Mutations in C-natriuretic peptide (*NPPC*): a novel cause of autosomal dominant short stature

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Purpose: C-type natriuretic peptide (CNP) and its principal receptor, natriuretic peptide receptor B (NPR-B), have been shown to be important in skeletal development. CNP and NPR-B are encoded by natriuretic peptide precursor-C (*NPPC*) and natriuretic peptide receptor 2 (*NPR2*) genes, respectively. While *NPR2* mutations have been described in patients with skeletal dysplasias and idiopathic short stature (ISS), and several *Npr2* and *Nppc* skeletal dysplasia mouse models exist, no mutations in *NPPC* have been described in patients to date.

Methods: *NPPC* was screened in 668 patients (357 with disproportionate short stature and 311 with autosomal dominant ISS) and 29 additional ISS families in an ongoing whole-exome sequencing study.

Results: Two heterozygous *NPPC* mutations, located in the highly conserved CNP ring, were identified. Both showed significant

reductions in cyclic guanosine monophosphate synthesis, confirming their pathogenicity. Interestingly, one has been previously linked to skeletal abnormalities in the spontaneous *Nppc* mouse long-bone abnormality (*lbab*) mutant.

Conclusions: Our results demonstrate, for the first time, that *NPPC* mutations cause autosomal dominant short stature in humans. The *NPPC* mutations cosegregated with a short stature and small hands phenotype. A CNP analog, which is currently in clinical trials for the treatment of achondroplasia, seems a promising therapeutic approach, since it directly replaces the defective protein.

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INTRODUCTION

C-type natriuretic peptide (CNP) and its principal receptor, natriuretic peptide receptor B (NPR-B), have been shown to be important in cartilage homeostasis and endochondral bone formation.^{1–4} Binding of CNP to the NPR-B receptor induces cyclic guanosine monophosphate (cGMP) synthesis, activating an intracellular signaling cascade that mediates its effects.^{5,6} In humans, CNP and NPR-B are encoded by the natriuretic peptide precursor C (*NPPC*; MIM 600292) and natriuretic peptide receptor 2 (*NPR2*; MIM 108961) genes, respectively. Homozygous defects in *NPR2* cause a severe skeletal dysplasia, acromesomelic dysplasia Maroteaux type (MIM 602875), which is characterized by severe short stature (<5 standard deviations from the mean (SDS) in adults), phalangeal–metacarpal abnormalities, and shortening and bowing of the limbs.⁴ Interestingly, heterozygous *NPR2* defects have been described in some idiopathic short stature (ISS; MIM 616255) cases, and in patients presenting with some clinical features similar to Léri–Weill

dyschondrosteosis (LWD; MIM 127300), such as shortening of the limbs and metacarpals, bowing of the radius, cleft palate and muscular hypertrophy, but lacking the characteristic Madelung deformity typically associated with LWD.^{7–10}

In contrast, no *NPPC* mutations have been observed in humans to date, although the importance of *Nppc* for skeletal growth has been clearly demonstrated in mice. The *Nppc* knockout mouse is characterized by a severe disproportionate form of dwarfism, associated with shortening of the limbs and endochondral ossification defects resulting in postnatal death.¹ Cnp overexpression leads to overgrowth in transgenic mice.¹¹ In humans, several genome-wide association studies have established a relationship between *NPPC* and height.^{12–14} Furthermore, it has been hypothesized that CNP haploinsufficiency may be the cause of the short stature observed in an individual with a 1.9 Mb deletion in chromosome 2q37, which spans 20 genes, including *NPPC*.¹⁵ Conversely, *NPPC* overexpression due to balanced translocations have been observed in individuals with tall stature and

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long hands, feet, and halluces, together with other skeletal defects.¹⁶

Since similar under- or overgrowth phenotypes have been observed in mice with *Nppc* or *Npr2* mutations, and *NPR2* mutations have been associated with short stature and skeletal defects in humans, we hypothesized that *NPPC* mutations might be associated with similar phenotypes in humans.

Here we present, for the first time, the identification of heterozygous mutations in *NPPC* in two families with short stature and small hands. Both mutations are located within the highly conserved ring structure of CNP. They failed to activate NPR-B in the homozygous state, and showed a significant reduction in cGMP synthesis in the heterozygous state. Interestingly, one of the identified *NPPC* missense mutations, p.Arg117Gly, is identical to that described in the spontaneous *Nppc* long-bone abnormality (*lbab*) mouse mutant, with shortening of long bones and smaller growth plates. A CNP analog that directly replaces the defective protein is currently in clinical trials for the treatment of achondroplasia and seems a promising therapeutic approach for patients with *NPPC* mutations causing short stature.

MATERIALS AND METHODS

Cohorts

We undertook *NPPC* mutation screening in two cohorts of short stature patients. All participants provided informed consent for the performed studies and ethical approval was obtained from the respective institutions.

The first cohort consisted of 668 patients with disproportionate short stature ($n=357$) or a diagnosis of ISS (i.e., proportionate short stature and no hormone deficiencies; $n=311$). *SHOX* and *NPR2* alterations were previously excluded. A second cohort consisting of 29 families with ISS in which *SHOX* and *NPR2* alterations had also been previously excluded was screened in the context of an ongoing whole-exome sequencing study.

Family descriptions

Family 1

In family 1, the proband was a 12-year-old Spanish girl (III.1), who was referred to her local endocrinology clinic for short stature (-2.3 SDS; **Figure 1**). Bone age was concordant for chronological age and her insulin-like growth factor 1 (201 ng/ml; -1.27 SDS) and insulin-like growth factor binding protein 3 (-0.72 SDS) levels were normal. She had short hands and feet, a short fourth metacarpal, and hypertelorism. Her mother had a height of 148.6 cm (-2.6 SDS) with small hands, and her father also had short stature (164.3 cm; -2.0 SDS), but normal hand length. The younger sister, aged 11.5 years, had normal height (147.0 cm; -0.4 SDS) and hand length. All relatives had normal sitting height: height ratios and armspan:height ratios, indicating that they all had proportionate stature. At 13.5 years of age, the proband still presents with proportionate short stature (-2.3 SDS) and the same dysmorphic features described at presentation. Further clinical details and anthropometric

measurements of the proband and family members are shown in **Table 1**, **Figure 1** and **Supplementary Figure S1** online.

Family 2

In family 2, the proband was a Brazilian girl (II.1), now aged 21, with short stature (145.0 cm; -2.9 SDS) and small hands after undergoing normal puberty. She is the older sister of a Brazilian male (II.2) who was referred to an endocrinology clinic for proportional short stature and delayed puberty. He was diagnosed with constitutional delay of growth (height: 141.0 cm; -2.77 SDS) and puberty, and treated with testosterone to induce puberty at the age of 14.6 years (50 mg of testosterone cypionate intramuscularly every 4 weeks for 3 months). His current height at the age of 17.2 years is 161.5 cm (-2.0 SDS) and he continues to grow. Their father has short stature (158.8 cm; -2.4 SDS) and small hands, while their mother has normal height (157.5 cm; -0.8 SDS) and no other relevant clinical history. Further clinical details and anthropometric measurements of the proband and family members are provided in **Table 1** and **Supplementary Figure S1**.

Genetic analysis

In cohort 1, the coding sequences and intron:exon boundaries of *NPPC* (NM_024409.3) were analyzed by Sanger sequencing (oligonucleotides and PCR conditions are available on request). Conservation and pathogenicity prediction analysis of the identified *NPPC* variants was carried out using combined annotation-dependent depletion (CADD) version 1.3 (<http://cadd.gs.washington.edu/>) and Alamut version 2.8 software (Interactive Biosoftware, France), which included sorting intolerant from tolerant (SIFT; <http://sift.bii.a-star.edu.sg>), Polyphen (<http://genetics.bwh.harvard.edu/pph2>), MutationTaster (<http://www.mutationtaster.org>) and splicing predictor analyses. Population frequencies of the detected variants were assessed using the Exome Aggregation Consortium (ExAC) browser (<http://exac.broadinstitute.org>), 1000 Genomes data (<http://www.internationalgenome.org>) and 264 Spanish normal-height controls (Banco de ADN, Salamanca University).

In cohort 2, exome sequencing (SureSelect Target Enrichment System, Agilent Technologies, Santa Clara, CA) was undertaken according to standard protocols and sequences were generated on an Illumina HiSEQ 2500 platform running on paired-end mode. Reads were aligned to the hg19 assembly of the human genome with the BWA-MEM aligner. Duplicated reads were flagged with biobambam2. Variant calling was performed with freebayes and the resulting variant call formats were annotated with ANNOVAR (<http://annovar.openbioinformatics.org>). Nonsynonymous or splice-site variants were then selected based on the pedigree and the familial short stature. Subsequently, our variant filtering focused on variants absent in controls (ExAC, 1000 Genomes and our in-house databases), highly conserved (GERP++; <http://mendel.stanford.edu/SidowLab/downloads/gerp>) and

Figure 1 Growth curve of the female proband from family 1. The growth curve is based on 2010 Spanish height data (<http://www.aeped.es/noticias/estudios-espanoles-crecimiento-2010>). The colored lines represent the growth percentiles.

predicted to be pathogenic by multiple *in silico* programs: Polyphen2, Mutation Assessor (<http://mutationassessor.org/r3>), SIFT, PROVEAN (<http://provean.jcvi.org/index.php>) and CADD version 1.3 (**Supplementary Figure S1**). Variants were validated by Sanger sequencing.

Plasmid constructions

The Myc-tagged expression constructs containing the detected *NPPC* mutants were generated by site-directed mutagenesis according to the manufacturer (QuikChange Site-Directed Mutagenesis Kit; GE Healthcare, Uppsala, Sweden) of pMyc-CNP (NM_024409; Origene Technologies, Rockville, MD). Mutagenesis primers (mutated base underlined) for the p.Arg117Gly (R117G) mutation were 5'-GGCCTCAAGCTGGACGGAATCGGCTCCATGAGC-3', and for the p.Gly119Cys (G119C) mutation they were 5'-GGCCTCAAGCTGGACCGAATCTGCTCCATGAGC-3'. All constructs were verified by sequencing.

cGMP synthesis

COS-7 cells were transfected with 500 ng of the complementary DNA of *NPR2* wild type (pHA – NPR-B) or empty vector (pHA) and 500 ng of the complementary DNA of *NPPC* wild type, one of the two mutations, or a combination of the wild type and one of the mutations (250 ng + 250 ng), thus mimicking the heterozygous state, using a 3:2 FuGene 6 (Promega, Madison, WI):DNA ratio according to the manufacturer's instructions. After 48 h posttransfection, 0.1 M hydrogen chloride was added to stop endogenous phosphodiesterase activity, to stabilize the released cGMP, and to lyse the cells. Cell lysates were centrifuged at 600 g for 10 minutes and the supernatants were isolated.

The cGMP levels in the supernatants were measured using the cGMP Complete ELISA kit (ENZO Life Sciences, Farmingdale, NY). Each construct combination was transfected in triplicate, each transfection was assayed in triplicate, and three biological replicates were performed. Student's *t*-tests, using SPSS version 15.0 for Windows, were carried out

Table 1 Anthropometric and clinical features of the individuals from the two families with mutations in *NPPC*

<i>NPPC</i> genotype	Family member	Height in cm (SDS)	Weight in kg (SDS)	Arm span (cm)	Arm span height	Sitting height (cm)	Sitting height	Hand length in cm (SDS)	Other clinical features
Family 1: p.Arg117Gly									
Heterozygous	Proband (III.1) (13.5 years, Tanner 5)	144.7 (−2.3)	54.5 (+0.2)	142.4	0.984	79.0	0.546	16.5 (−3.7)	Small hands and feet, short fourth metacarpal, cubitus and genu valgus, lower-limb hypertrophy, and hypertelorism
Heterozygous	Mother (II.5)	148.6 (−2.6)	64 (+0.8)	144.4	0.971	80.0	0.539	16.0 (−4.5)	Small hands and feet
Wild type	Father (II.4)	164.3 (−2.0)	90 (+1.4)	172.0	1.046	87.5	0.533	19.0 (−1.1)	Hypertelorism, bulky aspect
Wild type	Sister (III.2) (11.5 years, Tanner 3)	147.0 (−0.4)	52.5 (+1.0)	145.5	0.990	76.3	0.524	15.5 (−1.4)	
Wild type	Maternal grandfather (I.3)	146.5 ^a (−4.8)	58 (−1.5)	156.0	1.065	81.0	0.553	15.0 ^b (−4.2)	Arthritis
Heterozygous	Maternal grandmother (I.4)	138.5 ^a (−4.3)	57 (−0.1)	144.0	1.040	71.0	0.513	15.5 (−5.2)	Small hands
Heterozygous	Maternal Aunt (II.6)	146.5 (−2.9)	50 (−0.9)	149.0	1.017	79.0	0.539	15.0 (−6.0)	Small hands
Family 2: p.Gly119Cys									
Wild type	Proband (II.2) (17.2 years, Tanner 4)	160.5 ^c (−2.1)	47.5 (−2.9)	161.5	1.022	78.5	0.496	18.4 (−1.3)	Delayed puberty
Heterozygous	Sister (II.1)	145.0 (−2.9)	40 (3.0)	145.8	1.006	75.0	0.517	17.2 (−2.1)	Small hands
Heterozygous	Father (I.1)	158.8 (−2.4)	65.9 (−0.4)	153.3	0.965	81.0	0.510	17.7 (−2.1)	Small hands
Wild type	Mother (I.2)	157.5 (−0.8)	72 (+1.1)	156.0	0.990	79	0.502	17.9 (−1.6)	

SDS values are according to age and sex for the national standards. Family 1 was Spanish, while family 2 was Brazilian. All patients and their family members had proportionate growth. Children's ages and Tanner stages are indicated.

^aAge influenced height loss, thus ratios must be taken with precaution. ^bHand length is inaccurate due to arthritis. ^cThe patient is still growing and has been diagnosed with constitutional delay of growth and puberty. He started puberty after a short-term treatment with testosterone cypionate (50 mg per month for 3 months) at the age of 14.9 years.

to compare the differences between wild type and mutants. A *P* value of <0.05 was considered statistically significant.

RESULTS

Two heterozygous *NPPC* variants, p.Arg117Gly and p. Gly119Cys, were detected in two unrelated short stature individuals (Figure 2 and Table 2). The *NPPC* variant, p. Arg117Gly (family 1) was detected in the autosomal dominant ISS cohort. It affects an amino acid within the ring structure of CNP, which is highly conserved between the three natriuretic peptides, CNP, brain natriuretic peptide (BNP), and atrial natriuretic peptide (ANP; Figure 2).¹⁷ In silico analysis predicts the variant to be deleterious (Table 2). The variant is absent from the large population databases (Exome Aggregation Consortium and 1000 Genomes) and from 264 Spanish individuals with normal stature (−2 to +2 SDS) from the DNA bank at the University of Salamanca (<http://www.bancoadn.org/>). No further mutations were identified after additional screening of this patient using a targeted next-generation sequencing skeletal dysplasia panel (SKELETALSEQ.V4; *n* = 327 genes).

The heterozygous *NPPC* variant c.349C > G (p.Arg117Gly) was identified in the proband of family 1 (III.1), who had short stature (−2.3 SDS), small hands and feet, shortening of the fourth metacarpals, and hypertelorism. Subsequent analysis confirmed the presence of the *NPPC* mutation in the similarly affected mother, maternal grandmother and aunt, while it was absent in the examined family members with normal stature (Figure 2a and Table 1).

The second heterozygous *NPPC* variant, c.355G > T (p.Gly119Cys), was detected by WES in a Brazilian adult female (II.1) and her father (family 2), both of whom presented with short stature and small hands (Figure 2b and Table 2). The variant was absent from the normal stature mother and the short stature brother (II.2), who was diagnosed with constitutional growth and pubertal delay and who has a predicted adult height within the normal range (Supplementary Figure S3). Both the mother and the brother had normal hand size. The p.Gly119Cys variant also affects a highly conserved amino acid located in the CNP ring, two amino acids adjacent to the mutation present in family 1 (Figure 2c). This amino acid is conserved between CNP and ANP, but not BNP. The c.355G > T variant is absent from the large population databases and *in silico* analyses predict the variant to be pathogenic (Table 2). No additional pathogenic variants were identified by WES to explain the short stature in the proband or her family (Supplementary Figure S2, Tables S1 and S2).

In order to functionally confirm the predicted pathogenicity of the identified *NPPC* variants, we analyzed their ability to activate the NPR-B receptor by assessing the CNP-dependent guanylate cyclase. Both *NPPC* variants, p.Arg117Gly and p. Gly119Cys, failed to activate NPR-B in the homozygous state and showed a significant reduction in cGMP synthesis in the heterozygous state when cotransfected (ratio 1:1) with the wild-type construct (Figure 3).

Figure 2 Pedigrees and cosegregation analysis of the two families carrying the *NPPC* variants. Cosegregation analysis of the heterozygous *NPPC* variants, c.349C > G (p.Arg117Gly) from family 1 (a) and c.355G > T (p.Gly119Cys) from family 2 (b). *NPPC* genotype (N: wild type and M: mutated allele) and height SDS are indicated below each individual. The height SDS for individual II.2 (b) is currently on rhGH treatment. Individuals with short stature and small hands or short stature only are indicated with black and gray symbols, respectively. Probands are indicated by arrows. (c) Schematic representation of the CNP-22 functional ring showing the localization of the two mutated amino acids, p.Arg117Gly and p.Gly119Cys, in red and blue, respectively. Arg117 is conserved in all three natriuretic peptides (ANP, BNP and CNP), while Gly119 is conserved in CNP and ANP.

DISCUSSION

Our results demonstrate, for the first time, that *NPPC* mutations cause autosomal dominant short stature in humans. In both families, the *NPPC* mutation cosegregated with the short stature and small hands phenotype, and was absent in unaffected individuals, thus indicating complete penetrance. The profound negative effect on cGMP generation shown by the functional assessment of both mutations further corroborates these observations.

Interestingly, one of the identified *NPPC* missense mutations, p.Arg117Gly, is identical to that described in the spontaneous *Nppc* long-bone abnormality (*lbab*) mouse mutant.¹⁸ The *lbab/lbab* mouse is characterized by dwarfism due to the shortening of long bones and growth plate narrowing, especially in the proliferative and hypertrophic zones.^{19,20} Although the heterozygous mice (+/*lbab*) appeared to show no remarkable phenotype, a closer examination of the growth plate revealed a slight reduction in size,²⁰ thus correlating with the mild phenotype observed in humans.

NPPC encodes the 126-amino acid pre-pro CNP. The 23-residue signal peptide is proteolytically cleaved, giving rise to the pro-CNP peptide, which is further processed by the prohormone convertase furin into two secreted peptides: the propeptide 1-50 and CNP-53.²¹ In some tissues, CNP-53 is

then subsequently cleaved by an unknown extracellular enzyme to generate CNP-22. These mature peptides contain a conserved 17-amino acid disulfide-linked ring, necessary for correct functioning. Although CNP-22 and CNP-53 have very similar NPR-B activation and binding profiles, their tissue expression differs. While CNP-53 is the predominant form in the brain, endothelial cells, and heart, CNP-22 is higher in the plasma and cerebral spinal fluid.²¹

Both mutations are located within the ring structure of CNP, which is highly conserved between the three natriuretic peptides CNP, BNP, and ANP. Molecular modeling of the p.Arg117Gly mutation suggested that the presence of a glycine at codon 117 provides CNP with higher conformational flexibility, thus impeding the interaction between CNP and its receptor, NPR-B.²²

The prevalence of *NPPC* mutations in the entire cohort of short stature individuals ($n = 340$) was ~0.6%. The prevalence of *NPPC* mutations might be lower in an unselected cohort of ISS children, thus the screening of a large cohort is required to determine a more accurate estimate of the prevalence. Moreover, a previous report indicated that *NPPC* large deletions may be associated with short stature,¹⁵ thus suggesting that the calculated prevalence might be underestimated since deletions were not screened in the current

Table 2 Genetic details and *in silico* analysis of the *NPPC* variants

Pt no.	cDNA	Exon	Amino acid	Domain	Country	Cosegregation ^a	GERP++	SIFT	Mutation taster	Polyphen	CADD v1.3
1	c.349C > G	2	p.Arg117Gly	Ring	Spain	Yes	4.4	Del (0)	Disease causing (1)	Damaging (1.000)	Del (31)
2	c.355G > T	2	p.Gly119Cys	Ring	Brazil	Yes	5.29	Del (0)	Disease causing (1)	Damaging (1.000)	Del (35)

cDNA, complementary DNA; Del, deleterious.
All variants were absent from the ExAc database, 1000 Genomes and 264 normal-height Spanish individuals (DNA bank, University of Salamanca).
GERP++ values >2.45 are deemed conserved. CADD version 1.3 values >20 are classified as pathogenic. The probability of pathogenicity for Polyphen and MutationTaster are on a scale of 0–1, with 1 indicating deleterious. In contrast, SIFT values are indicated on a scale of 0–1 with 0 being deleterious and 1 indicating a polymorphism.
aCosegregation details are shown in **Figure 2**.

Figure 3 Functional characterization of the CNP variants. Analysis of the capacity of CNP wild type or the two mutants to activate NPR-B-mediated cGMP synthesis. cGMP activity of COS-7 cells transfected with NPR-B wild type (+) or empty (–), together with CNP wild type (WT) or mutants p.Arg117Gly (R117G) or p.Gly119Cys (G119C) and a 1:1 ratio of CNP wild type and each mutant was measured. cGMP values are expressed as a percentage relative to CNP/NPR-B-stimulated WT +/– SD. Each construct combination was transfected in triplicate, and each transfection was assayed in triplicate. Three biological replicates were performed. Thus, the statistics was determined for 27 ELISA assay results per construct combination. **P* < 0.01. Both mutants showed a drastic reduction in CNP-dependent cGMP synthesis in the heterozygous state and total ablation in the homozygous state.

study. The estimated incidence of *NPR2* mutations is five times higher, with mutations being detected in ~3% of children initially classified as ISS and ~3% of disproportionate short stature cases.^{8,9} Indeed, *NPPC* is considerably smaller than *NPR2*, thus the probability of a random mutation is far less, but this difference in incidence may also be due to other factors such as mutational hotspots or reproductive fitness.

Patients with heterozygous mutations in the gene encoding the ligand (*NPPC*) or in the gene encoding its receptor (*NPR2*) share certain clinical features, such as short stature, small hands, or brachydactyly. Some individuals also present with cubitus valgus and a high-arched palate. However, individuals with *NPR2* mutations may show additional clinical features resembling those observed in acromesomelic dysplasia Maroteaux type individuals, including frontal bossing and mesomelic shortening of the forearm. Although clinical heterogeneity is indeed observed, the principal

difference appears to be that individuals with *NPR2* mutations present with disproportionate growth while *NPPC* mutations to date have been observed only in individuals with proportionate growth. However, further cases are required for a more precise phenotypic evaluation.

A novel therapeutic approach for patients with *NPPC* mutations may be available in the near future. Phase II clinical trials (ClinicalTrials.gov identifier: NCT02055157) are currently in progress to test the efficacy of a CNP analog (BMN111, Biomarín Pharmaceutical) in patients with achondroplasia (MIM 100800), the most common type of dwarfism in humans. BMN111 acts as a key regulator of longitudinal bone growth by downregulating the mitogen-activated protein kinase pathway, which is activated by the FGFR3 (MIM 134934) gain-of-function mutation, p.Gly380Arg, present in 99.9% of individuals with achondroplasia. This results in the restoration of chondrocyte proliferation and differentiation, leading to bone growth. Preliminary data show promising improvements in height and other clinical characteristics associated with this pathology, as previously observed in the preclinical studies, where the CNP analog rescued the skeletal phenotype in *Fgfr3* Y367C/+ mice.^{23,24} In the context of an *NPPC* mutation, the CNP analog seems a promising therapeutic approach, since it directly replaces the defective protein.

In summary, we have identified the first *NPPC* mutations, in patients with proportionate short stature and small hands. The incidence of *NPPC* mutations in our short stature cohort was 0.6%. We also wait with great interest for the results of the CNP analog clinical trial as this would be a direct therapeutic approach for patients with CNP mutations.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

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DISCLOSURE

The authors declare no conflict of interest.

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