Heterozygous Mutations in Natriuretic Peptide Receptor-B (NPR2) Gene as a Cause of Short Stature in Patients Initially Classified as Idiopathic Short Stature


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Context: Based on the stature observed in relatives of patients with acromesomelic dysplasia, type Maroteaux, homozygous for mutations in natriuretic peptide receptor B gene (NPR2), it has been suggested that heterozygous mutations in this gene could be responsible for the growth impairment observed in some children with idiopathic short stature (ISS).

Objective: The objective of the study was to investigate the presence of NPR2 mutations in a group of patients with ISS.

Patients and Methods: The NPR2 coding region was directly sequenced in 47 independent patients with ISS. The functional consequences of NPR2 nonsynonymous variations were established using in vitro cell-based assays.

Results: Three novel heterozygous NPR2 mutations were identified: c.226T>C (p.Ser76Pro), c.788G>C (p.Arg263Pro), and c.2455C>T (p.Arg819Cys). These allelic variants were not found in our controls or in the 1000 Genomes database. In silico analysis suggested that the three missense mutations are probably damaging. All of them were selected for in vitro functional evaluation. Cells transfected with the three mutants failed to produce cyclic GMP after treatment with C-type natriuretic peptide. Cells cotransfected with mutant and wild-type-NPR-B (1:1) showed a significant decrease in cGMP levels after C-type natriuretic peptide stimulation in comparison with cells cotransfected with empty vector and wild type, suggesting a dominant-negative effect. These three mutations segregated with short stature phenotype in an autosomal dominant pattern (height SD score ranged from −4.5 to −1.7). One of these patients and two relatives have disproportional short stature, whereas in another patient a nonspecific skeletal abnormality was observed. All three of these patients were treated with recombinant human GH (33–50 μg/kg-d) without significant height SD score change during therapy.

Conclusions: We identified heterozygous NPR2 mutations in 6% of patients initially classified as ISS. Affected patients have mild and variable degrees of short stature without a distinct phenotype. Heterozygous mutations in NPR2 could be an important cause of nonsyndromic familial short stature. (J Clin Endocrinol Metab 98: E1636–E1644, 2013)
Longitudinal bone growth occurs at the growth plate by endochondral ossification. This process, involving chondrocyte proliferation and differentiation and extracellular matrix secretion, is regulated by many hormonal and local factors (1). Over the past several years, C-type natriuretic peptide (CNP) and its receptor [natriuretic peptide receptor-B (NPR-B); online inheritance in man (OMIM) *108961] system has emerged as an important regulator of endochondral bone growth (2). NPR-B is a homodimeric receptor that is characterized by a modular structure: an extracellular ligand-binding domain, a transmembrane region, an intracellular kinase homology domain, and carboxyl-terminal guanylyl cyclase domain (3). CNP is the main endogenous ligand of NPR-B, and this high-affinity binding produces cytoplasmic cGMP from GTP (4). The NPR-B gene (NPR2) is expressed in a variety of tissues, including the brain, the pituitary, the adrenal gland, the kidney, the lung, the uterus, the cartilage, and the growth plate (2).

Animal models showed a CNP/NPR-B role in increasing chondrocyte proliferation, matrix synthesis, and cell hypertrophy in the growth plate (5–7). In humans, genome-wide association studies have included the CNP system in the etiology of height variation (8–10). Additionally, a heterozygous gain-of-function mutation of the NPR2 causes an overgrowth disorder (11). Conversely, homozygous mutations in the NPR2 cause acromesomelic dysplasia, type Maroteaux (AMDM; OMIM *602875), a skeletal dysplasia with extreme short stature (12).

Parents of patients with AMDM, heterozygous for NPR2 mutations, were noted to be shorter than expected for their population of origin (13, 14). In one study, the average height among 30 adult heterozygous carriers of NPR2 mutations was 5.7 cm lower than that of population-matched controls (12). Another study that evaluated a single family from a patient with AMDM showed that family members heterozygous for NPR2 mutations had a height SD score (SDS) 1.4 lower than noncarrier family members (n = 16 vs 23; 95% confidence interval for a difference of means: −2.0 to −0.8) (15). Based on their results, the authors suggested that approximately 1 in 30 individuals with idiopathic short stature may carry heterozygous mutations in NPR2 (15). This suggestion was reinforced by the identification of a NPR2 mutation with a dominant-negative effect, which can cause a short stature phenotype in the heterozygous state in one AMDM family (16).

Thus, the objective of this study was to investigate the presence of NPR2 mutations in a group of patients with idiopathic short stature (ISS) unrelated to patients with acromesomelic dysplasia, and determine their phenotypic features.

Patients and Methods

Subjects

This study was approved by the local ethics committee, and the patients or guardians gave their written informed consent. Subjects in the present study included 47 independent Brazilian patients with ISS (30 males) who fulfilled the following diagnostic criteria: prenatal short stature, height SDS > −2 or less (17), unremarkable medical history, and absence of abnormal findings on clinical examination or in laboratory tests that could account for short stature (18). Allelic variants were evaluated in a sample of 72 control individuals (31% males) with normal stature (height SDS of 1.2 ± 0.7) from the same ethnic background.

Anthropometric measurements

The clinical assessment included measurements of height, sitting height, and body weight. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. The results were converted to Z-scores using age- and gender-specific normal values (19). Disproportional short stature was defined as the sitting height to height ratio (SH/H) greater than 2 SDS above the mean for age and sex (20).

Molecular studies

Genomic DNA was extracted from peripheral blood leukocytes using standard techniques. The primers were designed to amplify all exons and exon-intron boundaries according to the published NPR2 genomic DNA sequences (primer sequences and amplification protocols are available upon request) (GenBank accession number NC_000009.11 for genomic and NM_003995.3 for mRNA sequences). PCR products were bi-directionally sequenced with the dideoxy chain termination method using a dye terminator kit and analyzed in an ABI Prism 3100 automated sequencer (Applied Biosystems Inc). Defects in short stature homeobox-containing gene (SHOX, NM_000451) were ruled out according to previous published protocols in patients with NPR2 allelic variation (21). The frequency of NPR2 variants in patients with ISS was compared with that of the 1000 Genomes database ([http://www.1000genomes.org/](http://www.1000genomes.org/), May 5, 2013) by χ², and the level of significance was set at 0.05.

In silico prediction of mutation effects

To identify the potential effects of sequence variants identified in NPR2 on protein function or structure, the wild-type (WT) and variant sequences were submitted to Mutation Taster ([http://www.mutationtaster.org](http://www.mutationtaster.org)) (22) and the PolyPhen method ([http://genetics.bwh.harvard.edu/pph2](http://genetics.bwh.harvard.edu/pph2)) (23).

Functional studies

In vitro studies were performed at Keio University (Tokyo, Japan). A hemagglutinin (HA)-tagged human NPR-B construct (HA-WT-NPR-B) has been described previously (16). Mutant expression constructs (S76P, R263P, R819C) were synthesized by site-directed mutagenesis (QuickChange XL site-directed mutagenesis kit; Agilent Technologies). Mutagenic primer sequences are available on request. Sequences of the constructs were verified by direct sequencing. COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were transiently transfected with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol.
COS-7 cells, seeded in 12-well plates, were transiently transfected with WT-NPR-B, mutant NPR-Bs, or empty vector. Forty-eight hours after transfection, cells were lysed with M-PER protein extraction reagent (Pierce) in the presence of 1% protease inhibitor cocktail. The samples were loaded onto 10% SDS-PAGE gels and transferred to polyvinylidene difluoride transfer membranes. HA-NPR-B was detected by immunoblotting using a rat anti-HA antibody (1:500, 60 min at room temperature, clone 3F10; Roche Applied Science), incubated with a secondary antibody (anti-rat IgG-horseradish peroxidase conjugate, 1:2500, 30 min at room temperature), and was imaged with a molecular imager (BioRad Laboratories).

COS-7 cells, seeded in 12-well plates, were transiently transfected with WT, mutant, or empty vector (200 ng of plasmid per well). To evaluate a putative dominant-negative effect of the identified NPR-B mutants, COS-7 cells were cotransfected with constant amounts of HA-WT NPR-B cDNA (100 ng/well) plus empty vector or plus each mutant NPR-B cDNAs at a WT to mutant ratio of 1:1. Forty-eight hours after transfection, the cells were incubated with or without 100 nM CNP-22 (Bachem, Ltd) in OptiMEM (Invitrogen) for 20 minutes at 37°C. Then 0.1 M HCl was added to stop the reaction, and cells were centrifuged at 600 × g for 10 minutes. cGMP in the supernatant was measured by competitive enzyme immunoassay according to the manufacturers’ protocol (cGMP Complete; Enzo Life Science). Statistical significance was analyzed using Welch’s t test; \( P < .05 \) was considered statistically significant. All experiments were performed more than three times in triplicates.

Confocal microscopy

COS-7 cells were seeded in 12-well plates containing sterile coverslips and were transfected with either WT or mutants HA-NPR-B. Forty-eight hours after transfection, cells were washed with PBS and were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Fixed cells were incubated with a rat anti-HA antibody (clone 3F10; Roche Applied Science) at 1:100 for 30 minutes. After washing with PBS, cells were incubated with the secondary antibody (Alexa Fluor 568 goat antirat IgG; Life Technologies) at 1:100 for 30 minutes. The coverslips were then placed onto a clean slide and mounted with VECTASHIELD mounting medium with 4′,6-diamino-2-phenylindole (Vector Laboratories). Fluorescent images were taken using a confocal microscope TCS-SP5 (Leica).

Results

Patients’ characteristics

The cohort was characterized by a male predominance (64%). Seventy percent of patients had at least one parent with height SDS less than −2.0 (mean target height SDS −1.5 ± 0.7). At the age of the first evaluation (9.0 ± 3.5 y), patients had delayed bone age (7.5 ± 3.2 y), short stature (height SDS −2.8 ± 0.6), and normal BMI (BMI SDS = −0.4 ± 1.2).

Molecular results

Three different heterozygous mutations in NPR2 were identified in three male patients. The three variations are missense, and all of the variations predict amino acid changes in highly conserved residues in NPR-B. Two of them are located in the extracellular region at the ligand (CNP) binding domain [p.Ser76Pro (c.226T>C), p.Arg263Pro (c.788G>C)]. The third mutation is located between the kinase homology and the guanylyl cyclase domains [p.Arg819Cys (c.2455C>T)] (Figure 1). These allelic variants were found in neither our controls (144 alleles) nor the 1000 Genomes database (2184 alleles). An in silico analysis suggested that the three missense mutations are probably damaging. All of the identified NPR2 mutations were selected for in vitro functional evaluation. In addition, we identified eight polymorphisms that had been reported in the 1000 Genomes database (Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org).

In vitro functional assay

COS-7 cells transfected with HA-WT-NPR-B, mutant constructs (HA-S76P, HA-R263P, and HA-
R819C), or empty vector (mock) and the whole-cell extracts were resolved by SDS-PAGE and Western blotting. Western blot analysis confirmed that the three mutant proteins had comparable expressions to wild type at approximately 120 kDa (Figure 2A). It is noteworthy that the expressed variants HA-S76P and HA-R263P NPR-B are detected as a single band, whereas wild-type HA-NPR-B and HA-R819C are detected as distinct doublets.

We examined cGMP production in the cells transfected with HA-WT-NPR-B or HA mutants. Treatment with CNP at a dose of 100 nM increased intracellular cGMP levels by more than 100-fold in HA-WT-transfected cells relative to mock transfected cells. cGMP production after stimulation with CNP was significantly decreased in cells transfected with the three mutants and similar to mock-transfected cells (Figure 2B). To investigate the molecular mechanism underlying the dominant mode of inheritance of the short stature phenotype, the cGMP production was assessed by cotransfection of equal amounts of the plasmids encoding the WT and each mutant NPR-B proteins or the empty vector. Cells cotransfected with mutant cDNA showed a significant decrease in cGMP levels after CNP stimulation in comparison with cells transfected with WT receptor and empty vector (Figure 2C), thus suggesting a dominant-negative effect of all mutants over the WT.

**Confocal microscopy**

To test the hypothesis that these three missense mutations possibly cause defects in protein trafficking, the localization of NPR-B was examined in fixed COS-7 cells (Figure 3). Wild-type NPR-B was observed in cell membrane (red) as well as R819C mutant. On the other hand, S76P and R263P NPR-B were not localized in the cell membrane.

**Phenotype/genotype relationships**

**Patient with p.Ser76Pro mutation**

At the first appointment at 12.9 years, he had mild disproportionate short stature, normal BMI, bone age was compatible with chronological age and he had already started puberty (Tanner 3 pubertal stage) (Table 1). His hormonal evaluation showed normal IGF-I and IGF bind-
ing protein (IGFBP)-3 levels and a normal GH response to the clonidine test, and gonadotropin and T levels were at the pubertal range. The patient was treated with recombinant human GH (rhGH; 50 μg/kg/d) and letrozole (2.5 mg/d) since he was 13 years old (Figure 4A). His height SDS remained stable and his adult height prediction based on his bone age improved from 156 cm to 167.4 cm during therapy. The p.Ser76Pro was also identified in his short father (145 cm) and older sister (139 cm) (Figure 5A), who also has mild disproportional short stature (SH/H SDS 2.0). The patient’s mother, at 153 cm, had a normal NPR2 genotype.

**Patient with p.Arg263Pro mutation**

At his first evaluation, he was 9.4 years old and had proportionate short stature with normal BMI and delayed bone age (Table 1). He had hypothyroidism due to a sublingual thyroid, which had been correctly treated since he was 5 years old. His IGF-I and IGFBP-3 levels were within the reference range and GH peak after clonidine stimulation was normal. After treatment with depot GnRH agonist (GnRHa) for 1.5 years and rhGH (33 μg/kg/d) for 3.3 years, his final height was 158 cm (height SDS -2.1) (Figure 4B). The patient’s father (156 cm) and grandfather (150 cm) also had short stature and both were also heterozygous for the p.Arg263Pro mutation. The patient’s mother (156 cm) and sister (126 cm with 9.4 y) had a normal NPR2 genotype (Figure 5B).

**Patient with p.Arg819Cys mutation**

At his first appointment at 11.1 years, he had proportionate short stature and normal BMI, and his bone age was compatible with his chronological age (Table 1). His hormonal evaluation showed normal IGF-I and IGFBP-3 levels. A skeletal survey revealed shortened metacarpal (Supplemental Figure 1). He started puberty at the age of 12.1 years, with a height of 131 cm, an adult height prediction of 160 cm, and a combined therapy with rhGH (50 μg/kg/d or 0.15 U/Kg/d) and GnRHa was initiated. After 2.7 years of treatment, depot GnRHa was discontinued and he presented normal pubertal development. At the last clinical visit, he was 17.1 years old, his height velocity was 6.4 cm/y, and his height was 158.3 cm (height SDS 2.4 SDS), and he is still on rhGH treatment (Figure 4C). His adult height prediction improved to 163.5 cm. The patient’s father (height 175 cm) had a normal NPR2 genotype. His mother (height 152 cm), who has altered body proportions (SH/H SDS +3.3), had the p.Arg819Cys mutation (Figure 5C).

**Discussion**

Approximately 80% of the variation in human height is explained by heritable factors (24). Although common polymorphisms are significantly associated with height in healthy individuals, there is evidence that relatively rare genetic variants are involved with larger effects on stature
Figure 4. Growth chart of the patients with heterozygous NPR2 mutations: p.Ser76Pro (A), p.Arg263Pro (B), and p.Arg819Cys (C). The rhGH, letrozole, and/or GnRH analog therapies are indicated.
Up to now, defects in few genes were associated with idiopathic short stature phenotype (20, 26–30). Several lines of evidence indicate that the CNP/NPR-B pathway plays an important role in chondrocyte development and linear growth (2, 5–7, 11).

The action of the natriuretic peptide system on longitudinal growth is partially explained by the capacity of NPR-B signal transduction to inhibit fibroblast growth factor receptor-3 (OMIM 134934) downstream signaling at the level of the MAPK cascade (31). Activating mutations in FGFR3, which promote a sustained activation of the MAPK pathway, are responsible for achondroplasia (OMIM 100800), a common short-limb dwarfism, and its milder form, hypochondroplasia (OMIM 146000) (32). CNP and its analogs are under investigation in animal models of achondroplasia to improve the dwarfism and skeletal clinical features, with promising results (33, 34). Additionally, SHOX (OMIM 312865) strongly induces the expression of the NPPB gene, which encodes brain natriuretic peptide, another important ligand of NPR-B (35). Isolated SHOX haploinsufficiency is observed in 1%–14% of children with ISS (36). These data suggest an involvement of natriuretic peptide system on well-established pathways involved in different short stature conditions.

Based on the short stature of relatives of patients with AMDM, it has been suggested that heterozygous mutations in NPR2 could be responsible for the short stature observed in some children classified as ISS (15, 16). In the present study, we identified functionally relevant NPR2 mutations in 3 of 47 ISS children. The rate of probably damaging NPR2 variant detection in the cohort of patients classified as ISS (3 of 47 patients, 6.4%) was significantly higher than the rate in the 1000 Genomes database (10 of 1092 subjects, 0.9%; \( P = 0.006 \)). A loss of NPR-B function caused by these three missense mutations was confirmed by the lack of elevation of guanylate cyclase activity after CNP stimulation (Figure 2B). Furthermore, an effect of a heterozygous state was confirmed based on the dominant-negative effect demonstrated by the cotransfection WT/mutant (Figure 2C). Confocal microscopy analysis demonstrated that p.Arg819Cys mutant seems to have at least some normal trafficking to the plasma membrane, whereas p.Ser76Pro and p.Arg263Pro loss of function possibly results from the endoplasmic reticulum retention of receptor, a mechanism
that was already shown for other missense NPR2 mutations (37). In our experiments, WT NPR-B is expressed as a doublet band (Figure 2A), which reflects different glycoforms (38, 39). Conversely, mutant p.Ser76Pro and p.Arg263Pro NPR-B were expressed as a single band, suggesting an impairment of glycosylation. The truncated form of NPR-B lacking the catalytic domain (NPR-B-490), which is partially glycosylated, decreases the WT receptor activity in a negative dominant fashion, probably preventing the WT receptor to reach the cell surface (38). It is possible that the same could explain the negative dominant effect of p.Ser76Pro and p.Arg263Pro mutants. Additionally, we could postulate that the p.Arg819Cys mutant forms a heterodimer with WT NPR-B, thereby interfering its signal transduction in a dominant-negative fashion, as previously suggested for a mutation localized in the kinase homology domain (16). However, further studies are needed to fully understand the mechanisms of the dominant-negative effect of these NPR2 mutations.

The identified NPR2 mutations segregate with short stature phenotype in a dominant inheritance pattern (Figure 5). A variable phenotype considering the differences among short stature severity, body proportion, and some nonspecific skeletal abnormalities was observed, even within members of the same family. This variable phenotype is also observed in families with isolated SHOX deficiency (36). As an example, within the family with the NPR2 p.Arg819Cys mutation, the patient has proportional short stature, whereas his mother, who has height at the low normal limit, has altered body proportions. It is noteworthy that all three patients did not have significant height SDS change during therapy with rhGH (Table 1 and Figure 4). Nevertheless, all these patients started rhGH therapy around the age of 13 years when puberty had already started, which could contribute to the observed poor response during treatment. Previously, rhGH therapy was described in one patient homozygous for NPR2 mutation, whose height SDS decreased from –6.3 to –7.2 during 4 years of rhGH therapy (50 µg/kg/d) (15).

We identified heterozygous NPR2 mutations in 6% of patients initially classified as ISS. In contrast with homozygous mutations in this gene, which produces a severe short stature and distinguishable phenotype, heterozygous mutations in NPR2 are associated with mild and variable growth impairment without distinct phenotype. Our results suggest that heterozygous mutations in NPR2 could be an important cause of nonsyndromic familial short stature. Although our study was based on a relatively small number of patients, the present results raise the possibility to explain the postnatal growth impairment observed in several children classified as ISS. Analyses of larger cohorts are needed to explore the role of heterozygous NPR2 defects in patients with ISS and its impact on growth promoting therapies.

Acknowledgments

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