystems, Foster City, Calif., USA). Sequencing products were analysed on an ABI Prism 310 sequencer (PE Applied Biosystems).

Methylation Analysis of the GNAS Locus by Methyl-DNA Immunoprecipitation

Differential methylation analysis of DMRs within the *GNAS* gene A/B, NESP55, NESPas, and XLas was performed in a group of patients as described before [28]. Briefly, 5.5 µg of DNA was sonicated to produce a random fragment size of 500 bp. After denaturation for 10 min at 95°C, immunoprecipitation was performed overnight at 4°C with 10 µl monoclonal antibody against 5-methylcytidine (Eurogentec, Cologne, Germany) in a final volume of 500 µl IP buffer (10 mM sodium phosphate, pH 7.0, 140 mM NaCl, 0.05% Triton X-100). The reaction was incubated with 30 µl of blocked Dynabeads M-280 sheep antibody to mouse IgG (DynaL Biotech, Hamburg, Germany) for 2 h at room temperature and washed three times with 700 µl of IP buffer. After incubation with proteinase K for 3 h at 55°C, methylated DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation.

PCR primers for the amplification of the four different *GNAS* DMRs were previously described [29]. The primers were adapted for methyl-DNA immunoprecipitation (MeDIP) analysis as following: A/B DMR forward 5'-ATGGCCATGAAGCTCAAAGC-3' and reverse 5'-GCCGTCTTCTCTCGCTCT-3'; NESPas DMR forward 5'-GGGCTAAAGGAGCTGACTGA-3 and reverse 5'-T AGGGTGGTTTCGAGACTGG-3'; XLas DMR forward 5'-GAC GACACTCCCGTCAACAT-3' and reverse 5'-TTCCATCTCTG CTGCTTCCT-3'; NESP55 DMR forward 5'-CTTCCAAAAAG GGACCCATC-3' and reverse 5'-TGGGCAACTAACCTGAAT CC-3'. Additionally the CpG island up- and downstream of *GNAS*-AB was analysed using the following primers: AB_1 forward 5'-CCCCCAATTCTCTCTCTTTC-3' and reverse 5'-TGGCA GGAGTCTGTTTACCC-3'; AB_2 forward 5'-TTCCTTTTCG GTTGCTCTGT-3' and reverse 5'-AGAGCCAGAGAGCAGG AGTG-3'; and AB_3 forward 5'-TGCCTCGGGAACAGTAAG AC-3' and reverse 5'-GTCCTTCTGCAGCTGCTTCT-3'.

Real-time PCR on MeDIP DNA was carried out in triplicate using an ABI PRISM 7700 PCR System (Applied Biosystems) and AbBsoLute SYBR Green ROX Mix (ABgene, Lafayette, Colo., USA).

Statistical Analysis

Statistical analysis was performed using Kruskal-Wallis and Scheffé's test (StatView; SAS Institute, Cary, N.C., USA). A *p* value <0.05 was considered significant.

Results

A total number of 571 patients (301 males, 270 females) with different aetiologies of short stature were identified (fig. 1). Significant differences regarding chronological age, bone age, and height SDS were noticed between different groups of short stature patients, showing that patients with GHD and SGA were significantly younger compared to patients with ISS (data not shown). Concerning height SDS, patients with ISS had a less severe height SDS deficit compared to patients with SGA, TS, and GHD, but comparable to that observed in patients with PHP-Ia (data not shown). Further, no typical signs of AHO (as

Fig. 2. Radiographs of the left hand of patients with (a) PHP-Ia (ratio of height SDS to mean 4th and 5th metacarpal length SDS 0.31) and (b) short stature (ratio of height SDS to mean 4th and 5th metacarpal length SDS 0.79).

round face, obesity, subcutaneous calcification, or intellectual disabilities) or abnormalities in the serum calcium or phosphate metabolism were documented. No significant differences concerning length of the metacarpal bones and their ratio to height SDS were noticed between these groups (data not shown). Measurements of the metacarpal bones in all patients with short stature showed a mean reduction of the length of the 4th and 5th metacarpals of -1.16 ± 1.4 SDS and a mean height SDS of -3.29 ± 0.91 . When considering all analysed patients as a homogeneous population of patients with short stature, the loss in the length of metacarpal bones could be globally explained by the loss in height SDS. Therefore, we selected a subgroup of patients who presented a more pronounced loss of the length of 4th and 5th metacarpal bones in relation to their height. These patients were considered as presenting a disproportionate metacarpal shortening (mean 4th and 5th metacarpal length SDS <-2 , height SDS <-2 , and ratio of height SDS to mean 4th and 5th metacarpal length SDS ≤ 1) and were further analysed (fig. 1). Representative radiographs of a patient with PHP-Ia (fig. 2a) and a patient with short stature and a ratio of height SDS to mean 4th and 5th metacarpal length SDS ≤ 1 are shown (fig. 2b). Meeting inclusion criteria of disproportionate

Table 1. Auxological data of patients with short stature and short metacarpals in relation to their height

Patients	Male	Female	CA, years	BA, years	Height SDS	Mean metacarpal 4th and 5th length SDS	Ratio of height SDS to mean metacarpal 4th and 5th length SDS
ISS (n = 36)	22	14	11.2±5.4	11.1±5.8	-2.6±0.5	-3.2±0.8	0.8±0.1
SGA (n = 5)	2	3	12.3±3.8	12.1±3.5	-2.8±0.4	-3.1±0.4	0.9±0.1
TS (n = 4)		4	9.3±3.1	9.3±3.9	-2.8±0.4	-3.6±0.4	0.8±0.2
GHD (n = 6)	3	3	6.3±3.7	6.1±5.2	-3.1±0.9	-3.9±1.2	0.8±0.2
PHP-Ia (n = 4)	2	2	12.3±6.4	n.d.	-2.8±1.5	-6.4±2.2	0.4±0.1

CA = Chronological age; BA = bone age (determined according to the method of Greulich and Pyle); n.d. = not done. No statistical differences were noted as analysed by Kruskal-Wallis test between different diagnoses of short stature patients regarding age, bone age, height SDS, length of metacarpal bones, and their ratio to height SDS.

metacarpal shortening, 51 patients with short stature (27 males, 24 females) were identified with the following diagnosis: ISS (n = 36), SGA (n = 5), TS (n = 4), and GHD (n = 6) (fig. 1; table 1). This represents 8.9% of all analysed patients. Their auxological data are presented in table 1. No significant difference was observed between these groups comparing chronological ages, bone ages, height SDS, means of the 4th and 5th metacarpal length SDS, and ratios of height SDS to mean 4th and 5th metacarpal length SDS ≤ 1 .

GNAS gene sequencing and Gsa protein analysis were performed (fig. 1) in 20 randomly selected patients and in 4 patients with PHP-Ia who presented consecutively in our outpatient department with short stature and ratios of height SDS to a mean 4th and 5th metacarpal length of SDS ≤ 1 . Their auxological data are presented in table 2: ISS (n = 13), SGA (n = 1), GHD (n = 3), TS (n = 3), and PHP-Ia (n = 4). Chronological age varied from 2.0 to 17.7 years, and bone age from 1.5 to 17.0 years (table 2). No significant differences concerning chronological age and bone age between the groups were noticed. With the exception of 1 patient (No. 21) with PHP-Ia (height SDS -1.14), all patients presented with a height SDS < -2 (range -2.2 to -4.6) and a mean of the 4th and 5th metacarpal length SDS < -2 (range -2.2 to -8.7), without any significant differences between the distinct groups ($p = 0.48$ for height SDS; $p = 0.41$ for mean metacarpal length SDS).

Mutations in the *GNAS* coding exons were identified in 2 out of 4 PHP-Ia patients. Patient No. 21 harboured a mutation in exon 5 in codon 115, a nucleotide sequence insertion from CCC→CTCC, causing a frameshift mutation with a premature stop codon at position 139 and patient No. 22 presented a 4-bp deletion ΔGACT in codon

189/190 with a stop codon at position 202 in exon 7. No further mutations in the *GNAS* coding exons were detected in this cohort of patients with short stature and disproportionate metacarpal shortening (table 2). In patient No. 3, two silent mutations, one in exon 7, codon 185 (ATC→ATT) and one in exon 13, codon 371 (AAC→AAT) were present. In patient No. 9 we identified one base substitution from G→A in intron 7.

Further we investigated in our selected patients with disproportionate metacarpal shortening the Gsa protein activity in freshly isolated platelets. As expected, all examined patients with PHP-Ia presented a reduction of Gsa protein activity (fig. 3). Interestingly, 4 out of 16 investigated patients with short stature and disproportionate metacarpal shortening (representing 25%) also showed a reduction of the Gsa function comparable to that of patients with PHP-Ia (fig. 3). This group included 2 patients with ISS, 1 with TS, and 1 with GHD. Gsa activation was also measured in the 4 healthy patients serving as negative controls (fig. 3) and showed normal activity. By this analysis, we identified a group of patients with short stature and significant short 4th and 5th metacarpals, presenting a reduction in the Gsa protein activity, without coding mutations in the *GNAS* exons.

Next we decided to analyse DMRs within the *GNAS* gene of the 4 patients with short stature and disproportionate metacarpal shortening, presenting Gsa hypofunction and of the 4 negative controls. Compared to our controls, a tendency towards reduced methylation was observed in our short stature patients, without reaching statistical significance (data not shown). Furthermore, knowing that in PHP-Ib patients the reduced Gsa expression in the proximal renal tubule is due mainly to methylation deficiencies in the A/B locus, we analysed

Table 2. Auxological data and GNAS analysis of patients with short stature and metacarpal dysmorphism and patients with PHP-Ia

Patient	Diagnosis	CA, years	BA, years	Height, SDS	GH	Mean metacarpal 4th and 5th length SDS	Ratio of height SDS to mean metacarpal 4th and 5th length SDS	GNAS variations	T393C polymorphism	Gsa activity
1	ISS	14.9	15	-2.3	no	-2.4	1.0	no mutation	TC	n.d.
2	ISS	15.1	15	-2.2	no	-2.2	1.0	no mutation	TT	reduced
3	ISS	2.2	1.5	-4.6	no	-5.0	0.9	ATC→ATT, exon 7 and AAC→AAT, exon 13, silent mutation	TT	normal
4	ISS	2.4	2.5	-2.7	no	-2.9	1.0	no mutation	TT	normal
5	ISS	13.1	11.5	-2.7	no	-2.8	1.0	no mutation	CC	normal
6	ISS	17.7	17.0	-2.8	no	-3.2	0.9	no mutation	CC	normal
7	ISS	2.4	2.0	-3.0	no	-3.8	0.8	no mutation	TT	n.d.
8	ISS	14.8	14	-3.44	no	-3.6	1.0	no mutation	TC	reduced
9	ISS	10.1	9	-2.6	no	-5.0	0.5	intron 7, 1 bp G→A	TC	n.d.
10	ISS	11.6	11.5	-2.2	no	-2.4	0.9	no mutation	TC	normal
11	ISS	2.9	2.0	-2.4	no	-3.1	0.8	no mutation	CC	normal
12	ISS	11.8	11	-2.7	no	-2.9	0.9	no mutation	TT	n.d.
13	ISS	12.5	11	-2.8	no	-3.1	0.9	no mutation	TC	normal
14	TS	11.2	11.0	-3.2	yes	-3.4	1.0	no mutation	TC	normal
15	TS	5.3	4	-2.9	yes	-3.3	0.9	no mutation	CC	reduced
16	TS	8.6	9	-2.8	yes	-4.3	0.7	no mutation	CC	normal
17	SGA	10.5	10.5	-2.9	no	-3.0	1.0	no mutation	TC	normal
18	GHD	2.1	1	-2.7	yes	-3.2	0.9	no mutation	TC	normal
19	GHD	7.2	6	-2.5	yes	-2.7	0.9	no mutation	TC	reduced
20	GHD	5.5	4	-3	yes	-3.2	1.0	no mutation	TC	normal
21	PHP-Ia	8.7	8.5	-1.14	no	-3.7	0.3	CCC→CTCC, exon 5, codon 115	TT	reduced
22	PHP-Ia	2.0	n.d.	-2.26	no	-5.7	0.4	4 bp deletion (ΔGACT) exon 7, codon 189/190	TT	reduced
23	PHP-Ia	16.2	n.d.	-3.23	no	-8.7	0.4	no mutation	TC	reduced
24	PHP-Ia	14.4	n.d.	-4.66	no	-7.8	0.4	no mutation	TT	reduced

CA = Chronological age; BA = bone age (determined according to the method of Greulich and Pyle); GH = growth hormone treatment; n.d. = not done. No significant differences regarding age and bone age between the groups with different diagnoses were noticed as analysed by Kruskal-Wallis test ($p = 0.23$, respectively $p = 0.2$).

the CpG island upstream of the *GNAS*-A/B locus in more detail. Our study showed that a region further upstream of the *GNAS*-A/B DMR within this CpG island is methylated in all investigated patients, whereas no methylation was detected within the CpG island further downstream of the *GNAS* A/B DMR (data not shown).

Discussion

In this study we identified a phenotype consisting of short stature associated with disproportionate metacarpal shortening (ratios of height SDS to mean 4th and 5th metacarpal length SDS ≤ 1). This distinct phenotype was

present in 8.9% of a large cohort of patients with short stature ($n = 571$). It is not uncommon that short stature is associated with dysmorphism of the metacarpal bones as is the case in patients with dyschondrosteosis, TS, hypochondroplasia, or brachydactyly type E [30, 31]. We assume that the complex regulated *Gsa* pathway is more frequently involved in the aetiology of short stature with disproportionate metacarpal shortening than currently appreciated. Indeed, we could demonstrate that in 4 of 16 analysed patients with disproportionate metacarpal shortening a reduction of *Gsa* protein activity was present in platelets. Because mutations of *GNAS* were detected in patients with PHP-Ia and PPHP who presenting typically AHO, we screened our cohort for mutations

Fig. 3. Platelet $G\alpha$ aggregation-inhibition test. Dose-dependent inhibition of collagen-induced platelet aggregation ($2 \mu\text{g/ml}$) by the indicated concentrations of the $G\alpha$ -coupled receptor agonist prostaglandin E_1 (ng/ml) for 4 control patients, 4 patients with PHP-Ia, and the 4 identified patients with short stature and distinct shortening of the 4th and 5th metacarpals.

within the *GNAS* gene [32]. However, no loss-of-function mutations in the *GNAS* exons (1–13) were detected.

Previously described mutations were even only identified in 50% of the PHP-Ia patients [7]. This is in accordance with prior data from patients with AHO, who presented in 85% of the cases a reduction of $G\alpha$ protein activity, whereas only 50% of these patients showed *GNAS* coding mutations [10]. One explanation could be the presence of a genetic defect outside the coding region of the *GNAS* gene, probably in the promoter region or in other regulatory regions leading to $G\alpha$ deficiency.

In patients presenting an AHO phenotype a broad epigenetic *GNAS* defect without a *GNAS* coding mutation was

detected [17, 19]. Therefore, we performed *GNAS* methylation studies in the 4 patients with reduced $G\alpha$ protein activity. In our analysis, although a slight tendency of reduced methylation in short stature patients compared to control was observed, it was not statistically significant. In our opinion, these findings cannot completely explain the reduction of $G\alpha$ activity in our patients. As we focused on regions which were previously described to be important in *GNAS* epigenetics [18], we cannot exclude that other methylation changes outside of the analysed regions could be associated with the loss of $G\alpha$ activity in our patients.

We hypothesize that patients with short stature and disproportionate short metacarpals and a reduction of

the Gsa protein activity could be part of the wide range of the different types of PHP/PPHP and a genetic overlap may exist. This hypothesis is supported by the fact that until now Gsa hypofunction has been reported to different extents in patients with PHP-Ia, PHP-Ib or PPHP [17, 25, 26]. Many authors demand a new classification of the different groups of PHP [18], which is also supported by Fernandez-Rebollo et al. [33] who reported a patient with a clinical diagnosis of PHP-Ia, reduced Gsa activity, no coding mutation on Gsa exons (1–13), and a loss of methylation in exon A/B. These investigations for the first time ruled out a large intragenic deletion within the *GNAS* locus, involving not only Gsa coding exons, but also exon A/B located upstream of Gsa [33]. By this complex deletion, the methylation defect on exon A/B, the clinical AHO phenotype, and the reduced Gsa function could be explained indicating an overlap between molecular and clinical features of the different types of PHP/PPHP. Such complex genetic changes in the *GNAS* cluster could also be involved in our patients and need to be further investigated.

An unexpected finding was that we identified Gsa hypofunction in 1 female with TS (No. 15) (table 2). In patients with TS, SHOX haploinsufficiency is thought to explain short metacarpals [34]. The disproportionate shortening of the metacarpals in this individual was not as severe as it was in patient No. 16 with normal Gsa

function (table 2). Therefore, we hypothesize that SHOX haploinsufficiency and Gsa hypofunction are not additively causing short metacarpals and presumably both signalling pathways lead to one unknown mechanism. This theory must however be confirmed by experimental evidence.

To summarize, we describe a distinct clinical phenotype characterized by short stature and pronounced shortening of the 4th and 5th metacarpal length in relation to their height. We hypothesize that these patients could be part of the wide variety of PHP/PPHP, because Gsa hypofunction was detected in 25% of the patients. Analysis of the 1–13 coding exons of the *GNAS* gene and epigenetic analysis do not reveal any mutations or significant epigenetic changes in these patients. However, we think it is worthwhile analysing in patients with short stature and disproportionate metacarpal shortening the Gsa protein activity, *GNAS* gene, epigenetic alteration, and intragenic deletion in the *GNAS* cluster to further elucidate the phenotype and genotype of Gsa dysfunction.

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