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Mutações em heterozigose no gene do receptor tipo B dos peptídeos natriuréticos (*NPR2*) são causa de baixa estatura inicialmente classificada como idiopática

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Programa de Endocrinologia

Orientação: Prof. Dr. Alexander Augusto de Lima Jorge

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Apêndice B - Vasques GA, Arnhold IJP, Jorge AAL. Role of the natriuretic peptide system in normal growth and growth disorders. *Horm Res Paediatr.* 2014;82(4): 222-9. doi: 10.1159/000365049

Apêndice C - Hisado-Oliva A, Garre-Vázquez AI, Santaolalla-Caballero F, Belinchón A, Barreda-Bonis AC, Vasques GA, Ramirez J, Luzuriaga C, Carlone G, González-Casado I, Benito-Sanz S, Jorge AA, Campos-Barros A, Heath KE. Heterozygous *NPR2* mutations cause disproportionate short stature, similar to Léri-Weill dyschondrosteosis. *J Clin Endocrinol Metab.* 2015 Aug; 100(8):E1133-42. doi: 10.1210/jc.2015-1612

Listas

LISTA DE ABREVIATURAS

AMDM	displasia acromesomélica do tipo Maroteaux
BEI	baixa estatura idiopática
CALR	calreticulina
COS-7	linhagem celular semelhante a fibroblastos derivada de tecido renal de macacos
cDNA	ácido desoxirribonucleico complementar
cGMP	monofosfato de guanosina cíclico
CNP	peptídeo natriurético tipo C
DAPI	<i>4'6-diamidino-2-phenylindole</i>
DMEM	meio de Eagle modificado por Dulbecco
et al	e outros
ExAC	<i>Exome Aggregation Consortium</i>
FGFR3	receptor tipo 3 de fatores de crescimento de fibroblastos
GnRHa	análogo do hormônio liberador de gonadotrofina
HA	hemaglutinina
HEK293A	linhagem celular derivada de células renais embrionárias humanas
IGF-1	fator de crescimento insulina-símile 1
IGFBP-3	proteína 3 de ligação a fator de crescimento insulina-símile
MAPK	proteína quinase ativada por mitógeno
MgCl₂	cloreto de magnésio
NPPB	gene que codifica o peptídeo natriurético do tipo B
NPR-B	receptor tipo B dos peptídeos natriuréticos
NPR-C	receptor tipo C dos peptídeos natriuréticos
NPR2	gene que codifica o receptor tipo B dos peptídeos natriuréticos
OMIM	<i>Online Mendelian Inheritance in Men</i>

PCR	reação de polimerização em cadeia
RCF	força centrífuga relativa
rhGH	hormônio de crescimento recombinante humano
<i>SHOX</i>	<i>short stature homeobox containing gene</i>
U2OS	linhagem celular de osteossarcoma humano
WT	homozigoto para o alelo selvagem
Z	escore de desvio padrão para idade e sexo

LISTA DE SÍMBOLOS

=	Igual a
<	Menor que
≤	Menor ou igual a
>	Maior que
±	Mais ou menos
cm	Centímetro
g	Gravidade
µg/Kg/d	Micrograma por quilograma por dia
µg/ml	Micrograma por mililitro
µl	Microlitro
µM	Micromolar
mg/d	Micrograma por dia
mg/ml	Miligramma por mililitro
ml	Mililitro
mm	Milímetros
mM	Milimolar
M	Molar
ng	Nanograma
ng/ml	Nanograma por mililitro
nM	Nanomolar
pmol	Picomol
UI/µl	Unidades internacionais por microlitro

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Resumo

Vasques GA. *Mutações em heterozigose no gene do receptor tipo B dos peptídeos natriuréticos (NPR2) são causa de baixa estatura inicialmente classificada como idiopática* [Tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2015.

Nos últimos anos, o sistema do peptídeo natriurético do tipo C (CNP) e seu receptor (NPR-B) foi apontado como um importante regulador do processo de ossificação endocondral. Vários estudos em animais evidenciam o seu papel de estímulo à proliferação e diferenciação de condrócitos e secreção de matriz extracelular. Mutações bialélicas com perda de função do gene do NPR-B (*NPR2*) levam a uma doença denominada displasia acromesomélica do tipo Maroteaux (AMDM), uma displasia esquelética caracterizada por baixa estatura extrema. Observa-se que familiares de pacientes com AMDM carreadores de mutação no *NPR2* têm estatura abaixo da média da população a qual pertencem, sugerindo um papel de mutações em heterozigose do *NPR2* como causadoras de baixa estatura idiopática (BEI). Os objetivos deste estudo foram avaliar a presença de mutações no gene *NPR2* em um grupo de pacientes com BEI e correlacionar os achados moleculares com o fenótipo dos pacientes e familiares. A região codificadora do gene *NPR2* foi sequenciada pelo método de Sanger em 60 pacientes com diagnóstico de BEI. Foram identificadas cinco diferentes variantes alélicas *missense* em heterozigose no *NPR2*, cada uma em um único paciente. Essas variantes foram submetidas à análise funcional *in vitro* para avaliação da atividade da guanililciclase e microscopia confocal para localização intracelular dos receptores NPR-B. As variantes c.226T>C / p.Ser76Pro, c.788G>C / p.Arg263Pro e c.2455C>T / p.Arg819Cys segregam com o fenótipo de baixa estatura dentro das famílias e determinam um prejuízo funcional ao NPR-B. As três variantes geram proteínas que exercem efeito dominante negativo e os receptores NPR-B com as mutações p.Ser76Pro e p.Arg263Pro não se localizam na membrana celular. As variantes c.491C>G / p.Ala164Gly e c.1636A>T / p.Asn546Tyr não segregam com o fenótipo de baixa estatura nas famílias e não se evidenciou um efeito dominante negativo. O escore-Z da altura dos indivíduos carreadores das variantes funcionalmente deletérias variou de -4,5 a -1,7. Um dos pacientes e dois familiares apresentam desproporção corporal e um paciente tem metacarpos curtos. Como conclusão, mutações em heterozigose no gene *NPR2* são causa de baixa estatura em 3 de 60 pacientes com diagnóstico inicial de BEI (5% da nossa casuística). Os indivíduos afetados têm graus variados de baixa estatura, sem um fenótipo característico.

Descritores: Peptídeo Natriurético Tipo C/deficiência; Peptídeo Natriurético Tipo C/genética; Lâmina de Crescimento/crescimento & desenvolvimento; Estatura/genética; Insuficiência de Crescimento/genética; Biologia Molecular/métodos; Nanismo/genética.

Summary

Vasques GA. *Heterozygous mutations in natriuretic peptide receptor-B gene (NPR2) are cause of short stature initially classified as idiopathic* [Thesis]. São Paulo: “Faculdade de Medicina, Universidade de São Paulo”; 2015.

Over the past several years, C-type natriuretic peptide (CNP) and its receptor (NPR-B) system has emerged as an important regulator of endochondral bone growth. Animal models showed a CNP/NPR-B role in promoting chondrocyte proliferation and differentiation and matrix synthesis. Biallelic loss-of-function mutations in NPR-B gene (*NPR2*) cause acromesomelic dysplasia type Maroteux (AMDM), a skeletal dysplasia with extreme short stature. Relatives of patients with AMDM, heterozygous for *NPR2* mutations, were noted to be shorter than expected for their population of origin, suggesting that heterozygous mutations in *NPR2* could be a cause of idiopathic short stature (ISS). The objective of this study was to investigate the presence of *NPR2* mutations in a group of patients with ISS and to correlate molecular findings with phenotype. The *NPR2* coding region was sequenced by Sanger’s method in 60 patients with ISS. Five different heterozygous missense variants in *NPR2* were identified in five patients. The functional consequences of those variants were established using in vitro cell-based assay to determine guanylate cyclase activity and confocal microscopy to determine intracellular localization of NPR-B. The variants c.226T>C / p.Ser76Pro, c.788G>C / p.Arg263Pro and c.2455C>T / p.Arg819Cys segregated with short stature phenotype and were functionally deleterious. NPR-B receptors with these three variants have a dominant-negative effect and p.Ser76Pro and p.Arg263Pro NPR-B were not localized in the cell membrane. Cosegregation analysis of the variants c.491C>G / p.Ala164Gly and c.1636A>T / p.Asn546Tyr was inconclusive and they did not have a dominant negative effect. Carriers of functionally deleterious variants have a height SD score that ranged from -4.5 to -1.7. One of these patients and two relatives have disproportionate short stature and one has shortened metacarpal. In conclusion, heterozygous mutations in *NPR2* gene are cause of short stature in 3 of 60 patients initially classified as ISS (5% of our cohort). Affected individuals have variable degrees of short stature without a distinct phenotype.

Descriptors: Natriuretic Peptide, C-type/deficiency; Natriuretic Peptide, C-type/genetics; Growth Plate/growth & development; Body Height/genetics; Failure to Thrive/genetics; Molecular Biology/methods; Dwarfism/genetics.

1 Introdução

1 INTRODUÇÃO

O crescimento é um processo complexo que envolve múltiplos fatores genéticos e ambientais. Polimorfismos em dezenas de genes foram associados à variabilidade de altura entre os indivíduos saudáveis ^(1, 2). Entre esses genes associados à estatura, há aqueles cuja ação se dá na cartilagem de crescimento, promovendo a ossificação endocondral e consequente crescimento longitudinal. Três trabalhos recentes de estudos genômicos de associação global demonstraram que variantes alélicas comuns presentes em genes envolvidos na via de sinalização do peptídeo natriurético tipo C (CNP) estão relacionadas à determinação da altura de indivíduos saudáveis ⁽¹⁻³⁾.

Nos últimos anos, o sistema do peptídeo natriurético tipo C (CNP) e seu receptor (NPR-B) foi identificado como importante regulador da proliferação e maturação da cartilagem de crescimento ⁽⁴⁾. Mutações em homozigose em camundongos ^(5, 6) e mutações bialélicas em humanos ⁽⁷⁾ do gene que codifica o NPR-B (*NPR2*) são responsáveis por displasia esquelética grave com importante comprometimento estatural. Adicionalmente, a análise de uma família afetada por mutação no *NPR2* ⁽⁸⁾ e estudo em animais heterozigotos para mutações no *Npr2* ⁽⁹⁾ sugeriram um papel significativo de defeitos neste gene em heterozigose no comprometimento do crescimento, sem causar displasia esquelética aparente.

Os resultados destes estudos apontam o *NPR2* como um importante gene candidato para baixa estatura não síndrômica. Por estas razões, o objetivo do presente estudo foi investigar a presença de mutações no gene *NPR2* em indivíduos com baixa estatura idiopática.

1.1 A cartilagem de crescimento

A cartilagem de crescimento está localizada entre a epífise e a metáfise na extremidade distal dos ossos longos. O crescimento longitudinal de um indivíduo depende do processo denominado ossificação endocondral que ocorre na cartilagem de crescimento. Esse processo inicia-se com a diferenciação de células mesenquimais em condrócitos. Estes, por sua vez, distribuem-se em três regiões principais: zona de repouso, zona proliferativa e zona hipertrófica. A zona de repouso contém condrócitos com baixa taxa de replicação, funcionando como um reservatório para a zona proliferativa. Na zona proliferativa, essas células se replicam rapidamente, dispondo-se em colunas paralelas ao eixo longitudinal do osso. Na medida em que se distanciam da zona de repouso, os condrócitos param de se replicar e aumentam de tamanho, passando a fazer parte da zona hipertrófica. Os condrócitos hipertróficos formam a matriz extracelular e, antes de sofrerem apoptose, secretam fatores que atraem vasos sanguíneos e células precursoras de osteoblastos ⁽¹⁰⁾. O processo de proliferação e hipertrofia dos condrócitos, além da secreção de matriz extracelular, denomina-se condrogênese.

A condrogênese é regulada por múltiplos fatores genéticos e hormonais que podem agir de maneira endócrina, parácrina e autócrina ⁽¹¹⁾ (Figura 1). O principal regulador da cartilagem de crescimento é o hormônio *Indian hedgehog* (Ihh), sintetizado por condrócitos localizados entre a zona proliferativa e início da zona hipertrófica. Ele tem ação parácrina, coordenando a proliferação e diferenciação de condrócitos e a diferenciação de osteoblastos ⁽¹²⁾. Ihh age diretamente nos condrócitos, estimulando a sua proliferação, e indiretamente através do estímulo ao peptídeo relacionado ao paratormônio (PTHrP), com o qual forma uma alça de *feedback* negativo. O PTHrP mantém os condrócitos na fase proliferativa, inibindo a sua diferenciação ⁽¹²⁾. A expressão do Ihh é inibida pelos fatores de crescimento de fibroblasto (FGFs), família de proteínas que através da ligação aos seus receptores (FGFRs), diminuem a proliferação dos condrócitos e aceleram o processo de hipertrofia. As proteínas ósseas morfogenéticas (BMPs) também são reguladoras da cartilagem de crescimento e agem de maneira oposta aos FGFs, aumentando a proliferação dos condrócitos e a expressão do Ihh. Tanto os FGFs quanto as BMPs agem em vias paralelas que interagem com a sinalização Ihh/PTHrP ⁽¹¹⁾.

O sistema hormônio de crescimento (GH) / fator de crescimento insulina símile tipo 1 (IGF-1) é o principal regulador endócrino do crescimento ósseo longitudinal pós-natal, e tem um papel permissivo na proliferação e diferenciação dos condrócitos. Os hormônios tireoidianos, glicocorticóides e esteróides sexuais apresentam ações modulatórias na síntese e ação do GH e do IGF-1, além de influenciarem diretamente os

fatores locais que agem na cartilagem de crescimento ⁽¹⁰⁾. Outro sistema regulador do crescimento endocondral que vem ganhando importância é o que envolve os peptídeos natriuréticos e seus receptores.

Figura 1 - Regulação da cartilagem de crescimento. Ihh (*Indian hedgehog*) é expresso por condrócitos entre as zonas proliferativa e hipertrófica. Ele estimula a proliferação dos condrócitos diretamente e através do estímulo ao PTHrP (*PTH-related peptide*). O PTHrP inibe a hipertrofia dos condrócitos e forma uma alça de *feedback negativo* com o Ihh. BMPs (*bone morphogenetic proteins*) estimulam Ihh e a proliferação dos condrócitos, enquanto os FGFs (*fibroblast growth factors*) agem de maneira oposta e aceleram o processo de hipertrofia. O CNP (*C-type natriuretic peptide*) estimula a proliferação, diferenciação e alongamento dos condrócitos na zona hipertrófica. O GH (*growth hormone*) recruta células da zona de repouso e, através do IGF-1 (*insulin-like growth factor-1*), estimula a proliferação e crescimento dos condrócitos na zona hipertrófica. O VEGF (*vascular endothelial growth factor*) é expresso na zona hipertrófica e é responsável pela invasão de vasos provenientes da metáfise.

1.2 Sistema do peptídeo natriurético tipo C (CNP) e seu receptor (NPR-B)

A família dos peptídeos natriuréticos é composta de: peptídeo natriurético atrial (ANP), peptídeo natriurético encefálico (BNP), peptídeo natriurético tipo C (CNP) e peptídeo natriurético tipo D (DNP) ⁽¹³⁾. O DNP é o membro mais recentemente descoberto e o seu papel ainda é pouco conhecido ⁽¹⁴⁾. O ANP e o BNP têm efeitos diurético, natriurético e hemodinâmicos (anti-hipertroóficos, anti-proliferativos e relaxamento de vasos) ⁽¹³⁾. O CNP não tem ação natriurética clinicamente relevante e desempenha um papel importante na regulação da ossificação endocondral ⁽¹⁵⁾. Ele está presente principalmente na zona hipertrófica da cartilagem de crescimento. O CNP estimula a proliferação dos condrócitos e aumenta o número e a altura dessas células na zona hipertrófica. Além disso, tem como função primordial estimular a síntese de matriz cartilaginosa ⁽⁴⁾.

As ações biológicas dos peptídeos natriuréticos são mediadas pela ativação de dois subtipos de receptores: receptor tipo A (NPR-A) e receptor tipo B (NPR-B). Eles são homodímeros de membrana caracterizados estruturalmente por: um domínio extracelular de ligação, um domínio transmembrana, um domínio intracelular de homologia à quinase e um com atividade guanililciclase ⁽¹⁶⁾. O ANP e o BNP são os ligantes endógenos do NPR-A, enquanto o CNP se liga preferencialmente ao NPR-B. Após a ligação CNP/NPR-B, ocorre ativação da guanililciclase, com formação de GMP cíclico (cGMP) (Figura 2) e ativação de proteínas quinases dependentes de cGMP I e II (cGK-I e cGK-II), desencadeando uma cascata

de fosforilação intracelular responsável por intermediar as ações biológicas no CNP ⁽¹⁷⁾.

O gene responsável por codificar o receptor NPR-B (*NPR2* – OMIM 108961) está localizado no 9p13.3, estende-se por 17.317 pares de base e é organizado em 22 éxons que codificam uma proteína de 1.047 aminoácidos. O gene *NPR2* é expresso em diversos tecidos, incluindo o cérebro, hipófise, suprarrenal, rim, pulmão, útero e cartilagem de crescimento ⁽⁴⁾.

Há ainda o receptor tipo C (NPR-C), que não apresenta atividade guanililciclase e participa da internalização e degradação dos ligantes, tendo um papel de regulador negativo das ações dos peptídeos natriuréticos ⁽⁴⁾.

Figura 2 - Representação esquemática do homodímero NPR-B. Após a ligação do CNP, ocorre um acúmulo intracelular de cGMP

1.3 Modelos animais de alteração na interação CNP e NPR-B

Camundongos nocaute para o gene do CNP (*Nppc*) apresentam um comprometimento importante do crescimento linear devido a alterações na ossificação endocondral. As espessuras das zonas proliferativa e hipertrófica da cartilagem de crescimento desses animais encontram-se reduzidas ⁽¹⁸⁾. Por outro lado, camundongos transgênicos com hiperexpressão do *Nppc* apresentam crescimento linear exagerado, com expansão das zonas proliferativa e hipertrófica na cartilagem de crescimento ⁽¹⁹⁾.

Em 2005, estudos de *linkage* possibilitaram a identificação de uma mutação em homozigose no gene *Npr2* de camundongos com condrodissplasia (*Npr2^{cn}*). Eram animais espontaneamente mutantes que apresentavam nanismo, com encurtamento de membros e cauda. Foi identificada uma mutação *missense*, c.2654T>G, com troca de leucina por arginina no códon 885 (p.Leu885Arg), o que altera o domínio da guanililciclase. Foi constatado que o nível de cGMP não aumentou após estímulo com CNP em condrócitos de camundongos mutantes, demonstrando que a mutação diminuiu a atividade da guanililciclase do NPR-B ⁽⁵⁾.

Em 2007, outro grupo relatou que a presença de uma mutação em homozigose no *Npr2* era responsável pelo fenótipo de outra linhagem espontânea de camundongos pequenos e com membros curtos (*Npr2^{slw}*) ⁽⁶⁾. Esses animais apresentavam ossificação endocondral deficiente, demonstrada pelo número reduzido de condrócitos nas fases proliferativa e

hipertrófica e redução da espessura total das cartilagens de crescimento das tíbias ⁽⁶⁾.

Os estudos envolvendo linhagens de animais com mutação espontânea do *Npr2* não avaliaram o efeito da heterozigose neste gene. Entretanto, um estudo envolvendo camundongos heterozigotos para nocaute do *Npr2* gerados em laboratório (*Npr2*^{+/-}) mostrou que esses animais tinham o comprimento significativamente menor que camundongos selvagens (*Npr2*^{+/+}) ⁽⁹⁾.

1.4 A importância do sistema CNP/NPR-B em humanos

Em 2004, Bartels *et al.* relataram que mutações bialélicas com perda de função no gene *NPR2* eram causadoras de uma forma de displasia esquelética humana, conhecida como displasia acromesomélica tipo Maroteaux (AMDM - OMIM 602875) ⁽⁷⁾. Essa é uma doença descrita no início da década de 70 ⁽²⁰⁾, com herança autossômica recessiva e prevalência estimada em 1:1 milhão de pessoas. É uma causa de baixa estatura extrema pós-natal, sendo a altura final de indivíduos afetados abaixo de 5 desvios-padrão da média ⁽⁷⁾. A suspeita clínica de AMDM é geralmente feita até 1 ano de idade e a radiografia do esqueleto é típica ao redor dos 2 anos. Os segmentos médios e distais dos membros são os mais afetados. A radiografia das mãos revela metacarpos curtos e falanges com epífises em forma de cone. Os ossos longos vão se tornando progressivamente mais

curtos. Os corpos vertebrais são ovais na infância e, com o passar dos anos, as margens dorsais ficam menores do que as ventrais ⁽²¹⁾.

Até o momento, foram descritas 27 mutações diferentes em 32 famílias com casos de AMDM, sendo 27 famílias consanguíneas. A maioria dos pacientes descritos tem mutação em homozigose no *NPR2*, enquanto 3 indivíduos são heterozigotos compostos. Foram reportadas 15 mutações *missense*, 5 *frameshift*, 4 *nonsense* e 3 em sítios de *splice* ^(7, 8, 22-24). A maioria das mutações *missense* gera receptores que ficam retidos no retículo endoplasmático e, conseqüentemente, não atingem a membrana plasmática, o que diminui o número de receptores disponíveis para a ligação do CNP ⁽²⁵⁾. Também pode ocorrer comprometimento da ligação ao CNP ⁽²⁵⁾ ou da indução da atividade guanililciclase ⁽²²⁾.

Desde 1983, observa-se que os pais de pacientes com AMDM são menores que os seus pares ⁽²⁶⁾. Com a identificação das mutações bialélicas no *NPR2* como causa desta displasia esquelética, foi observado que não só os pais, como também irmãos dos pacientes índices, heterozigotos para mutações neste gene, eram 5,9 cm menores que controles da mesma população ⁽⁷⁾. Em 2006, Olney *et al.* ⁽⁸⁾ fizeram uma associação entre baixa estatura e mutações em heterozigose no *NPR2*. Foi relatado, na família de um paciente com AMDM, que familiares portadores da mutação p.I364fsX376 eram significativamente menores que os familiares não portadores (escore-Z de altura $-1,8 \pm 1,1$ vs. $-0,4 \pm 0,8$; $p < 0,0005$) ⁽⁸⁾.

Levando-se em consideração o banco de dados norte-americano *Little People of America*, tem-se que um em cada 2 milhões de indivíduos são

afetados pela AMDM. Admitindo-se o equilíbrio de Hardy-Weinberg, um em cada 700 indivíduos teria uma mutação em heterozigose no *NPR2*. E, considerando-se indivíduos com baixa estatura idiopática aqueles com escore-Z de altura inferior a -2,25, Olney *et al.* ⁽⁸⁾ calcularam que um em cada 30 indivíduos com baixa estatura idiopática seria portador de mutação no *NPR2* ⁽⁸⁾. Porém, esta hipótese não foi comprovada naquele momento.

Por outro lado, três mutações em heterozigose com ganho de função no *NPR2* (p.Ala488Pro, p.Arg655Cys, p.Val883Met) foram recentemente apontadas como causa de alta estatura ⁽²⁷⁻²⁹⁾. Estudos funcionais com células HEK293A transfectadas com cada uma das três sequências mutantes indicaram ativação constitutiva do NPR-B. Adicionalmente, após estímulo com CNP, as células com NPR-B mutante tiveram uma produção de cGMP significativamente maior do que células com NPR-B selvagem. Os pacientes e familiares carreadores das mutações p.Ala488Pro e p.Val883Met apresentam, além do crescimento excessivo, escoliose, aracnodactilia e hálux desproporcionalmente amplo ^(27, 29). Esse fenótipo já havia sido descrito anteriormente em uma paciente com hiperexpressão do gene que codifica o CNP (*NPPC*) devido a uma translocação balanceada t(2;7), reforçando o papel do sistema CNP/NPR-B como regulador importante do crescimento ⁽³⁰⁾.

2 Objetivos

2 OBJETIVOS

Pesquisar mutações no gene do receptor tipo B dos peptídeos natriuréticos (*NPR2*) em indivíduos com baixa estatura idiopática.

Avaliar o impacto funcional das mutações identificadas no *NPR2*.

Determinar o fenótipo dos pacientes e familiares com mutações em heterozigose no *NPR2*.

3 Casuística

3 CASUÍSTICA

3.1 Considerações éticas

Este estudo foi conduzido de acordo com princípios éticos seguindo as orientações contidas na declaração de Helsinki (adotada na 18ª Assembleia Médica Mundial, Helsinki, Finlândia, 1964). O projeto foi aprovado pela Comissão de Ética para Análise de Projetos de Pesquisa (CAPPesq- HCFMUSP), protocolo de número 0475/11. Consentimento por escrito foi obtido de todos os pacientes maiores de 18 anos ou pais/tutores, assim como assentimento por escrito dos pacientes entre 7 e 17 anos.

3.2 Seleção de pacientes

Foram selecionadas 60 crianças com baixa estatura idiopática acompanhadas no ambulatório da unidade de Endocrinologia do Desenvolvimento do Hospital das Clínicas da FMUSP.

Os pacientes foram selecionados seguindo os seguintes critérios:

Inclusão:

- Baixa estatura de início pós-natal.
- Escore de desvio padrão da altura (Z da altura) inferior a -2,0 em relação média de estatura da curva de crescimento de referência

(31).

Exclusão:

- Deficiência de hormônio de crescimento (DGH)
- Causas conhecidas de baixa estatura após o nascimento como presença de distúrbios genéticos e cromossômicos, desnutrição, doenças hepáticas, patologias sistêmicas, ósseas ou endocrinológicas ⁽³²⁾.
- Presença de mutações nos genes *SHOX* ou *FGFR3*, pesquisadas nos pacientes com baixa estatura desproporcional.

Como controles das variantes alélicas encontradas, foram selecionados indivíduos adultos saudáveis com escore-Z da altura superior a zero.

4 Métodos

4 MÉTODOS

4.1 Estudo Molecular

4.1.1 Extração do DNA Genômico de Linfócitos Periféricos

As amostras de DNA foram obtidas a partir de leucócitos de sangue periférico dos indivíduos arrolados na pesquisa. Dez mililitros de sangue venoso foram coletados em ácido etileno diaminotetracético (EDTA 25 mM). O botão leucocitário foi obtido a partir da lise dos glóbulos vermelhos utilizando-se um volume de solução de lise (NH_4Cl a 114 mM, NH_4HCO_3 a 1 mM) duas vezes maior que o volume de sangue, incubado a 4°C por 30 minutos. O material foi centrifugado a 4°C durante 15 minutos com uma RCF de 1600 x g (Sorvall, RT7, Germany), sendo desprezado o sobrenadante. O procedimento da lise de glóbulos vermelhos foi repetido uma vez.

O botão de células brancas foi suspenso em nove ml de solução de lise de glóbulos brancos (NaCl 150 mM, Tris-HCl 10 mM, pH 8,0; EDTA 10 mM pH 8,0) com 180 μL de dodecilsulfato de sódio a 10% (SDS) (Sigma, St. Louis, MO, USA) e 150 μL de proteinase K na concentração de 10 mg/mL (Gibco BRL, Gaithersburg, MD, USA), e incubado a 37°C por 18 horas. Após este período, 3,6 ml de solução saturada de NaCl a 6 M foi adicionada, seguido de agitação do conjunto vigorosamente durante 15 segundos. O material foi centrifugado por 15 minutos a 1600 x g. O sobrenadante foi transferido para outro tubo e o DNA foi precipitado

acrescentando-se dois volumes de etanol absoluto gelado, homogeneizando-se cuidadosamente por inversão. O DNA precipitado foi retirado do tubo e em seguida lavado em etanol a 70% durante 5 minutos, repetindo-se a operação por mais três vezes. Finalmente, o DNA foi lavado em etanol absoluto, seguido de secagem por centrifugação a vácuo (Eppendorf, Concentrator 5301, Germany). Após o procedimento, o DNA foi ressuspensão em tampão TE a 10:0,1 (Tris-HCl 10 mM, pH 8,0; EDTA 0,1 mM, pH 8,0).

4.1.2 Reação de Polimerização em Cadeia (PCR)

As reações foram realizadas em um volume final de 25 µL. Para cada reação, foram utilizados 100 a 200 ng de DNA genômico, 200 µM de cada desoxinucleotídeo (dNTP), 10 pmol de cada um dos oligonucleotídeos iniciadores, 0,25µL de Go Taq DNA Polimerase (Promega), 5,0 µL de Green Go Taq Buffer (Promega), 1µL de DMSO (Sigma, USA) e adicionado MgCl₂ (Invitrogen, Brasil) para se obter concentrações finais variáveis, descritas na Tabela 1. As sequências dos pares de oligonucleotídeos iniciadores utilizados para a amplificação do gene *NPR2* encontram-se na Tabela 1. O protocolo de amplificação consistiu das seguintes etapas: desnaturação inicial a 95°C por 5 minutos, seguida de 35 ciclos de desnaturação a 94°C por 30 segundos, anelamento a 55°C por 30 segundos e extensão a 72°C por 1 minuto, finalizando com a extensão a 72°C por 5 minutos. Todas as amplificações foram acompanhadas de um controle negativo. As amostras amplificadas foram identificadas em gel de agarose a

1%, com o uso do corante Blue green Loading Dye I, e observadas em transiluminador ultravioleta.

4.1.3 Sequenciamento Automático

Os produtos de amplificação foram submetidos à purificação enzimática com a combinação das enzimas fosfatase alcalina de camarão (2 UI/ μ L) e exonuclease I (10 UI/ μ L) (PCR product pre-sequencing kit, Amersham Life Science, Cleveland, OH, USA) com incubação a 37°C por 15 minutos seguido de 80°C por mais 15 minutos. A reação de sequenciamento foi realizada utilizando o kit ABI Prism™ BigDye Terminator (Perkin Elmer) e quantidades variáveis de produto de PCR (10 a 100 ng), de acordo com o tamanho do fragmento. Os produtos desta reação foram submetidos à eletroforese em sequenciador automático ABI Prism Genetic Analyzer 3100 automatic DNA sequencer (Perkin Elmer, Foster City, CA). As sequências obtidas foram comparadas com as sequências depositadas na base de dados do *National Center for Biotechnology na Information (NCBI)*.

Tabela 1 - Oligonucleotídeos iniciadores utilizados para a amplificação do gene *NPR2*

Região amplificada	Oligonucleotídeos iniciadores específicos	MgCl ₂ (mM)*
Éxon 1	1F: 5' CTG GTA GCC CAC TCC TTG C 3' 1R: 5' GAT CCC AAC CAC CAC ATA CA 3'	1,5
Éxon 2	2F: 5' TCT GCT TTG GGG TTG ATA GG 3' 2R: 5' TTC ATT TTA CTC CTG TGG CTC A 3'	3,5
Éxons 3 e 4	3-4F: 5' CAC ACA CAC ACG CAG AGA GA 3' 3-4R: 5' GTG GGC AAC TCT GCC ATA TT 3'	2,5
Éxons 5 a 7	5-7F: 5' GAT CGG GGA AGG GTA GAC TC 3' 5-7R: 5' TGG ATC TAA GAG ACA ACC AGC A 3'	2,5
Éxons 8 a 10	8-10F: 5' CTT CCA GCC CTG TCT CTC AG 3' 8-10R: 5' CCA CTA CAT AAG AGG ACC TTT CTC 3'	2,5
Éxons 11 e 12	11-12F: 5' AAA TTG GCT AGA TGG GCA AG 3' 11-12R: 5' CCA TGG GAA TAA GGG GAG AT 3'	2,5
Éxons 13 e 14	13-14F: 5' GCT TAT TAT TTT TGT GGA CCC AAG 3' 13-14R: 5' CCA TTT CGT ACC TTC TGG ACA 3'	3,5
Éxons 15 e 16	15-16F: 5' CAC TTC GCA GTG GTC CTT TC 3' 15-16R: 5' GCA GCA TAG CTG ATG TGG AG 3'	3,5
Éxons 17 e 18	17-18F: 5' GTC TTC CCA GCC ACT TTC CT 3' 17-18R: 5' CGA AGT ACA CTC CAG CAC CA 3'	2,5
Éxons 19 e 20	19-20F: 5' TCT GGT CTC CAG CAT GTC AG 3' 19-20R: 5' AAA ATA TGC CCG AGG ATG C 3'	3,5
Éxons 21 e 22	21-22F: 5' ATT GGG AGG TTG GGC ATA TT 3' 21-22R: 5' GCA GAG GGC TAC AAC TTC CA 3'	2,5

* - Concentração final de MgCl₂ na solução para PCR

4.2 Estudo *in silico*

Os dados de frequência das variantes alélicas observadas nos pacientes foram obtidos junto ao banco de dados *Exome Aggregation Consortium* (ExAC) no dia 23 de julho de 2015 na página eletrônica <http://exac.broadinstitute.org/> ⁽³³⁾. As identidades das variantes já catalogadas como SNPs foram obtidas na página eletrônica do dbSNP, <http://www.ncbi.nlm.nih.gov/snp>.

As variantes alélicas encontradas foram avaliadas quanto ao seu impacto funcional através dos seguintes modelos de bioinformática: *Mutation Taster* (<http://www.mutationtaster.org>) ⁽³⁴⁾ e *PolyPhen-2* (<http://genetics.bwh.harvard.edu/pph2>) ⁽³⁵⁾.

4.3 Estudo Funcional

As cinco variantes alélicas *missense* em heterozigose foram submetidas a estudo funcional.

A análise funcional das três primeiras variantes identificadas foi realizada na Universidade de Keio, em Tóquio, Japão, com colaboração do Dr. Tomonobu Hasegawa. As outras duas variantes encontradas posteriormente foram avaliadas funcionalmente na Universidade Autônoma de Madri, Espanha, com colaboração da Dra. Karen Heath.

4.3.1 Ensaios *in vitro*

A sequência de cDNA do NPR-B foi submetida a PCR com oligonucleotídeos específicos para a introdução de *HindIII* na extremidade 5' e *XhoI* na extremidade 3'. O produto desta PCR foi submetido à digestão pelas enzimas *HindIII* e *XhoI* para ligação ao vetor pcDNA3.1, de modo a gerar pcDNA3.1/NPR-B selvagem (WT). Para a síntese de vetores com epítipo de hemaglutinina (HA), mutagênese sítio dirigida foi utilizada para a inserção de *SacII* e *HpaI* entre os nucleotídeos +55 a +60 e +67 a +72, respectivamente. A sequência que codifica o epítipo de HA foi inserida entre os sítios de restrição *SacII* e *HpaI*.

Os receptores NPR-B contendo as mutações S76P, R263P e R819C foram sintetizados por mutagênese sítio dirigida, de acordo com instruções do fabricante (QuickChange XL Site-Directed Mutagenesis Kit; Agilent Technologies, Santa Clara, CA). Os receptores NPR-B com as mutações A164G e N546Y foram produzidos com instruções de outro fabricante (QuickChange Site-Directed Mutagenesis Kit, GE Healthcare, Uppsala, Sweden). A inserção de todas as mutações foi confirmada por sequenciamento direto. A síntese dos vetores com HA foi realizada seguindo as mesmas etapas descritas acima para o receptor selvagem.

Células COS-7 e U2OS, colocadas em placas de 12 poços, foram cultivadas em meio DMEM, com adição de soro fetal bovino a 10% e penicilina/estreptomicina a 1%. Essas células foram transfectadas com cDNA de NPR-B-WT e de cada um dos mutantes, além do vetor vazio, na quantidade de 200 ng de plasmídeo por poço. Foi utilizada Lipofectamina

2000 de acordo com o protocolo do fabricante (Life Technologies, Carlsbad, CA). Após 48 horas da transfecção, as células transfectadas com cDNA de NPR-B-WT e com cada um dos mutantes S76P, R263P e R819C foram lisadas com o reagente para extração de proteínas M-PER (Pierce, Rockford, IL), na presença de uma solução inibidora de protease a 1%. As amostras foram colocadas em géis de dodecil-sulfato de sódio de poliacrilamida a 10% e submetidos à eletroforese (SDS-PAGE), sendo posteriormente transferidas para membranas de difluoreto de polivinilideno. A detecção de HA-NPR-B foi feita por *immunoblotting*, com o uso de anticorpo anti-HA de ratos (1:500; clone 3F10; Roche Applied Science, Indianápolis, IN) e anticorpo secundário anti-IgG de rato (1:2500). As imagens foram feitas com aparelho apropriado (BioRad, Hercules, CA). Não foi realizada análise por *Western blot* dos mutantes A164G e N546Y.

Para avaliação do potencial efeito dominante negativo das mutações encontradas, as células foram cotransfectadas com quantidades equivalentes de cDNA selvagem (HA-NPR-B-WT) adicionadas ao vetor vazio ou a cada um dos cinco mutantes (100 ng de cada plasmídeo por poço).

Após 48 horas da transfecção, as células foram incubadas com e sem 100 nM de CNP-22 (Bachem, Ltd, UK) em meio Optimem (Invitrogen, Carlsbad, CA), por 20 minutos a 37°C. Foi adicionado 0,1 M de HCl a fim de parar a reação e as células foram centrifugadas a 600 x g por 10 minutos. O GMP cíclico foi medido por ensaio imunoenzimático competitivo de acordo com o protocolo do fabricante (cyclic GMP Complete, Enzo Life Science, Farmingdale, NY). Todos os experimentos foram realizados mais que duas

vezes em triplicata. Diferença estatisticamente significativa foi considerada com valores de $p \leq 0,05$, utilizando-se teste t de Welch.

4.3.2 Microscopia confocal

Células COS-7, cultivadas em placas com 12 poços, foram transfectadas com cDNA de NPR-B-WT e de cada um dos três mutantes (S76P, R263P, R819C). As células U2OS foram cotransfectadas com a sequência selvagem ou com cada uma das sequências mutantes (A164G, N546Y) e com um vetor que expressa um marcador de retículo endoplasmático (pRED-Calreticulin/CALR, Origene Technologies Inc, Rockville, MD). Após 48 horas da transfecção, as células foram lavadas com solução tampão de fosfato e fixadas em paraformaldeído a 4% por 10 minutos em temperatura ambiente.

As células COS-7 fixadas foram incubadas com anticorpo anti-HA de ratos na concentração de 1:1000 por 30 minutos. Após lavagem com solução tampão de fosfato, as essas células forma incubadas com um anticorpo de cabra anti-IgG de ratos (Alexa Fluor 568, Life Technologies, Carlsbad, CA), a 1:1000 por 30 minutos. As lamelas foram colocadas em uma lâmina limpa e montadas com o meio de montagem VECTASHIELD® com DAPI (Vector Laboratory, Burlingame, CA). As imagens fluorescentes foram tiradas com o uso de microscópio confocal TCS-SP5 (Leica, Mannheim, Germany).

Em relação às células U2OS, foram utilizados anticorpos monoclonais anti-HA de camundongos (Sigma-Aldrich, San Louis, MO) e anticorpos de cabra anti-IgG de camundongos (Alexa Fluor 488, Life Technologies), ambos diluídos a 1:2000. As células foram contrastadas com DAPI (Sigma-Aldrich) e montadas com o meio DePeX (BDH, Merck Chemicals Ltd, UK). As localizações intracelulares de NPR-B, CALR e DAPI foram obtidas utilizando-se o microscópio fluorescente Leica DM5500B (Leica Microsystems GmbH, Germany). Os experimentos foram feitos em replicata.

5 Resultados

5 RESULTADOS

5.1 Características da casuística

A casuística estudada se caracteriza por um predomínio de indivíduos do sexo masculino (67%). Na maioria das famílias (72%), pelo menos um dos pais tem escore-Z da altura menor que -2,0 (média do escore-Z da altura alvo: $-1,5 \pm 0,7$), caracterizando uma alta prevalência de baixa estatura familiar. Os pacientes iniciaram o acompanhamento com 9,4 anos na média, sendo que a idade de início variou de 1,3 a 16,5 anos. A média do escore-Z da altura inicial foi de -2,8. Baixa estatura desproporcional, definida por escore-Z da altura sentada dividida pela altura total maior que 2,0, foi encontrada em 32% da casuística. Baseado nesta característica, dividimos os pacientes como tendo BEI proporcional ou desproporcional. A única característica que diferiu entre os dois grupos foi o escore-Z do IMC, que foi mais elevado no grupo de baixa estatura desproporcional ($0,3 \pm 0,7$ *versus* $-0,8 \pm 1,3$; $p = 0,001$). As características clínicas da casuística encontram-se resumidas na Tabela 2.

Foram estudados 72 indivíduos controles, sendo 31% do sexo masculino, com escore-Z da altura de $1,2 \pm 0,7$.

Tabela 2 - Características clínicas da casuística de pacientes com BEI

	Baixa estatura idiopática		
	Proporcional	Desproporcional	Total
Número de pacientes	41	19	60
Sexo Masculino (%)	63	74	67
História familiar de BEI (%)	66	84	72
Z estatura alvo	-1,5 ± 0,7	-1,7 ± 0,7	-1,5 ± 0,7
Dados da avaliação inicial			
Idade cronológica (anos)	9,2 ± 3,7	9,9 ± 2,8	9,4 ± 3,5
Z da altura	-2,8 ± 0,6	-2,7 ± 0,5	-2,8 ± 0,6
Z da altura sentada/altura total	-0,1 ± 1,2	2,6 ± 0,6*	0,8 ± 1,7
Z do IMC	0,8 ± 1,3	0,3 ± 0,7*	0,6 ± 1,3

BEI = baixa estatura idiopática, Z = escore de desvio padrão para idade e sexo

* - $p \leq 0,001$ em relação aos pacientes com BEI proporcionais

5.2 Resultados Moleculares

Os achados moleculares do sequenciamento do gene *NPR2* estão resumidos na Tabela 3. Foram encontradas 7 variantes alélicas em heterozigose, sendo 5 não sinônimas.

Três variantes (c.226T>C / p.Ser76Pro, c.491C>G / p.Ala164Gly e c.788G>C / p.Arg263Pro) localizam-se no domínio de ligação ao CNP. A variante c.1636A>T / p.Asn546Tyr localiza-se no domínio de homologia à quinase e a variante c.2455C>T / p.Arg819Cys localiza-se entre este domínio e o domínio guanililciclase. Cada uma destas variantes foi identificada em um único paciente. Nestes pacientes, mutações nos genes *SHOX* e *FGFR3* foram excluídas em estudos prévios.

As 5 variantes não sinônimas não foram encontradas em pelo menos 140 alelos controles de nossa população. Não há descrição das variantes c.226T>C / p.Ser76Pro e c.788G>C / p.Arg263Pro no banco de dados do *ExAC*. As demais variantes alélicas não sinônimas identificadas em nossos pacientes foram encontradas em uma frequência significativamente menor ($p < 0,001$) do que a observada no *ExAC* (Tabela 3).

Tabela 3 - Achados moleculares do sequenciamento do gene *NPR2*

Localização	Gene	Proteína	dbSNPs	Frequência alélica	
				Estudo atual	<i>ExAC</i>
Éxon 1	c.18T>C	p.Leu6Leu	rs2228580	25%	24%
	c.226T>C	p.Ser76Pro		0,8%	Não descrita
	c.491C>G	p.Ala164Gly	rs62637657	0,8%	0,003%
Éxon 2	c.788G>C	p.Arg263Pro		0,8%	Não descrita
Éxon 10	c.1636A>T	p.Asn546Tyr	rs138315850	0,8%	0,002%
Éxon 11	c.1794C>T	p.Tyr598Tyr	rs5812	25%	23%
Éxon 16	c.2455C>T	p.Arg819Cys		0,8%	0,002%

5.3 Resultados do estudo *in silico*

Dentre as cinco variantes alélicas *missense* avaliadas pelos estudos *in silico*, quatro foram preditas como potencialmente danosas à função do NPR-B (Tabela 4). Os escores de predição do efeito funcional de cada uma delas encontram-se resumidos na Tabela 4.

Tabela 4 - Escores de predição do efeito funcional das variantes *missense* em heterozigose no *NPR2*

Mutação	<i>Mutation Taster</i>		<i>Polyphen2</i>	
	Escore *	Predição	Escore*	Predição
p.Ser76Pro	0,95	Causadora de doença	0,97	Provavelmente danosa
p.Ala164Gly	0,56	Polimorfismo	0,06	Benigna
p.Arg263Pro	0,74	Causadora de doença	0,87	Possivelmente danosa
p.Asn546Tyr	1	Causadora de doença	0,88	Possivelmente danosa
p.Arg819Cys	1	Causadora de doença	1	Provavelmente danosa

*- Quanto mais próximo ao valor de 1, mais potencialmente danosa é a variante

5.4 Segregação das variantes alélicas *missense* no *NPR2* nas famílias dos pacientes afetados

As variantes p.Ser76Pro, p.Arg263Pro e p.Arg819Cys segregam claramente com o fenótipo de baixa estatura nas famílias (Figura 3). De maneira distinta, as variantes p.Ala164Gly e p.Asn546Tyr estão presentes nos pacientes, mas não nas suas mães que também apresentam baixa estatura (Figura 3).

Figura 3 - Segregação das variantes alélicas no *NPR2* nas famílias dos pacientes afetados. Os carreadores das variantes são denominados N/M. Os números abaixo dos indivíduos correspondem ao escore-Z da altura. As setas indicam os probandos de cada família. Os símbolos cheios referem-se a indivíduos com baixa estatura

5.5 Resultados do estudo funcional das variantes p.Ser76Pro, p.Arg263Pro e p.Arg819Cys no *NPR2*

5.5.1 Ensaaios *in vitro* de geração de cGMP

A análise por *Western blot* confirmou a expressão das três proteínas mutantes, comparável à expressão do NPR-B selvagem, a cerca de 120KD (Figura 4). Nota-se que as expressões das variantes HA-S76P e HA-R263P foram detectadas em apenas uma banda, enquanto as proteínas selvagem e HA-R819C foram detectadas em duas bandas distintas.

Figura 4 - *Western blot* do extrato de proteínas celulares de células transfectadas com HA-NPRB selvagem, HA-S76P, HA-R263P e HA-R819C e demonstração do vetor vazio. Utilizado como anticorpo primário anti-HA

A produção de cGMP foi avaliada em cada um dos três tipos de células transfectadas com as sequências mutantes, na célula com NPR-B selvagem e em células controle transfectadas com o plasmídeo vazio. Após tratamento com CNP na concentração de 100nM, a produção de cGMP aumentou em cem vezes nas células selvagens em comparação às células sem o *NPR2*. A produção de cGMP foi significativamente menor nos três tipos de células com *NPR2* mutante ($p < 0,001$), demonstrando um prejuízo funcional das proteínas p.Ser76Pro, p.Arg263Pro e p.Arg819Cys (Figura 5).

De modo a confirmar o efeito dominante negativo das três mutações, as células cotransfectadas com o gene selvagem e cada um dos mutantes mostraram uma diminuição significativa na produção de cGMP após estímulo com CNP em comparação às células transfectadas com o gene selvagem e o vetor vazio ($p < 0,001$) (Figura 6).

Figura 5 - Células COS-7 transfetadas com HA-NPR-B selvagem, cada um dos mutantes (HA-S76P, HA-R263P e HA-R819C) e com o vetor vazio foram incubadas com 100 nM de CNP por 20 minutos. Os resultados mostram produção diminuída de cGMP após estímulo com CNP em células transfetadas com os três diferentes mutantes

Figura 6 - Avaliação do efeito dominante negativo das três proteínas mutantes sobre a selvagem. Células COS-7 foram cotransfetadas com quantidades equivalentes de cDNA selvagem mais o vetor vazio ou mais cada cDNA mutante (relação cDNA selvagem: mutante de 1:1). Os resultados mostram produção diminuída de cGMP após estímulo com CNP em células cotransfetadas com cada um dos três mutantes

5.5.2 Microscopia confocal

A localização do NPR-B na membrana celular foi avaliada para testar a hipótese de que essas mutações potencialmente levariam a um defeito no tráfego dos receptores em direção à membrana. O NPR-B selvagem foi observado na membrana celular (visto em vermelho), assim como o mutante Arg819Cys. Por outro lado, os receptores mutantes Ser76Pro e Arg263Pro não foram localizados na membrana celular (Figura 7).

NPR-B selvagem

NPR-B S76P

NPR-B R263P

NPR-B R819C

Figura 7 - Avaliação da localização celular do NPR-B. As linhagens celulares COS-7 foram submetidas à imunofluorescência indireta, através do anticorpo primário de rato anti-HA e anticorpo secundário Alexa Flour 568 anti-rato (vermelho). O núcleo celular, corado com DAPI, é visto em azul

5.6 Resultados do estudo funcional das variantes p.Ala164Gly e p.Asn546Tyr no *NPR2*

5.6.1 Ensaaios *in vitro* de geração de cGMP

Células COS-7 transfectadas com as sequências selvagem, com o vetor vazio e com cada uma das sequências mutantes foram avaliadas quanto à produção de cGMP. Após estímulo com CNP, as células com as variantes p.Ala164Gly e p.Asn546Tyr apresentaram uma redução de 19% e 31%, respectivamente, na síntese de cGMP (Figura 8).

Entretanto, as células cotransfectadas com quantidades equivalentes de cDNA selvagem e cada um dos mutantes não mostraram uma redução significativa na produção de cGMP em relação a células com o cDNA selvagem e o vetor vazio (Figura 9). Isso sugere que as variantes p.Ala164Gly e p.Asn546Tyr não apresentam um efeito dominante negativo em heterozigose.

Figura 8 - Células COS-7 transfectadas com HA-NPR-B selvagem, cada um dos mutantes (HA-A164G e HA-N546Y) e com o vetor vazio foram incubadas com 100 nM de CNP por 20 minutos. Os resultados mostram produção diminuída de cGMP após estímulo com CNP em células transfectadas com os dois diferentes mutantes. * $p < 0,05$ e ** $p < 0,01$

Figura 9 - Ausência de efeito dominante negativo das duas proteínas mutantes sobre a selvagem. Células COS-7 foram cotransfectadas com quantidades equivalentes de cDNA selvagem mais o vetor vazio ou mais cada cDNA mutante (relação cDNA selvagem: mutante de 1:1). Os resultados mostram produção semelhante de cGMP após estímulo com CNP em células cotransfectadas com cada um dos dois mutantes

5.6.2 Microscopia confocal

A localização do NPR-B em células U2OS foi realizada para se avaliar o impacto das variantes p.Ala164Gly e p.Asn546Tyr no tráfego dos receptores até a membrana celular. Ambos receptores mutantes foram observados na membrana celular (visto em verde), assim como o NPR-B selvagem, e não se colocalizaram com o retículo endoplasmático (visto em vermelho) (Figura 10).

Figura 10 - Avaliação da localização celular do NPR-B. As linhagens celulares U2OS foram submetidas à imunofluorescência indireta, através do anticorpo primário de camundongo anti-HA e anticorpo secundário Alexa Flour 488 anti-camundongo (verde). O retículo endoplasmático, corado com CALR, é visto em vermelho. O núcleo celular, corado com DAPI, é visto em azul

5.7 Caracterização fenotípica dos pacientes heterozigotos para as variantes alélicas funcionalmente deletérias no *NPR2*

Os três pacientes carreadores das mutações são do sexo masculino. As características clínicas e laboratoriais desses pacientes encontram-se resumidas na Tabela 5.

5.7.1 Paciente com a mutação p.Ser76Pro

Em sua primeira consulta, com 12,9 anos, o paciente apresentava baixa estatura com discreta desproporção corporal, IMC normal, idade óssea compatível com idade cronológica e já havia iniciado a puberdade (estádio 3 de Tanner) (Tabela 5). A avaliação hormonal revelou uma resposta normal do GH ao estímulo com clonidina e níveis puberais de gonadotrofinas e testosterona. A radiografia de esqueleto não evidenciou alterações. Foi iniciado rhGH (50µg/Kg/d), mantido até os dias atuais, e letrozol (2,5mg/d), do qual o paciente fez uso por 2 anos (Figura 11). Com o tratamento, o IGF-1 chegou a 968ng/mL (escore-Z: +2,2) e o IGFBP-3 a 8,7µg/mL (escore-Z: +1,5). O escore-Z da altura se manteve estável durante o tratamento, mas a previsão da altura adulta baseada na idade óssea aumentou de 156 cm para 167,4 cm. Na última consulta, com 15,6 anos, apresentava escore-Z da altura de -2,1 e velocidade de crescimento de 6 cm/ano. A mutação p.Ser76Pro também foi identificada no pai do paciente (145 cm) e na irmã de 18 anos (139 cm), que notavelmente também apresenta desproporção corporal discreta (escore-Z da altura sentada/altura total: +2,0). A mãe do paciente, com 153 cm, tem o genótipo do *NPR2* normal (Figura 12).

Figura 11 - Curva de crescimento do paciente com a mutação p.Ser76Pro

Figura 12 - Heredograma da família do paciente com a mutação p.Ser76Pro. Os portadores da mutação são denominados N/M. Os números abaixo dos indivíduos correspondem ao escore-Z da altura. Os símbolos sólidos representam os indivíduos com baixa estatura

5.7.2 Paciente com a mutação p.Arg263Pro

O paciente apresentou-se aos 9,4 anos de idade, com baixa estatura proporcional, IMC adequado e idade óssea atrasada (Tabela 5). Ele tinha histórico de tireoide sublingual, com hipotireoidismo adequadamente tratado desde os 5 anos. Os exames hormonais mostraram níveis normais de IGF-1 e IGFBP-3 e resposta adequada do GH ao estímulo com clonidina. O paciente foi tratado com rhGH (33µg/Kg/d) por 3,3 anos e análogo de GnRH por 1ano e meio, tendo atingido altura adulta de 158 cm (escore-Z da altura final: -2,5) (Figura 13). Após 1 ano de tratamento, o IGF-1 foi 720ng/mL (escore-Z: +1,1), tendo atingido o valor de 1180ng/mL (escore-Z: +2,9) após a suspensão do análogo de GnRH. Tanto o pai do paciente (156 cm), quanto o avô paterno (150 cm), eram heterozigotos para a mutação p.Arg263Pro. Já sua mãe (156 cm) e irmã (126 cm com 9,4 anos) não apresentaram a mesma alteração no *NPR2* (Figura 14).

Figura 13 - Curva de crescimento do paciente com a mutação p.Arg263Pro

Figura 14 - Heredograma da família do paciente com a mutação p.Arg263Pro. Os carreadores da mutação são denominados N/M. Os números abaixo dos indivíduos correspondem ao escore-Z da altura. Os símbolos sólidos representam os indivíduos com baixa estatura

5.7.3 Paciente com a mutação p.Arg819Cys

Em sua primeira avaliação, com 11,1 anos, o paciente apresentava baixa estatura proporcional, IMC normal com leve hipertrofia muscular e idade óssea compatível com a idade cronológica (Tabela 5). As dosagens de IGF-1 e IGFBP-3 foram normais. A radiografia de esqueleto mostrou metacarpos curtos (Figura 15). Ele iniciou a puberdade aos 12,1 anos, com 131 cm e previsão de altura adulta baseada na idade óssea de 160 cm. Nesse momento, foi iniciado tratamento com rhGH (50µg/Kg/d) e análogo de GnRH. Durante o tratamento, o valor do IGF-1 chegou a 838ng/mL (escore-Z: +2,6). O análogo de GnRH foi suspenso após 2,7 anos, com altura de 147,1 cm, e a puberdade evoluiu normalmente. Na última consulta ambulatorial do paciente, com 19,2 anos e na qual foi suspenso o uso de rhGH, a velocidade de crescimento estava em 1,0 cm/ano e a sua altura era de 163,5 cm (escore-Z: -1,7) (Figura16). Nessa ocasião, observou-se acentuação do aspecto musculoso (Figura 17) apesar do hábito sedentário. O pai do paciente (175 cm) não tem a alteração no *NPR2*. A mãe, com altura no limite inferior da normalidade (152 cm), mas com proporção corporal alterada (escore-Z da altura sentada:altura total = +3,3), tem a mutação p.Arg819Cys (Figura 18). A radiografia das mãos da mãe não evidenciou metacarpos curtos.

Figura 15 - Radiografia da mão do paciente com a mutação p.Arg819Cys demonstrando metacarpos curtos

Figura 16 - Curva de crescimento do paciente com a mutação p.Arg819Cys

Figura 17 - Foto do tronco do paciente portador da mutação p.Arg819Cys aos 19 anos evidenciando hipertrofia muscular

Figura 18 - Heredograma da família do paciente com a mutação p.Arg819Cys. Os carreadores da mutação são denominados N/M. Os números abaixo dos indivíduos correspondem ao escore-Z da altura. Os símbolos sólidos representam os indivíduos com baixa estatura

Tabela 5 - Características clínicas e laboratoriais dos três pacientes com mutações em heterozigose no *NPR2*

Paciente	1	2	3
Mutação no <i>NPR2</i>	p.Ser76Pro	p.Arg263Pro	p.Arg819Cys
Sexo	Masculino	Masculino	Masculino
escore-Z da altura do pai	-4,5 ^a	-2,8 ^a	0
escore-Z da altura da mãe	-1,6	-1,0	-1,7 ^a
Primeira avaliação			
Idade (anos)	12,9	9,8	11,2
Idade óssea (anos)	13	7,5	10
escore-Z da altura	-2,1	-2,8	-2,8
escore-Z da envergadura	-3,7	-2,5	-4,0
escore-Z da mão	-1,8	ND	-2,7
escore-Z da altura sentada:altura total	2,3	0	1,3
escore-Z do IMC	0,4	0,4	1,6
Última avaliação			
Idade (anos)	15,6	20,8	19,2
Idade óssea (anos)	ND	19	
escore-Z da altura	-2,1	-2,5	-1,7
Avaliação laboratorial antes do tratamento			
Pico de GH no teste de estímulo (ng/ml) ^b	19,4	5,9	ND
escore-Z do IGF-1	ND	0,3	0,2
escore-Z do IGFBP-3	ND	-1,6	0

ND: não disponível

^a presença da mutação em heterozigose^b pico de GH considerado normal acima de 3,3 ng/ml ⁽³⁶⁾

6 Discussão

6 DISCUSSÃO

Fatores hereditários explicam aproximadamente 80% da variabilidade da altura em humanos ⁽³⁷⁾. Embora polimorfismos comuns sejam significativamente associados à altura de indivíduos saudáveis, a influência individual de cada variante polimórfica é pequena (ao redor de 4 mm). Há evidências de que variantes genéticas raras influenciam a estatura de modo mais expressivo ⁽³⁸⁾. Até o momento, defeitos em poucos genes foram associados ao fenótipo de baixa estatura idiopática ⁽³⁹⁻⁴⁴⁾.

Há ampla evidência científica que indica a importância da via CNP/NPR-B na condrogênese e no crescimento linear ^(4, 5, 9, 18, 27). A ação do sistema do peptídeo natriurético no crescimento longitudinal é parcialmente explicada pela capacidade da transdução do sinal pelo NPR-B de inibir a cascata da MAPK na via de sinalização do FGFR3 (OMIM 134934) ⁽⁴⁵⁾. Mutações ativadoras do *FGFR3*, que como consequência levam à ativação sustentada da via MAPK, são causadoras da acondroplasia (OMIM 100800) e hipocondroplasia (OMIM 146000) ⁽⁴⁶⁾, duas importantes displasias esqueléticas associadas à baixa estatura com encurtamento dos membros. O uso de CNP e de seus análogos foi estudado em modelos animais de acondroplasia, com melhora do crescimento e recuperação do fenótipo esquelético ^(47, 48). O CNP recombinante humano, denominado BMN-111 (vosoritide), está em fase 2 de estudo clínico, com o objetivo de se avaliar a segurança, a tolerabilidade e a eficácia em crianças com acondroplasia (*ClinicalTrials.gov Identifier*: NCT02055157).

Outro fator que reforça a influência da via CNP/NPR-B no crescimento é a sua relação com o gene *SHOX* (OMIM 312865), cuja haploinsuficiência é causa já bem estabelecida de baixa estatura ⁽⁴⁹⁾. Sabe-se que o *SHOX* induz à expressão do gene *NPPB*, que codifica o peptídeo natriurético encefálico, outro importante ligante do NPR-B ⁽⁵⁰⁾. Dessa forma, a ativação do *SHOX* pode estimular diretamente a via do NPR-B ou aumentar a concentração local de CNP através da saturação do receptor NPR-C.

Baseado na baixa estatura de familiares de pacientes com AMDM, foi sugerido que mutações em heterozigose no *NPR2* poderiam ser responsáveis pela baixa estatura de crianças classificadas como tendo BEI ^(8, 22). No estudo atual, nós identificamos 5 variantes alélicas *missense* em heterozigose em um grupo de 60 pacientes, e 3 foram classificadas como provavelmente patogênicas por múltiplas linhas de evidência.

A perda da função do NPR-B causada pelas variantes p.Ser76Pro, p.Arg263Pro e p.Arg819Cys foi confirmada pela ausência de elevação da atividade da guanililciclase após estímulo com CNP (Figura 5). Adicionalmente, após transfecção selvagem/mutante, observou-se que essas variantes em heterozigose levam a um efeito dominante negativo (Figura 6). A análise da microscopia confocal demonstrou que o mutante p.Arg819Cys apresenta, pelo menos em parte, um tráfego normal para a membrana celular. Por outro lado, a perda de função dos mutantes p.Ser76Pro e p.Arg263Pro pode ser devida à retenção desses receptores no retículo endoplasmático, um mecanismo já descrito para outras mutações *missense* no *NPR2* ⁽²⁵⁾. No nosso experimento de *Western blot*, o NPR-B

selvagem foi observado em duas bandas, assim como o p.Arg819Cys, o que reflete duas diferentes glicoformas^(51, 52). Já os mutantes p.Ser76Pro e p.Arg263Pro foram expressos em uma única banda, sugerindo um defeito na glicosilação dessas proteínas. Uma forma truncada do NPR-B (NPR-B-490) que também é menos glicosilada exerce efeito dominante negativo sobre o receptor selvagem. É postulado que a glicosilação incompleta impede que dímeros formados pelo receptor selvagem e mutado alcancem a membrana celular⁽⁵¹⁾. É possível que o mesmo mecanismo explique o efeito dominante negativo dos mutantes p.Ser76Pro e p.Arg263Pro. Em relação ao mutante p.Arg819Cys, postulamos que ele forma um heterodímero com o NPR-B selvagem e interfere na transdução do sinal de maneira dominante negativa, mecanismo previamente sugerido para uma mutação localizada no domínio de homologia à quinase⁽²²⁾. Entretanto, estudos adicionais são necessários para se compreender completamente os mecanismos responsáveis pelo efeito dominante negativo de cada mutação. Além disso, é importante ressaltar que os estudos funcionais realizados neste trabalho não permitem avaliar o efeito de haploinsuficiência.

Todas as mutações *missense* relatadas em pacientes com AMDM determinam uma diminuição drástica da atividade da guanililciclase após o estímulo com CNP^(22, 25), semelhante ao observado com as variantes p.Ser76Pro, p.Arg263Pro e p.Arg819Cys identificadas em pacientes com BEI. Em contraste, as variantes p.Ala164Gly e p.Asn546Tyr levam a uma discreta redução na síntese de cGMP (Figura 8), o que nos sugere que o impacto funcional dessas variantes não é significativo. Além disso, não foi

visto um efeito dominante negativo após transfecção selvagem/mutante (Figura 9) e os receptores com estas variantes se localizam, como esperado, na membrana plasmática (Figura 10).

Considerando a falta de segregação familiar conclusiva e a ausência de efeito funcional deletério na proteína ⁽⁵³⁾, as variantes p.Ala164Gly e p.Asn546Tyr são classificadas como benignas. Já as variantes p.Ser76Pro, p.Arg263Pro e p.Arg819Cys são classificadas como provavelmente patogênicas, pois são funcionalmente deletérias, apresentam segregação positiva, comprometimento em estudos *in silico* e sabe-se que variantes *missense* diversas no *NPR2* levam a AMDM em homozigose ⁽⁵³⁾.

A análise de segregação nas famílias com variantes alélicas provavelmente patogênicas no *NPR2* revela um padrão de herança autossômica dominante. Há um fenótipo variável em relação à intensidade da baixa estatura, proporções corporais e anormalidade esquelética inespecífica, mesmo considerando membros de uma mesma família. Por exemplo, na família com a mutação p.Arg819Cys, o paciente tem baixa estatura proporcional e aspecto musculoso, enquanto a mãe tem a altura no limite inferior da normalidade com desproporção corporal. Recentemente, essa mesma mutação foi descrita em um paciente espanhol com baixa estatura desproporcional e hipertrofia muscular ⁽⁵⁴⁾.

Esse fenótipo variável é observado também em famílias com deficiência isolada do *SHOX* ⁽⁴⁹⁾. E de forma interessante, há sobreposição de características clínicas entre pacientes heterozigotos para mutações no *SHOX* e *NPR2*. Recentemente, mutações em heterozigose no *NPR2* foram

identificadas em 3% de uma coorte de pacientes com baixa estatura desproporcional com suspeita de discondroesteose de Léri-Weill e sem defeitos no *SHOX* ⁽⁵⁴⁾.

É interessante notar que nenhum dos pacientes apresentou mudança no canal de crescimento após a introdução do rhGH. Somente o paciente com a mutação p.Arg819Cys teve uma melhora do escore-Z da altura, observada na última consulta. Há de se considerar que todos os pacientes iniciaram o tratamento já na puberdade, o que pode ter contribuído para a resposta insatisfatória. Previamente, há a descrição de um paciente com mutação em homozigose no *NPR2* que recebeu tratamento com rhGH (50µg/Kg/d) por 4 anos, cujo escore-Z da altura diminuiu de -6,3 para -7,2 ⁽⁸⁾. E, mais recentemente, há relatos de 5 pacientes com mutações em heterozigose no *NPR2* tratados com rhGH com respostas bastante variáveis ⁽⁵⁴⁻⁵⁶⁾.

Em resumo, nós identificamos mutações em heterozigose no *NPR2* em 5% dos pacientes inicialmente classificados como tendo BEI. Em contraste com mutações em homozigose neste gene, que levam à baixa estatura grave com fenótipo esquelético típico, mutações em heterozigose no *NPR2* estão associadas a alterações leves e variáveis no crescimento, sem fenótipo característico.

Após a publicação do nosso trabalho, dois outros estudos reforçaram o papel de mutações em heterozigose no *NPR2* como causa de baixa estatura. Um estudo japonês identificou 2 mutações em heterozigose no *NPR2* em uma coorte de 101 pacientes com BEI ⁽⁵⁵⁾. Em outro estudo, foram

encontradas mutações em heterozigose no *NPR2* em 1,9% de 320 pacientes com BEI e, considerando só os pacientes com baixa estatura familiar, essa prevalência foi de 13,6% ⁽⁵⁶⁾.

Nossos resultados e os estudos subsequentes indicam que mutações em heterozigose no *NPR2* são uma causa importante de baixa estatura não sindrômica familiar. E, no nosso serviço, com a maior disponibilidade do sequenciamento paralelo de larga escala, o gene *NPR2* foi incluído em painéis para o diagnóstico de crianças com baixa estatura idiopática. Com essa medida, objetivamos diminuir a realização de exames desnecessários, aliviar a ansiedade dos pais e dar continuidade aos estudos para se aprimorar a abordagem terapêutica de crianças com mutações em heterozigose no *NPR2*.

7 Conclusões

7 CONCLUSÕES

Foram identificadas mutações em heterozigose no gene *NPR2* em 3 de 60 pacientes com baixa estatura inicialmente classificada como idiopática.

Os receptores NPR-B com as mutações p.Ser76Pro, p.Arg263Pro e pArg819Cys exercem um efeito dominante negativo sobre o receptor selvagem e são responsáveis por um prejuízo funcional na via de sinalização do NPR-B.

Mutações em heterozigose no *NPR2* determinam baixa estatura com modo de herança autossômica dominante, com penetrância completa e fenótipo variável.

8 Anexos

ANEXO A - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA
UNIVERSIDADE DE SÃO PAULO-HCFMUSP

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**DADOS DE IDENTIFICAÇÃO DO SUJEITO DA PESQUISA OU RESPONSÁVEL LEGAL**

1. NOME: :.....

DOCUMENTO DE IDENTIDADE Nº : SEXO : .M ☐ F ☐

DATA NASCIMENTO:/...../.....

ENDEREÇO Nº APTO:

BAIRRO: CIDADE

CEP:..... TELEFONE: DDD (.....)

2. RESPONSÁVEL LEGAL

NATUREZA (grau de parentesco, tutor, curador etc.)

DOCUMENTO DE IDENTIDADE :SEXO: M ☐ F ☐

DATA NASCIMENTO.:/...../.....

ENDEREÇO: Nº APTO:

BAIRRO: CIDADE:

CEP: TELEFONE: DDD (.....).....

DADOS SOBRE A PESQUISA

1. **TÍTULO DO PROTOCOLO DE PESQUISA:** “Pesquisa de mutações no gene do receptor do peptídeo natriurético do tipo C (*NPR2*) em indivíduos com baixa estatura idiopática”.

PESQUISADOR : Alexander Augusto de Lima Jorge

CARGO/FUNÇÃO: Médico Responsável e Professor da Faculdade de Medicina –USP INSCRIÇÃO
CONSELHO REGIONAL Nº 80.218

UNIDADE DO HCFMUSP: Disciplina de Endocrinologia e Metabologia

3. AVALIAÇÃO DO RISCO DA PESQUISA:

RISCO MÍNIMO ☒ RISCO MÉDIO ☐

RISCO BAIXO ☐ RISCO MAIOR ☐

4. DURAÇÃO DA PESQUISA : 4 anos

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA
UNIVERSIDADE DE SÃO PAULO-HCFMUSP

- 1 – Desenho do estudo e objetivo(s): O senhor(a) e/ou seu filho(a) está sendo convidado a participar deste estudo que visa avaliar, sob o ponto de vista genético, crianças com baixa estatura sem causa definida. O estudo tem como objetivo identificar e avaliar possíveis alterações no gene NPR2 que é responsável por regular o crescimento do esqueleto – principalmente dos ossos longos.
- 2 – Descrição dos procedimentos que serão realizados, com seus propósitos e identificação dos que forem experimentais e não rotineiros: nenhum.
- 3 – Relação dos procedimentos rotineiros e como são realizados – Serão coletados 2 tubos pequenos ou dez mililitros (10ml) de sangue venoso para obter material genético (DNA) que será utilizado para o estudo.
- 4 – Descrição dos desconfortos e riscos esperados nos procedimentos dos itens 2 e 3; Apenas os relacionados à coleta de sangue simples: dor mínima no local da punção e risco mínimo de formar mancha roxa local (equimose) ou de infecção local.
- 5 – Benefícios para o participante: O estudo não traz benefícios diretos aos envolvidos. Porém, nosso estudo ajudará a identificar uma causa genética / familiar de baixa estatura.
- 6 – Relação de procedimentos alternativos que possam ser vantajosos, pelos quais o paciente pode optar; não existe
- 7 – Garantia de acesso: em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis pela pesquisa para esclarecimento de eventuais dúvidas. O principal investigador é o Prof. Dr *Alexander Augusto de Lima Jorge*, que pode ser encontrado no endereço - *Faculdade de Medicina da USP (LIM-25) - Av. Dr. Arnaldo, 455 5º andar sala 5340 CEP 01246-903 São Paulo – SP* Telefone(s) (55 11) 3061-7252. Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa (CEP) da FMUSP – Av. Dr, Arnaldo 455 – São Paulo – SP – CEP 01246-903, telefone: 3061-7412, – E-mail: cep.fmusp@hcnet.usp.br

8 – É garantida a liberdade da retirada de consentimento a qualquer momento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na Instituição;

09 – Direito de confidencialidade – As informações obtidas serão analisadas em conjunto com outros pacientes, não sendo divulgada a identificação de nenhum paciente;

10 – Direito de ser mantido atualizado sobre os resultados parciais das pesquisas, quando em estudos abertos, ou de resultados que sejam do conhecimento dos pesquisadores;

11 – Despesas e compensações: não há despesas pessoais para o participante em qualquer fase do estudo, incluindo exames e consultas. Também não há compensação financeira relacionada à sua participação. Se existir qualquer despesa adicional, ela será absorvida pelo orçamento da pesquisa.

12 - Compromisso do pesquisador de utilizar os dados e o material coletado somente para esta pesquisa.

Acredito ter sido suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo “Pesquisa de mutações no gene do receptor do peptídeo natriurético do tipo C (*NPR2*) em indivíduos com baixa estatura idiopática”. Eu discuti com o Dr. Alexander Augusto de Lima Jorge sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido, ou no meu atendimento neste Serviço.

Assinatura do paciente/representante
legal

Data ____ / ____ / ____

Assinatura da testemunha

Data ____ / ____ / ____

para casos de pacientes menores de 18 anos, analfabetos, semi-analfabetos ou portadores de deficiência auditiva ou visual.

(Somente para o responsável do projeto)

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste paciente ou representante legal para a participação neste estudo.

Assinatura do responsável pelo estudo

Data ____ / ____ / ____

9 Referências

9 REFERÊNCIAS

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10 Apêndices

APÊNDICE A

Vasques GA, Amano N, Docko AJ, Funari MFA, Quedas EPS, Nishi MY, Arnhold IJP, Hasegawa T, Jorge AAL. Heterozygous Mutations in Natriuretic Peptide Receptor-B (*NPR2*) Gene as a Cause of Short Stature in Patients Initially Classified as Idiopathic Short Stature. J Clin Endocrinol Metab. 2013 Oct;98(10):E1636-44. doi: 10.1210/jc.2013-2142.

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Advances in Genetics—Endocrine Care

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 disproportionate 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 $\mu\text{g/kg-d}$) 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)

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Abbreviations: AMDM, acromesomelic dysplasia, type Maroteaux; BMI, body mass index; CNP, C-type natriuretic peptide; GnRH, GnRH agonist; HA, hemagglutinin; ISS, idiopathic short stature; *NPR2*, *NPR-B* gene; *NPR-B*, natriuretic peptide receptor-B; OMIM, online inheritance in man; rhGH, recombinant human GH; SDS, SD score; SHA, sitting height to height ratio; WT, wild type.

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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: postnatal 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 bidirectionally 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/>, May 5, 2013) by χ^2 , 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>) (22) and the PolyPhen method (<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 (antirabbit 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 OptiMax (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 into 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 antirabbit 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>).

Figure 1. Schematic representation of the homodimeric domain structure of NPR-B and the location of mutations within the receptor protein. Nonsense and frameshift mutations are located on the left, whereas the missense mutations are located on the right of the figure. The variants described in this study are in bold.

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.

Figure 2. In vitro functional assay. A, NPR-B protein expression in COS-7 cells transfected with HA-WT, HA-S76P, HA-R263P, HA-R819C, or empty vector (mock). Representative Western blots with anti-HA antibody are shown. B, COS-7 cells transiently transfected with empty vector (mock) or HA-WT NPR-B or each mutant (200 ng/well) were incubated with 100 nM CNP for 20 minutes. Results are expressed in cGMP measurement after CNP stimulation (mean $\pm$ 1 SEM). C, Evaluation of a putative dominant-negative effect of the identified NPR-B mutants on the WT NPR-B. COS-7 cells cotransfected with constant amounts of HA-WT NPR-B cDNA (100 ng/well) plus empty vector or plus each mutant NPR-B cDNAs at WT to mutant ratios of 1:1. Results are expressed in cGMP measurement after CNP stimulation (mean $\pm$ 1 SEM). Data are representative of three independent experiments (each performed in triplicate) with similar results.

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 binding

Figure 3. Immunolocalization of HA-tagged WT and each identified mutant receptor transiently expressed in COS-7 cells. The immunostaining of HA-tagged NPR-B was performed by means of an anti-HA monoclonal primary antibody incubated to visualize the receptors located at the cell surface in red. Nuclei stained with 4',6'-diamino-2-phenylindole are shown in blue.

Table 1. Clinical and Laboratorial Characteristics of the Three Patients With Functionally Significant Heterozygous *NPR2* Mutations

	Patient 1	Patient 2	Patient 3
<i>NPR2</i> mutation	p.Ser76Pro	p.Arg263Pro	p.Arg819Cys
Sex	male	male	male
Father's height SDS	−4.5 ^a	−2.8 ^a	0.0
Mother's height SDS	−1.6	−1.0	−1.7 ^a
First evaluation			
Age, y	12.9	9.8	11.2
Bone age, y	13	7.5	10
Height SDS	−2.1	−2.8	−2.8
Arm span SDS	−3.7	−2.5	−4.0
Sitting height to total height SDS	2.3	0.0	1.3
BMI SDS	0.4	0.4	1.6
Last observation			
Age, y	15.3	20.8	17.1
Bone age, y	13.5	19	15
Height SDS	−2.1	−2.5	−2.4
Pretreatment laboratory evaluation			
GH peak at stimulation test, $\mu\text{g/L}$ ^b	19.4	5.9	NA
IGF-I SDS	NA	0.3	0.2
IGFBP-3 SDS	NA	−1.6	0

Abbreviation: NA, not available.

^a Presence of the mutation in heterozygous state.^b normal GH peak greater than 3.3 $\mu\text{g/L}$ (40).

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 $\mu\text{g/kg}\cdot\text{d}$) 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 proportional 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 $\mu\text{g/kg}\cdot\text{d}$) for 3.3 years, his final height was 158 cm (height SDS −2.5) (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 $\mu\text{g/kg}\cdot\text{d}$ 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 (−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.

Figure 5. Pedigrees of the affected families carrying heterozygous *NPR2* mutations: p.Ser76Pro (A), p.Arg263Pro (B), and p.Arg819Cys (C). The arrows indicate the probands. The height SDS and corresponding genotype (N, wild type allele; M, mutant allele) for each family member are as indicated. Subjects with short stature are indicated by solid symbols, whereas individuals with normal stature are indicated as open symbols.

(25). 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 muta-

tions 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%; $P = .006$). 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 ($50 \mu\text{g/kg}\cdot\text{d}$) (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.

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APÊNDICE B

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Role of the Natriuretic Peptide System in Normal Growth and Growth Disorders

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Key Words

Growth · Natriuretic peptide · Short stature · Tall stature · Skeletal dysplasia

Abstract

The C-type natriuretic peptide (CNP) and its receptor (NPR-B) are recognized as important regulators of longitudinal growth. Animal models involving CNP or NPR-B genes (*Nppc* or *Npr2*) support the fundamental role of CNP/NPR-B for endochondral ossification. Studies with these animals allow the development of potential drug therapies for dwarfism. Polymorphisms in two genes related to the CNP pathway have been implicated in height variability in healthy individuals. Biallelic loss-of-function mutations in NPR-B gene (*NPR2*) cause acromesomelic dysplasia type Maroteaux, a skeletal dysplasia with extremely short stature. Heterozygous mutations in *NPR2* are responsible for nonsyndromic familial short stature. Conversely, heterozygous gain-of-function mutations in *NPR2* cause tall stature, with a variable phenotype. A phase 2 multicenter and multinational trial is being developed to evaluate a CNP analog treatment for achondroplasia. Pediatricians and endocrinologists must be aware of growth disorders related to natriuretic peptides, although there is still much to be learned about its diagnostic and therapeutic use.

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Introduction

Growth is an essential process for the development of a healthy adult and it is a sensitive marker of child health status. It comprises a dynamic, nonhomogeneous and complex process of replication and differentiation of cells from all tissues. It is generally assumed that growth is regulated by genetic, nutritional, environmental and hormonal factors. With respect to genes, adult height and growth pattern are largely genetically programmed. Studies in twins have indicated that the heritability of height is high (~80%), suggesting that genetic variation is the main determinant of stature [1]. Although rare genetic variants are involved with larger effects on stature, common polymorphisms are significantly associated with height in healthy individuals [2]. Among the identified genes associated with stature, there are several genes that act in the growth plate-promoting endochondral ossification [3].

Genome-wide association studies identified genetic loci related to human height variability mapped in natriuretic peptide system genes [2–7]. Since 1998, C-type natriuretic peptide (CNP) and its receptor (NPR-B) have been implicated as important regulators of endochondral bone growth [8, 9]. Animal models support the importance of this system for normal growth [10–12] and allow

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the development of studies involving new treatment perspectives [13, 14]. Additionally, it is known that homozygous or compound heterozygous loss-of-function mutations in *NPR2* cause acromesomelic dysplasia type Maroteaux (AMDM; OMIM*602875), a skeletal dysplasia with extremely short stature [15]. Recently, heterozygous loss-of-function mutations in *NPR2* have been implicated as a cause of short stature in patients initially classified as having idiopathic short stature [16, 17]. Conversely, some studies have pointed heterozygous gain-of-function mutations in *NPR2* as a cause of tall stature [18–20]. A similar phenotype had been observed in an individual with overexpression of the CNP caused by a balanced translocation [21, 22].

Due to the discovery of new mutations in the CNP/NPR-B system and emerging therapeutic perspectives related to CNP to increase height velocity, this review aims to disseminate the up-to-date knowledge to researchers and clinicians. For the present review, the PubMed database was searched for all publications relating the natriuretic peptide system to longitudinal growth or height.

Natriuretic Peptides and Their Receptors

The natriuretic peptide family consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), CNP and dendroaspis natriuretic peptide, a D-type natriuretic peptide (DNP) [23]. DNP is the newest member of the natriuretic peptide family and, despite causing vasorelaxation, little is known about its function [24]. ANP and BNP have hemodynamic and anti-remodeling actions in the cardiovascular system. They have diuretic, natriuretic, vasorelaxant, anti-proliferative, and anti-hypertrophic effects [23]. CNP, the third member of the natriuretic peptide family, lacks a significant natriuretic function and plays a key role in the regulation of endochondral bone growth [25]. CNP occurs in a wide variety of tissues, but it is notably present in the hypertrophic zone of the growth plate. CNP promotes synthesis of cartilage matrix and stimulates chondrocyte proliferation and differentiation in an autocrine/paracrine manner [25].

The biological actions of natriuretic peptides are mediated by activation of two subtypes of receptors: NPR-A and NPR-B [26]. They are homodimeric receptors that are characterized by a modular structure: an extracellular ligand-binding domain, a transmembrane region, an intracellular kinase homology domain and a carboxyl-terminal guanylyl cyclase domain [27]. NPR-A and NPR-B have a high degree of similarity: approximately 43% in the

ligand-binding domain, 72% in the kinase homology domain and 91% in the guanylyl cyclase domain [28]. NPR-A is encoded by *NPR1* gene (OMIM 108960) which is located on chromosome 1 (q21–q22), whereas NPR-B is encoded by *NPR2* gene (OMIM 108961) which is located on chromosome 9 (p12–21). Both genes contain 22 exons [28]. ANP and BNP are endogenous ligands for NPR-A, while CNP binds preferentially to NPR-B. After ligand binding, there is an intracellular accumulation of cyclic GMP (cGMP) that activates several intracellular target molecules, such as cGMP-dependent protein kinases I and II (cGKI and cGKII), cyclic nucleotide regulated ion channels and cGMP-regulated phosphodiesterases [9, 25, 29] (fig. 1). cGKII knockout mice develop dwarfism as a result of impaired endochondral ossification, indicating that cGKII is important for CNP-mediated endochondral ossification [30]. However, the growth plate histology of cGKII-deficient mice differs from that of *Nppc* or *Npr2* knockout mice, suggesting the involvement of other signaling pathways [30]. NPR-C is a natriuretic peptide receptor with no intracellular guanylyl cyclase domain and is responsible for receptor-mediated degradation of natriuretic peptides. Natriuretic peptides are also degraded by endopeptidases [25].

Animal Models Involving Natriuretic Peptide and Growth

Mice with targeted disruption of CNP (*Nppc*^{−/−}) had disproportionate dwarfism with short limbs because of impaired endochondral ossification [10]. This phenotype was similar to that previously observed in two strains of mice with a spontaneous autosomal recessive mutant gene, named *cn/cn* [11] and *slw/slw* (short-limb dwarfism) mice [31]. Using linkage analysis and a candidate gene approach, homozygous loss-of-function mutations in *Npr2* gene, that encodes NPR-B receptor, have been shown to be responsible for the growth phenotype of these mice [11, 31]. Histological analysis had demonstrated that the tibial growth plates in affected mice (*Npr2*^{cn/cn} and *Npr2*^{slw/slw}) were narrower than in normal mice, with a decreased number of proliferating and hypertrophic chondrocytes [11, 31]. Recently, mice with a new naturally occurring homozygous mutant of *Npr2* (*pwe* = peewee) exhibited disproportional dwarfism and female infertility was discovered [32]. *Npr2*^{pwe/pwe} oocytes had premature meiotic resumption with no progression to the two-cell embryo stage [32]. Interestingly, a transgenic female mouse lacking NPR-B (*Nppc*^{−/−}) had also infertility.

Fig. 1. Schematic representation of the homodimeric domain structure of NPR-B. After CNP binding, there is an intracellular accumulation of cGMP.

They had uterine atrophy, with thin endometrium and myometrium, and no estrus cycles [12]. The reason why some *Npr2* animal models (*Npr2^{we/pwe}* and *(Nppc^{-/-})* have infertility phenotype whereas others do not (*Npr2^{at/at}*) remains unknown, and this difference may be related to the genetic background of the strains [32]. Interestingly, it was demonstrated that CNP is expressed in ovarian granulosa cells and inhibits oocyte maturation by the activation of NPR-B [33]. Furthermore, treatment with CNP in infantile mice promotes ovarian growth, allowing successful ovulation induction by gonadotropins [34].

The effect of heterozygous *Npr2* mutation on length was evaluated in mice with one disrupted *Npr2* allele (*Npr2^{+/-}*). *Npr2^{+/-}* mice were significantly smaller in nasoanal length than wild-type ones [12]. In contrast, transgenic mice with overexpression of *Nppc* (col2-CNP-Tg and SAP-*Nppc*-Tg mice) had exaggerated linear growth with expansion of the proliferative and hypertrophic zones of the growth plate [13, 35]. A similar overgrowth phenotype was observed in mice lacking NPR-C, which is codified by *Npr3* gene (*Npr3^{-/-}*). As NPR-C is involved in the clearance of natriuretic peptides, its disruption can increase CNP available for binding to NPR-B [36, 37].

Polymorphisms in Natriuretic Peptide System and Normal Height Variability

Height is a complex quantitative trait, whose variability can be partially explained by the influence of several polymorphisms, each of them determining a small amount of this phenotype. In the last decade, a hundred single-nucleotide polymorphisms (SNP) capable of influencing adult height were identified by genome-wide association studies, including SNPs located near to *NPR3* and *NPPC* genes. Four SNPs (rs2679178, rs749052, rs2580816, rs6717918) located upstream of *NPPC* and five SNPs surrounding the *NPR3* gene (rs6450922, rs1173727, rs3792752, rs10472828, rs10472828) were related to adult height [2–7]. Each variant allele was associated with an estimated mean change of around 0.4 cm in height. There is little information about the interactions of these polymorphisms among themselves and their interaction with other SNPs already associated with height variability.

Mutations in Natriuretic Peptide System and Short Stature

Biallelic loss-of-function mutations in *NPR2*, the gene that encodes NPR-B in humans, cause AMDM (OMIM*602875), a rare disease described in 1971, with an estimated prevalence of <1/1,000,000 [15, 38]. It is a skeletal dysplasia characterized by an extremely disproportional short postnatal stature. AMDM can be suspected in affected patients by 1 year of age and skeletal survey is usually typical around 2 years of age. Predominantly, middle (forearms and forelegs) and distal (hands and feet) segments of the skeletal system are affected. Radiographs of the hands show short metacarpals and phalanges with cone-shaped epiphyses and early fusion of growth plates. Long tubular bones are normal in the first year of life and become progressively shorter [39]. The vertebral bodies are oval-shaped in childhood and there is posterior wedging of vertebral bodies, with shorter dorsal margins than the ventral ones, mainly in thoracic vertebrae [39]. Individuals with AMDM have an adult height SD score (SDS) <-5 and there are no reports of infertility in affected patients [15, 40].

Up to now, 25 different mutations in *NPR2* have been reported in 29 families with AMDM [15, 38, 40, 41]. Consanguinity was reported in 24 of 28 families. The majority of patients reported had homozygous loss-of-function mutations, whereas compound heterozygous mu-

Table 1. Loss-of-function mutations in *NPR2* found in patients with AMDM

Mutation	Protein	Exon/ intron	Domain	Status	Consan- guinity*	Ref.
c.94 C>A	p.P32T	exon 1	ligand binding	homozygous	yes	15
c.343T>G	p.W115G	exon 1	ligand binding	homozygous	yes	15
c.528T>A	p.D176E	exon 1	ligand binding	compound heterozygous	no	15
c.844C>T	p.Q282*	exon 2	ligand binding	homozygous	yes	15
c.890C>T	p.T297M	exon 3	ligand binding	homozygous	yes	15
c.1013A>G	p.Y338C	exon 4	ligand binding	homozygous	yes	15
c.1092delT	p.I364fs*376	exon 4	ligand binding	homozygous	no	38
c.1111C>T	p.R371*	exon 4	ligand binding	compound heterozygous	no	15
c.1162C>T	p.R388*	exon 5	ligand binding	compound heterozygous	no	15
c.1225G>A	p.A409T	exon 6	ligand binding	homozygous	yes	15
c.1238G>A	p.G413E	exon 6	ligand binding	homozygous	yes	15
c.1294delC	p.P432fs*476	exon 6	ligand binding	homozygous	yes	15
c.1314_1315delCT	p.P438fs*441	exon 6	ligand binding	compound heterozygous	no	15
c.1436+1G>T	p.K480*	intron 7	ligand binding	compound heterozygous	no	15
c.1498C>T	p.Q500*	exon 8	ligand binding	homozygous	yes	15
c.1887+2T>A	p.G630fs*?	intron 12	kinase homology	homozygous	yes	15
c.1972C>T	p.L658F	exon 13	kinase homology	homozygous	yes	41
c.2123A>G	p.Y708C	exon 14	kinase homology	homozygous	NA	15
c.2304_2307delTTGGinsCTGATGGA	p.W769*	exon 15	kinase homology	homozygous	yes	15
c.2326C>T	p.R776W	exon 15	kinase homology	homozygous	yes	15
c.2720C>T	p.T907M	exon 18	guanylyl cyclase	homozygous	yes	40
c.2869C>T	p.R957C	exon 19	guanylyl cyclase	homozygous	yes	15
c.2876G>C	p.G959A	exon 19	guanylyl cyclase	compound heterozygous	no	15
c.2986+2T>G	p.?	intron 20	guanylyl cyclase	homozygous	yes	40
c.3059delG	p.R1020fs*1025	exon 21	guanylyl cyclase	homozygous	yes	15

* History of consanguinity in the affected family. NA = Not available.

tations were identified in 3 individuals. The mutations included 4 nonsense, 5 frameshift, 3 splice site and 13 missense mutations in the 29 families with AMDM reported (table 1). The majority of missense mutations generated receptors that were retained in the endoplasmic reticulum, never reached the cell surface and consequently decreased the number of receptors available to extracellular ligands [42]. Other missense mutations lead to receptors with a compromised ligand binding [42] or with an impaired ligand-induced guanylyl cyclase activity [41].

Since 1983, parents of patients with AMDM have been noted to be shorter than expected for their population of origin [43]. One multiethnic study showed that the average height among 31 carrier adults of *NPR2* mutations (27 parents and 4 siblings) was 5.9 cm lower than that of population-matched controls ($p = 0.012$) [15]. In another study of one family of a patient with AMDM, relatives heterozygous for *NPR2* mutations had a height SDS 1.4 lower than family members homozygous for the wild-

type allele ($n = 16$ vs. 23; 95% confidence interval for a difference of means: -2.0 to -0.8) [38].

Recently, two studies implicated heterozygous mutations in *NPR2* on nonsyndromic common short stature. Our group identified missense heterozygous *NPR2* mutations (p.S76P, p.R263P and p.R819C; table 2) in 3 of 47 (6%) Brazilian children initially classified as idiopathic short stature [16]. A Japanese group also identified two missense heterozygous *NPR2* mutations (p.R110C and p.Q417E; table 2) in 2 of 101 patients with short stature [17]. These patients heterozygous for *NPR2* mutations had mild and variable degrees of short stature without a distinct phenotype. One patient and 2 relatives carrying *NPR2* mutations had disproportionate short stature, and another patient had short metacarpals observed radiologically. These five mutations segregated with short stature phenotype in an autosomal dominant pattern. A great variability in the degree of height deficit was observed among carriers of *NPR2* mutation, including relatives of patients with AMDM (height SDS ranged from -4.5 to -1.3). A similar

Table 2. Heterozygous loss-of-function *NPR2* mutations found in patients with nonsyndromic short stature

Mutation				Patient phenotype			Ref.
at cDNA level	at protein level	exons	domain	case	height SDS	other characteristics	
c.226T>C	p.S76P	1	ligand binding	index	-2.1	disproportionate short stature (SH/H SDS +2.3)	16
				father	-4.5	isolated short stature	
				sister	-3.8	disproportionate short stature (SH/H SDS +2.3)	
c.328C>T	p.R110C	1	ligand binding	index	-2.1	isolated short stature	17
c.788G>C	p.R263P	2	ligand binding	index	-2.8	isolated short stature	16
				father	-2.8	isolated short stature	
				grandfather	3.7	isolated short stature	
c.1249C>G	p.Q417E	6	ligand binding	index	-2.7	isolated short stature	17
c.2455C>T	p.R819C	16	between kinase homology and guanylyl cyclase domains	index	-2.8	short metacarpals	16
				mother	-1.7	abnormal body proportions (SH/H SDS +3.3)	

SH/H SDS = Sitting height to total height (SH/H) SDS for sex and age.

Table 3. Heterozygous gain-of-function *NPR2* mutations found in patients with tall stature

Mutation				Patient phenotype		Family members with heterozygous mutation		Ref.
at cDNA level	at protein level	exon	domain	height SDS	other characteristics	n	height SDS	
c.1462G>C	p.A488P	8	ligand binding	+5.2	marfanoid habitus, scoliosis, arachnodactyly, long hallux, ankle valgus deformity, unstable slipped capital femoral epiphysis	5	>+4	20
c.1963C>T	p.R655C	13	kinase homology	+5.2	long fingers, mild thoracic kyphosis, mild scoliosis and mild right hip dysplasia	NA	NA	19
c.2647G>A	p.V883M	17	guanylyl cyclase	+3.9	scoliosis, long hands and feet with arachnodactyly, long and wide great toes; family members with the same phenotype of the index case	2	+3.0 and +3.8	18

NA = Not available.

situation was observed in patients with mutations in *SHOX* gene (OMIM 312865) [44], a well-established cause of short stature. Interestingly, 4 patients (3 Brazilian and 1 Japanese) with heterozygous *NPR2* mutations were treated with recombinant human growth hormone. Their growth rate and height SDS have not substantially improved after recombinant human growth hormone treatment [16, 17].

Functional studies of these five mutations showed that cells co-transfected with mutants and wild-type NPR-B (1:1) had a significant decrease in cGMP levels after CNP stimulation in comparison with cells co-transfected with empty vector and wild-type, suggesting a dominant-negative effect [16, 17]. Confocal microscopy analysis demonstrated that p.S76P, p.R110C and p.R263P mutants were not expressed at the cell surface, whereas p.R819C and p.Q417E mutants showed clear cell surface expres-

sion [16, 17]. Further analysis showed that p.R110C receptor is not fully glycosylated and had defective trafficking from the endoplasmic reticulum to the Golgi apparatus. Co-immunoprecipitation revealed that a p.R110C receptor interacts with the wild-type receptor, explaining a dominant negative effect [17], a mechanism that had been explored in a previous study [45].

Mutations in Natriuretic Peptide System and Tall Stature

In 2007, a de novo balanced t(2;7)(q37.1;q21.3) translocation was reported in a 14-year-old girl with tall stature, marfanoid habitus and skeletal anomalies [21]. She had a height SDS of +3.3, scoliosis, arachnodactyly of

Fig. 2. CNP and FGF signaling pathways converge at the MAPK pathway. CNP binds to NPR-B and generates cGMP, which activates different signaling mediators, such as cyclic nucleotide phosphodiesterases (PDEs), cGMP-regulated ion channels (cGICs), and cGMP-dependent protein kinases (cGKI and cGKII). The

CNP/NPR-B effect on growth is mainly mediated by activation of cGKII. cGKII inhibits the activation of RAF-1, and consequently, the activation of MEK1/2 and ERK1/2. The effect of CNP on FGFR3 pathway leads to an increase in chondrocyte proliferation and differentiation and an increase in cartilage matrix synthesis.

hands, and feet with very long hallux. Two genes involved in bone remodeling, *COL1A2* on chromosome 7 (OMIM*120160) and *NPPC* on chromosome 2 (OMIM*600296) were mapped near the breakpoints. CNP-22 plasma concentration was increased in the patient and *NPPC* was overexpressed in her fibroblasts. The authors have proposed that CNP expression in the patient had been put under the control of the *COL1A2* enhancer, causing her overgrowth syndrome [21].

Over the past 2 years, three gain-of-function mutations of the *NPR2* gene were described (table 3) [18–20]. The families with the p.V883M and p.A488P mutations showed a similar phenotype, mainly characterized by scoliosis, arachnodactyly and long hallux [18, 20]. In one of these families, several affected members had coxa valga deformity with epiphyseal dysplasia of the femoral capital epiphyses and unstable slipped capital femoral epiphysis [20]. The patient with p.R655C had only mild skeletal abnormalities (table 3), suggesting that activating *NPR2* mutations should be suspected even in patients with isolated tall stature phenotype [19]. Two probands had a long birth length SDS (+3.1 and +6) [18, 19], whereas an-

other one was born with a normal length [20]. Tall stature was observed as an autosomal dominant trait and height SDS of carrier *NPR2* mutations individuals ranged from +3.0 to +5.2 (table 3). In both index patients from families with the p.V883M and p.A488P mutations, the bone mineral density Z-score of the lumbar spine was low when corrected for patients' height [18, 20], whereas the patients with p.R655C had normal bone mineral density [19]. The results of plasma concentrations of amino-terminal proCNP (NTproCNP) was variable among these patients, being slightly decreased in the first case [18], markedly decreased in the second one [19] and within the normal range in the third patient [20].

Functional studies indicated that these three are gain-of-function mutations with a constitutive activation of NPR-B. HEK293A cells transfected with each mutant cDNA had an increased cGMP production with or without CNP stimulation, in comparison with cells transfected with WT NPR-B [18–20]. Furthermore, co-immunoprecipitation showed that wild-type and mutant NPR-B-p.R655C can form stable heterodimers, suggesting a dominant positive effect [19].

Potential Therapeutic Use of CNP for Human Skeletal Dysplasias

Because of the importance of the CNP/NPR-B system as a growth stimulator, some animal models have been used to evaluate the therapeutic role of CNP in skeletal dysplasias, especially in achondroplasia (ACH, OMIM*100800). ACH is the most common human skeletal dysplasia and is characterized by disproportionate rhizomelic dwarfism. ACH is caused by a gain-of-function mutation in fibroblast growth factor receptor 3 gene (*FGFR3*, OMIM*134934), which leads to an impaired proliferation and terminal differentiation of chondrocytes and synthesis of the extracellular matrix in the growth plate [46]. *FGFR3* activation increases phosphorylation of the mitogen-activated protein kinase (MAPK) pathway. The MAPK signaling pathway is regulated by CNP. The binding of CNP to NPR-B generates cGMP that activates cGKII, which inhibits the activation of RAF-1, the activator of MEK1/2 [32] (fig. 2). The exact mechanism by which the cGKII inhibited RAF-1 is not well established, but probably involves the activation of p38 (or MAPK18) [47, 48]. Additionally, activation of MEK1/2 also inhibits cGMP generation by NPR-B after CNP stimulation [49, 50].

Overexpression of CNP in chondrocytes prevented the shortening of bones in a mouse model of ACH [13]. A recovery of extracellular matrix production through inhibition of *FGFR3*-mediated MAPK signaling pathway was observed [13]. In another study, ACH mice that overexpress CNP in the liver rescued the impaired bone growth phenotype [14]. This recovery was also observed after treatment with a continuous CNP infusion system [14].

Thus, these studies suggested that CNP could be a promising therapy for ACH [13, 14]. The challenge is to treat children with a drug with a short half-life (2 min after intravenous administration). Therefore, BioMarin Pharmaceutical Inc. has designed and manufactured a subcutaneous CNP analog, named BMN-111 [51]. BMN-111 has an extended half-life due to neutral endopeptidase resistance that allows once-daily subcutaneous administration. Daily subcutaneous injections of BMN-111 have previously been tested in mouse models of ACH, with improvement of dwarfism [51]. In humans, a phase 2 multicenter and multinational trial has been initiated, with estimated study completion in 2017 (ClinicalTrials.gov Identifier: NCT02055157).

Conclusion

The natriuretic peptide system has an important role in height determination in normal growth and growth disorders. In addition to AMDM caused by homozygous *NPR2* mutations, recent publications indicated that dominant negative and gain-of-function heterozygous *NPR2* mutations are responsible for short and tall stature phenotype, respectively. However, the small number of patients described to date does not allow to propose selection criteria to indicate *NPR2* molecular-genetic screening in patients with short or tall stature. Further studies are necessary to establish the frequency and to better characterize the phenotype of heterozygous *NPR2* mutation in individuals from the upper and lower distribution tails of height, as well as to investigate the influence of these mutations on the efficacy of growth hormone therapy. Additionally, ongoing and future clinical studies will determine the role of CNP analogs in growth promotion of patients with skeletal dysplasia and other causes of short stature.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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APÊNDICE C

Hisado-Oliva A, Garre-Vázquez AI, Santaolalla-Caballero F, Belinchón A, Barreda-Bonis AC, **Vasques GA**, Ramirez J, Luzuriaga C, Carlone G, González-Casado I, Benito-Sanz S, Jorge AA, Campos-Barros A, Heath KE. Heterozygous *NPR2* mutations cause disproportionate short stature, similar to Léri-Weill dyschondrosteosis. *J Clin Endocrinol Metab*. 2015 Aug;100(8):E1133-42. doi: 10.1210/jc.2015-1612.

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Advances in Genetics

Heterozygous *NPR2* Mutations Cause Disproportionate Short Stature, Similar to Léri-Weill Dyschondrosteosis

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Context: *SHOX* mutations have been detected in approximately 70% of Léri-Weill dyschondrosteosis (LWD) and approximately 2.5% of idiopathic short stature (ISS) cases, suggesting the implication of other genes or loci. The recent identification of *NPR2* mutations in ISS suggested that *NPR2* mutations may also be involved in disproportionate short stature.

Objective: The objective of the study was to investigate whether *NPR2* mutations can account for a proportion of the cases referred for LWD and ISS in whom no *SHOX* mutation was detected.

Patients and Methods: We undertook *NPR2* mutation screening in 173 individuals referred for suspected LWD and 95 for ISS, with no known defect in *SHOX* or its enhancers. Intracellular localization and natriuretic peptide precursor C-dependent guanylate cyclase activity were determined for the identified *NPR2* variants.

Results: Eight *NPR2* variants were identified in nine individuals, seven referred for suspected LWD and two for ISS. Six were demonstrated to affect NPR-B cell trafficking and/or its ability to synthesize cyclic GMP (cGMP) under response to natriuretic peptide precursor C/brain natriuretic peptide stimulation. All pathogenic mutations were detected in the suspected LWD referral group (~3%). Interestingly, one of these patients is currently being treated with recombinant human GH and in contrast to previous reports is showing a positive response to the treatment.

Conclusions: *NPR2* mutations account for approximately 3% of patients with disproportionate short stature and/or clinical or radiographic indicators of *SHOX* deficiency and in whom no *SHOX* defect has been identified. However, no patient has yet presented with Madelung deformity. Thus, *NPR2* should be screened in the *SHOX*-negative LWD referrals. (*J Clin Endocrinol Metab* 100: E1133-E1142, 2015)

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Abbreviations: AHI, arm span to height; AIDM, acromesomelic dysplasia, type Maroteaux; ANP, natriuretic peptide A; BNP, brain natriuretic peptide; CALR, calreticulin; cGMP, cyclic GMP; CNP, natriuretic peptide precursor C; DAPI, diamino-2-phenylindole; ER, endoplasmic reticulum; EV, empty vector; ExAC, Exome Aggregation Consortium; HA, hemagglutinin; HRM, high-resolution melting; ISS, idiopathic short stature; LWD, Léri-Weill dyschondrosteosis; MIM, Mendelian Inheritance in Man; NGS, next-generation sequencing; NPR2, natriuretic peptide receptor 2/guanylate cyclase B; NPR-B, natriuretic peptide receptor B; PAR1, pseudautosomal region 1; rhGH, recombinant human GH; SDS, SD score; SHH, sitting height to height ratio; *SHOX*, short stature homeobox containing gene; WT, wild type

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E1133

Léri-Weill dyschondrosteosis (LWD; Mendelian Inheritance in Man (MIM) 127300) is a skeletal dysplasia characterized by disproportionate short stature due to mesomelic limb shortening and the characteristic Madelung deformity, the bowing and shortening of the radius, the distal dislocation of the ulna, and pyramidal configuration of the carpal bones. Other minor abnormalities may include high-arched palate, cubitus valgus, short fourth metacarpal, micrognathia, and muscular hypertrophy (1). Most LWD cases are caused by heterozygous mutations in *SHOX* (short stature homeobox containing gene) or its regulatory regions, located on the pseudoautosomal region 1 (PAR1) (2–7). Heterozygous *SHOX* mutations are also observed in a small proportion (~2%–5%) of patients with idiopathic short stature (ISS; MIM 300582) (1, 8, 9). The penetrance of *SHOX* haploinsufficiency is high, but its clinical expression is very variable, with some LWD individuals having heights within the normal range and others lacking mesomelic shortening of the limbs or Madelung deformity (10). The clinical characteristics become more pronounced with age and are often more severe in females. Auxological analysis of the body proportions, the presence of minor abnormalities, and subtle radiographic signs can often be hard to detect; therefore, genetic analysis is required for confirmation.

Defects in both copies of *SHOX* are associated with the more severe skeletal dysplasia, Langer mesomelic dysplasia (LMD; MIM 249700), with disproportionate extreme short stature [–5.5 to –7.2 SD score (SDS)] and marked mesomelic and rhizomelic limb shortening (11–14).

As in the case of *SHOX*, homozygous mutations in *NPR2* (natriuretic peptide receptor B/guanylate cyclase B) cause a severe dysplasia: acromesomelic dysplasia, type Maroteaux (AMDM; MIM 602875), which is phenotypically similar to LMD in that patients have severe short stature (<–5 SDS in adults), shortening of the extremities, bowing of the forearm, and shortening and widening of metacarpals and phalanges (15, 16). Furthermore, the mouse model carrying a *Npr2* homozygous mutation also exhibited disproportionate dwarfism, short limbs, especially short bowed radius, short tail, and domed skull (17–19). Many *NPR2* mutations reported to date were shown to cause the retention of natriuretic peptide receptor B (NPR-B) in the endoplasmic reticulum (ER), promoting a reduced enzymatic activity due to impaired cellular trafficking (20). Interestingly, heterozygous *NPR2* mutation carrier parents were suggested to be shorter (21), with heights 5.7 cm shorter than population-matched controls (16). This was subsequently confirmed in a large multigenerational family with a mean –1.4 SDS height reduction in 16 carriers as compared with 23 noncarriers (22). However, no difference was observed for the sitting height

to height (SH/H) ratio between the *NPR2* mutation carriers and noncarriers (22). More recently, *NPR2* heterozygous missense mutations have been also identified in 2%–6% of ISS patients (23–25), suggesting that *NPR2* mutations may account for a significant proportion of the short stature population.

NPR2 codes for NPR-B, a transmembrane receptor related with endochondral bone growth (16, 22). NPR-B presents a modular structure as follows: 1) an extracellular ligand-binding domain that is able to bind to the natriuretic peptides natriuretic peptide precursor C (CNP), brain natriuretic peptide (BNP), and natriuretic peptide A (ANP); 2) a transmembrane region; 3) an intracellular kinase homology domain; and 4) a carboxyl-terminal guanylate cyclase domain, which, under response to natriuretic peptide stimulation, produces cyclic GMP (cGMP) from GTP (26). Natriuretic peptides participate in the regulation of endochondral bone ossification (CNP, BNP) and cardiovascular functions (BNP, ANP, CNP), binding to NPR-B with different efficiencies (CNP ≫ ANP ≧ BNP) (27, 28).

Although mutations in *SHOX* or its enhancers have been detected in approximately 70% of LWD cases (8, 29, 30), the molecular defect in the remaining approximately 30% is unknown, suggesting that other genes or loci could be implicated in this syndrome. In this study, we set out to determine whether heterozygous *NPR2* mutations could account for a proportion of patients referred for possible LWD and in whom no mutation was detected in *SHOX* or its enhancer regions. Similarly, we studied a cohort of ISS patients with no *SHOX* defect to investigate whether *NPR2* mutations are detected in LWD, ISS, or both pathologies. A total of 173 patients with suspected LWD and 95 with ISS were screened for mutations in *NPR2*. Functional studies were subsequently performed for the detected variants.

Materials and Methods

Cohort

A total of 173 patients with suspected LWD and 95 patients with ISS, with no detected alteration in *SHOX* or its regulatory regions, were included in the study. A suspected LWD diagnosis included two or more of the following features: 1) short stature less than –2 SDS (31); 2) Madelung deformity; 3) mesomelic shortening of the arms, determined visually; 4) presence of one or more secondary clinical characteristics of *SHOX* deficiency, as described previously (1). Arm span to height (A/H) and SH/H ratios were classified as abnormal if less than 0.965 and greater than 0.555, respectively, according to phenotyping scoring data determined for *SHOX* deficiency in 1608 unrelated individuals (1). ISS was defined as a height less than –2 SDS with normal GH or IGF levels and proportionate growth. A summary of the sex, age,

anthropometric, and clinical details of the two clinical cohorts is shown in Supplemental Table 1. All subjects had been extensively analyzed for mutations in *SHOX* or its enhancers using multiplex ligation-dependent probe amplification (MLPA) (P018E1/F1/G1; MRC Holland), self-designed MLPA assays, high-resolution melting (HRM), and sequencing (3, 4, 29, 32). All participants provided informed consent for the performed studies, and ethical approval was obtained from the respective institutions. Genomic DNA was isolated from peripheral blood (blood kit; QIAGEN; or Chemagig DNA extraction special; Chemagen, PerkinElmer Inc).

NPR2 mutation screening

The screening of the coding sequences and intron/exon boundaries of *NPR2* (NM_003995.3) was performed using HRM or next-generation sequencing (NGS). Primers were designed with the help of Oligo version 6 software (Molecular Biology Insights, Inc) and SNPcheck version 3 (<https://ngl.manchester.ac.uk/SNPcheckV3/snpcheck.htm>). PCR conditions are available in Supplemental Table 2. HRM was performed using a LightScanner 96 (BioFire Defense). All samples with an abnormal melting curve were subsequently sequenced using the BigDye Terminator version 3.1 kit (Life Technologies, Thermo Scientific Inc) and run on an ABI3730XL sequencer (Applied Biosystems). The NGS approach consisted of a custom-designed skeletal dysplasia panel (SkeletalSeq version 3) of 315 genes/regions using SeqCap EZ library enrichment (Roche Nimblegen) and run on a MiSeq platform (Illumina Inc). All variants were confirmed by direct sequencing, as was family testing.

In silico analysis

Conservation and pathogenicity prediction analysis of the identified *NPR2* variants was carried out using Alamut version 2.6-1 software (Interactive Biosoftware). Population frequencies of the detected variants were assessed using the Exome Aggregation Consortium (ExAC) data (<http://exac.broadinstitute.org/>).

Cell culture

COS-7 and human osteosarcoma (U2OS) cells were maintained in DMEM (Life Technologies), supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Life Technologies) at 37°C in 5% CO₂. Cells were transfected using FuGene 6 (Promega) according to the manufacturer's instructions.

Plasmid construction

The hemagglutinin (HA)-tagged NPR-B wild-type and p.Arg110Cys mutant expression vectors (33) were kindly donated by Dr N. Amano and Dr T. Hasegawa (Keio University School of Medicine, Tokyo, Japan). The HA-tagged expression vectors containing the detected NPR-B mutants were generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; GE Healthcare) following the manufacturer's recommendations. Mutagenesis primers can be found in Supplemental Table 3. All expression vectors were verified by sequencing.

Immunocytochemistry

Immunocytochemistry was undertaken as previously described (34). Basically, U2OS cells were transiently cotransfected

with pHA-NPR-B wild-type or mutants and the ER marker pRED-calreticulin (CALR; Origene Technologies Inc) expression vector. Anti-HA mouse monoclonal (Sigma-Aldrich) and Alexa fluor 488 goat antimouse (Life Technologies), both diluted at 1:2000, were used as primary and secondary antibodies, respectively. Cells were counterstained with diamino-2-phenylindole (DAPI; Sigma-Aldrich) and mounted with DePeX mounting medium (BDH, Merck Chemicals Ltd). NPR-B, CALR, and DAPI intracellular localizations were visualized using a Leica DM5500 B fluorescent microscope (Leica Microsystems GmbH). Two biological replicates were undertaken.

Enzyme-linked immunosorbent assay

ELISAs were carried out as previously described with a few modifications (23). COS-7 cells were seeded on 12-well plates. After 24 hours, cells were transiently transfected with pHA-NPR-B wild-type (WT), NPR-B mutants, or empty vector (EV). After 48 hours after transfection, cells were incubated with 100 nM of CNP-22 (Bachem) in DMEM for 20 minutes. Subsequently 0.1 M HCl was added to stop endogenous phosphodiesterase activity, stabilize the released cGMP, and lyse the cells. Cell lysates were centrifuged at 600 × g for 10 minutes, and the supernatants were isolated. The cGMP levels in the supernatant were measured using the cGMP Complete ELISA kit (ENZO Life Sciences Inc). Each mutant was analyzed in triplicate, and at least two biological replicates were performed. Student's *t* tests, using SPSS version 15.0 for Windows, were carried out to compare the differences between WT and mutants. A value of *P* < .05 was considered as statistically significant.

Results

NPR2 mutation screening

We analyzed the coding and intron-exon sequences of *NPR2* in 173 patients with suspected LWD and 95 with ISS with no known *SHOX*/PAR1 defect. Eight variants, seven missense and one in-frame codon deletion, were detected in nine individuals, seven LWD referrals, and two ISS (Table 1). Six of the detected variants have not been previously described in AMDM or ISS patients, whereas the p.Arg819Cys mutation has been previously reported as a pathogenic variant (23). The p.Asp256Tyr, p.Val548del, p.Glu991Gly, and p.Arg1020Trp variants have not been observed in the large population exome studies (ExAC), whereas the other four have been observed at extremely low allelic frequencies (Table 1). The conservation of the affected amino acids and the pathogenicity predictions are shown in Table 1. Cosegregation studies were undertaken where possible (*n* = 5, Supplemental Figure 1). The p.Asp256Tyr (proband 2), p.Val448del (proband 6), p.Arg819Cys (proband 7), and p.Glu991Gly (proband 8) variants cosegregated with the phenotype. The cosegregation analysis of the p.Asn546Tyr (proband 4) variant was inconclusive, whereas the p.Ala164Gly variant (proband 1) did not cosegregate with the short stature phenotype. The phenotypic characteristics of the

Table 1. Eight *NPR2* Variants Observed in Nine Individuals Referred for Suspected LWD (n = 173) and ISS (n = 95), All With No *SHOX* Defect

Proband	cDNA	Exon	Amino Acid	Domain	Country	Cosegregation ^a	SIFT	Mutation Tester	Polyphen
1 (ISS)	c.491C>G	1	p.Ala164Gly	Ligand binding	Brazil	No	0.05 Tol.	0.925 Polymorphism	0.065 Benign
2 (LWD)	c.766G>T	2	p.Asp256Tyr	Ligand binding	Spain	Yes	0 Del.	1 Disease causing	0.962 Damaging
3 (LWD)	c.1262C>T	6	p.Thr421Met	Ligand binding	Spain	ND	0 Del.	0.883 Disease causing	0.090 Benign
4 (LWD)	c.1636A>T	10	p.Asn546Tyr	Kinase homology	Brazil	NI	0 Del.	0.999 Disease causing	0.885 Damaging
5 (ISS)	c.1636A>T	10	p.Asn546Tyr	Kinase homology	Spain	ND	0 Del.	0.999 Disease causing	0.885 Damaging
6 (LWD)	c.1641_1643del	10	p.Val548del	Kinase homology	Spain	Yes	NA NA	NA NA	NA NA
7 (LWD)	c.2455C>T ^b	16	p.Arg819Cys	Inter-domains	Spain	Yes	0 Del.	1 Disease causing	1.000 Damaging
8 (LWD)	c.2972A>G	20	p.Glu991Gly	Guanylate cyclase	Spain	Yes	0 Del.	1 Disease causing	1.000 Damaging
9 (LWD)	c.3058C>T	21	p.Arg1020Trp	Guanylate cyclase	Spain	ND	0 Del.	1 Disease causing	1.000 Damaging

Abbreviations: NA, not applicable; ND, not determined; NI, noninformative; SIFT, Sorting Intolerant From Tolerant; Del, deleterious; Tol, tolerated. The protein domain, cosegregation analysis, and pathogenicity predictions are indicated for each variant. All variants were localized at highly conserved amino acids. Frequencies of the variant in the large sequencing project data [ExAC: 60 706 European (non-Finnish and Finnish), African, Asian, and Latino individuals]. Variants c.766G>T, c.1636A>T, c.2972A>G, and c.3058C>T have not been observed. Variant c.491C>G (rs62637657) has been observed in 3 of 66 522 European (non-Finnish) and 1 of 10 378 African American chromosomes (total 4 of 121 138). Variant c.1262C>T (rs140361919) has been observed in 5 of 66 712 European non-Finnish, 4 of 10 402 African, and 1 of 16 510 Asian chromosomes (total 10 of 121 322). Variant c.2455C>T has been observed in 2 of 66 740 European non-Finnish and 1 of 11 574 Latino chromosomes (total of all studied populations 3 of 121 406).

^a Cosegregation details are shown in Supplemental Figure 1.

^b This mutation has been previously described in one Brazilian family and its pathogenicity has been previously demonstrated (23).

probands with *NPR2* variants and their relatives are shown in Table 2.

Functional characterization of NPR-B variants

To functionally evaluate the pathogenicity of the identified variants, we first determined whether they affected NPR-B cell trafficking. For this purpose, we transiently transfected the WT and variant cDNAs and subcloned them into an HA-expression vector in human osteosarcoma cells (U2OS). Their intracellular localization was determined by immunocytochemistry using antibodies against NPR-B and the ER marker, CALR. The NPR-B variants p.Ala164Gly, p.Asn546Tyr, p.Val548del, and p.Glu991Gly were located at the cell surface, whereas three mutants, p.Asp256Tyr, p.Thr421Met, and p.Arg1020Trp, colocalized with the ER (Figure 1).

Second, we analyzed the receptor activity of all identified variants by assessing the CNP-dependent guanylate cyclase activity. Four of the analyzed mutants, p.Asp256Tyr, p.Val548del, p.Glu991Gly, and p.Arg1020Trp, showed a total absence of cGMP synthesis, similar to that of unstimulated wild type and the control mutant p.Arg110Cys (Figure 2A), whereas p.Thr421Met showed a 61% activity decrease. The p.Ala164Gly and p.Asn546Tyr mutants showed only moder-

ate reductions in cGMP synthesis, 19% and 31%, respectively (Figure 2A). Because we had detected the variants in a heterozygous state, we also analyzed whether they had a dominant-negative effect by cotransfecting each mutant with NPR-B WT at a ratio of 1:1. We observed a highly significant reduction of CNP-stimulated cGMP synthesis as compared with cells cotransfected with WT and EV with six of the NPR-B mutants tested, indicating a dominant-negative effect. However, the p.Ala164Gly and p.Asn546Tyr mutants did not show a significant reduction when cotransfected with the WT (Figure 2B), suggesting that they did not have a dominant-negative effect in the heterozygous state. Thus, functional analysis confirmed that six of the *NPR2* mutations, including the previously reported mutation, were pathogenic mutations (Table 3).

Discussion

A total of eight *NPR2* variants were identified in nine individuals, seven of the 173 patients referred for suspected LWD and two from the cohort of 95 ISS, all of whom had been previously excluded for *SHOX*/PAR1 defects. First, the pathogenicity of the identified *NPR2* variants was assessed using in silico prediction tools. Seven of

Table 2. Anthropometric and Phenotypic Characteristics of the Probands and Their Family Members

NPR-B Mutation/ Variant	Family Member	Referral LWD/ISS	Gender (M/F)	Age, y	Mutation/ Variant (Yes/No)	Anthropometric Measurements (SDS)	Dis. or Prop.	Other Clinical Characteristics
p.Ala164Gly	Proband 1	ISS	M	1.3	Y	H: 72 (−2.5) A/H: ND SH/H: ND	Prop	ND
	Mother		F	Adult	N	H: 144 (−3.0) A/H: ND SH/H: ND	Prop	ND
p.Asp256Tyr	Proband 2	LWD	M	6.6	Y	H: 108 (−2.5) A/H: 1.00 SH/H: 0.52	Prop	High-arched palate, cubitus valgus, muscular hypertrophy cone-shaped epiphysis
	Brother		M	Adult	Y	H: 155 (−3.5) A/H: 1.04 SH/H: ND	ND	ND
	Father		M	Adult	Y	H: 157 (−2.9) A/H: 1.04 SH/H: 0.51	ND	Cone-shaped epiphysis
p.Thr421Met	Proband 3	LWD	M	18.5	Y	H: 156 (−3.2) A/H: ND SH/H: ND	Dis	Short forearms, peculiar phenotype with long narrow face
p.Asn546Tyr	Proband 4	LWD	M	12.5	Y	H: 128 (−2.8) A/H: 0.99 SH/H: 0.56	Prop	Delayed bone age (1 y)
	Mother		F	Adult	N	H: 148 (−2.4) A/H: ND SH/H: 0.57	Dis	ND
p.Asn546Tyr	Proband 5	ISS	M	10.3	Y	H: 124 (−3.1) A/H: 0.94 SH/H: ND	Dis	Cubitus valgus and muscular hypertrophy
	Father		M	Adult	ND	H: (3.1) A/H: ND SH/H: ND	ND	ND
p.Val548del (II.1)	Proband 6 (II.1)	LWD	M	13.8	Y	H: 140 (−2.9) A/H: 0.973 SH/H: 0.51	Dis	Bowing of radius, mesomelic shortening of limbs, short fourth metacarpal and delayed bone age (2 y)
	Father (I.1)		M	Adult	N	H: 168 (−1.5) A/H: ND SH/H: ND	ND	ND
	Mother (II.2)		F	Adult	Y	H: 145.6 (−2.7) A/H: ND SH/H: ND	ND	ND
p.Arg819Cys	Proband 7	LWD	M	10.2	Y	H: 126 (−2.3) A/H: 0.95 SH/H: 0.54	Dis	Muscular hypertrophy
	Mother		F	Adult	Y	H: 144 (−3.0) A/H: 0.94 SH/H: 0.56	Dis	ND
p.Glu991Gly (II.1)	Proband 8 (II.1)	LWD	F	6.4 ^a	Y	H: 107 (−2.3) ^a A/H: ND* SH/H: ND*	Prop	High-arched palate, short fifth phalange, muscular hypertrophy, dental malpositioning, cone- shaped epiphysis
	Brother (II.2)		M	7.8	Y	H: 120 (−2.3) A/H: 0.98 SH/H: 0.51	Prop	High-arched palate, short fifth phalange, muscular hypertrophy, and severely delayed bone age (2 y)

(Continued)

Table 2. (Continued)

NPR-B Mutation/Variant	Family Member	Referral LWD/ISS	Gender (M/F)	Age, y	Mutation/Variant (Yes/No)	Anthropometric Measurements (SDS)	Dis. or Prop.	Other Clinical Characteristics
p.Arg1020Trp	Sister (II.3)		F	6.8	Y	H: 123.8 (−0.7) A/H: 0.973 SH/H: 0.51	Prop	Short fourth to fifth metacarpals
	Father (I.1)		M	Adult	N	H: 169.8 (−1.2) A/H: ND SH/H: ND	Prop	ND
	Mother (I.2)		F	Adult	Y	H: 155.5 (−1.4) A/H: ND SH/H: ND	Prop	High arched palate, bowing of radius, ankylosing spondylitis
	Proband 9	LWD	F	9.0	Y	H: 124.5 (−1.5) A/H: 0.956 SH/H: ND	Dis	Mesomelic limb shortening; lower leg bowing; limited extension of the elbows; brachydactyly, especially of fifth digit; flattened forehead; hypertelorism and strabismus; high-arched palate; dental malpositioning
	Mother		F	Adult	ND	H: 155 (−1.1) A/H: 0.965 SH/H: ND	Dis	Deafness

Abbreviations: Dis, disproportional growth; F, female; H, height; M, male; ND, not documented; Prop, proportional growth; SH, sitting height. Functionally confirmed pathogenic mutations are indicated in bold.

* Data at 6.4 years, prior to GH treatment. Further data are available in Supplemental Data

the eight variants were predicted to be pathogenic, whereas only the p.Ala164Gly mutation was classified as nonpathogenic. Only one of the identified variants, p.Arg819Cys, had been previously reported in an ISS patient and functionally demonstrated to be pathogenic (23). Therefore, functional studies were undertaken for the characterization of the additional seven *NPR2* variants identified.

Six of the *NPR2* variants were confirmed to be pathogenic mutations, and three were shown to cause the retention of NPR-B in the ER, resulting in a reduced enzymatic guanylate cyclase activity due to impaired cellular trafficking. However, the p.Thr421Met mutant behaved differently from the other two ER-retained mutants, showing 39% of WT cGMP activity, suggesting that some residual trafficking may still exist, despite not being observed in the immunochemical assay. Three *NPR2* mutations did not affect the ability to translocate to the plasma membrane but showed reduced cGMP synthesis. All six mutations were shown to have a dominant-negative effect over the WT form. Only three NPR-B mutants forms showed impaired cellular trafficking, a frequency similar to that previously observed in short-stature individuals (three of five), although very different from that reported in AMDM patients, in whom most *NPR2* missense mutants (11 of 12) were retained in the ER and showed reduced enzymatic activity (20, 23, 24). Although the cel-

lular localization has been studied only in a total of 23 NPR-B mutants so far (20, 23, 24), there is no apparent correlation between the domain location of the mutations and their ability to translocate to the plasma membrane, whereas all mutants caused a reduced capacity to synthesize cGMP.

The remaining two NPR-B variants, p.Ala164Gly and p.Asn546Tyr, identified in two ISS patients, did not show any dominant-negative effect when cotransfected with the WT construct. Both were transported to the cell membrane and showed a milder reduction of cGMP synthesis in the homozygous state. This emphasizes the utility of the cotransfection assays for the functional assessment of the dominant-negative effect of novel NPR-B variants on cGMP synthesis capacity, emulating the heterozygous state. Moreover, cosegregation analysis did not confirm cosegregation of the p.Ala164Gly variant with the short-stature phenotype within the examined family or in a second family, in which this variant was recently reported as a variant of unknown significance (25). Hence, in contrast to previous studies (23, 24), we did not find any *NPR2* pathogenic defects in patients classified as ISS, probably because we used a more stringent definition of ISS, according to Cohen et al (2006) (35) to determine which patient should be tested for *NPR2* mutations.

The previously reported p.Arg819Cys mutation (23) was also identified in proband 7. At age 10 years, he had

Figure 1. Intracellular localization of the NPR-B mutants. U2OS cells were transfected with NPR-B WT (A), p.Arg110C (R110C) mutant (B) as an ER retention control (24), or each mutant identified in our cohort (C–I). Intracellular localization of NPR-B (green), ER marker CALR (red), and nuclei with DAPI (blue) are shown. The three fluorescent images are merged in the fourth column. Images are at $\times 20$ magnification. The NPR-B mutants, p.Ala164Gly (A164G), p.Asn546Tyr (N546Y), p.Val548del (V548del), and p.Glu991Gly (E991G), all showed cell surface localization, whereas the p.Asp256Tyr (N256Y), p.Thr421Met (T421M), and p.Arg1020Trp (R1020W) mutants were retained in the ER. Identical results were obtained in two independent experiments.

Figure 2. CNP-dependent guanylate cyclase activity of WT NPR-B and mutants. A, Guanylate cyclase activity of COS-7 cells transfected with NPR-B WT or mutants (A164G, D256Y, T421M, N546Y, V548del, E991G, and R1020W) and the positive control (R110C), stimulated (+) or unstimulated (–) with CNP. CNP-dependent receptor activity is expressed as a percentage relative to CNP-stimulated NPR2-B WT. All mutants showed a reduction in CNP-dependent cGMP synthesis, to different degrees. B, cGMP activity of COS-7 cells transfected with a 1:1 ratio of NPR-B WT and EV or mutants and treated with CNP. CNP-stimulated cGMP values are expressed as a percentage relative to CNP-stimulated WT/EV. Five mutants showed a dominant-negative effect. Bars are SD of the mean. Each assay was undertaken in triplicate and two biological replicates were performed. Significant *P* values are indicated with asterisks (*, *P* < .05; **, *P* < .01).

a height SDS of -2.3 , an A/H ratio of 0.95 , an SH/H ratio of 0.54 , and muscle hypertrophy, ie, disproportionate short stature and shortening of the upper limbs. His carrier mother also has disproportional short stature (-3.0 SDS), and A/H and SH/H ratios of 0.94 and 0.56 , respectively, indicative of shortening of the upper and lower limbs. The previously reported Brazilian patient, aged 17 years, had proportional short stature (height -2.4 SDS, SH/H 1.3 SDS) and shortened metacarpals, whereas his mother had normal height (-1.7 SDS) but had an altered SH/H ratio

Table 3. Functional Characterization of the NPR-B Mutants

NPR-B Mutant	Retained in the ER	cGMP Synthesis (% of WT)	Dominant-Negative Effect on cGMP Synthesis	Pathogenic Summary
A164G	—	81	—	Nonpathogenic
D256Y	+	1.15	+	Pathogenic
T421M	+	39	+	Pathogenic
N546Y	—	69	—	Nonpathogenic
V548del	—	0.20	+	Pathogenic
R819C ^a	—	~2	+	Pathogenic
E991G	—	0.03	+	Pathogenic
R1020W	+	0.05	+	Pathogenic

Cellular localization studies were undertaken using immunocytochemistry in U2OS cells, and cGMP synthesis capacity was assessed by ELISA.

^a Functional studies were previously reported (23).

of 0.57, suggesting shortening of the lower limbs. Thus, the phenotypic expression of the p.Arg819Cys mutation is highly variable in the two examined families.

Interestingly, probands 2 and 8 presented with cone-shaped epiphyses, probands 8 and 9 presented with short phalanges, and proband 8 also presented with a broad forehead, all of which are clinical and radiological features described in patients with homozygous *NPR2* mutations (AMDM). Dental malpositioning was also described in probands 8 and 9, a feature previously not reported in patients with *NPR2* or *SHOX* mutations, ie, expanding the phenotypic spectrum of clinical features associated with *NPR2* mutations, although the implication of other factors is not excluded.

Hence, in this study, disproportionate short stature and/or phenotypic or radiographic indicators of *SHOX* deficiency were observed in all individuals with pathogenic *NPR2* mutations. This observation is not surprising because AMDM patients with *NPR2* homozygous mutations have severe disproportional short stature. Nevertheless, due to the high clinical heterogeneity observed in individuals with *SHOX* haploinsufficiency, it is quite understandable why these patients were referred as suspected LWD cases. Moreover, previous reports have shown that *SHOX* activates the *NPPB* promoter, causing an increase in BNP (36), which may either directly influence NPR-B signaling or indirectly increase local CNP levels by saturating the NPR-C receptor. *SHOX* also activates aggrecan (*ACAN*) and *FGFR3* expression in human U2OS cells (37, 38) and *Col2A1* and *ACAN* in chicken micromasses (39) while down-regulating *FGFR3* in chicken micromasses (38). Likewise, CNP enhances the synthesis of collagens (*Col2A1*) and proteoglycans (aggrecan) and influences cell proliferation, indicating a switch from proliferative to differentiated and hypertrophic chondrocytes, mediated by NPR-B (40). Therefore, *SHOX* and NPR-B and their downstream targets appear to be implicated in common growth pathways, which

could explain why similar phenotypes are observed in individuals with *SHOX* and *NPR2* mutations.

Interestingly, none of the patients with *NPR2* mutations presented to date with the Madelung deformity, the most characteristic abnormality of LWD. This absence is a phenotypic feature that may be helpful for the differential diagnosis of disproportionate short stature caused by autosomic dominant *NPR2* mutations. Nevertheless, we have to keep in mind that Madelung deformity may not be observed before 6 years of age (41), although the condition can become apparent earlier in some cases. Therefore, we should wait for the prepubertal patients with *NPR2* mutations to complete their puberty to definitely confirm the absence of the Madelung deformity. For now, our opinion is that these patients have phenotypic characteristics of the heterozygous form of AMDM, which is similar to that observed in many LWD individuals but it cannot be classified as LWD because no individual has Madelung deformity.

Recombinant human growth hormone (rhGH) is indicated for patients with *SHOX* haploinsufficiency. Improvements in height (+1.3 SDS) was observed in most individuals and 57% of them achieved a final height greater than -2 SDS (42). In contrast, few patients with *NPR2* mutations have been treated with rhGH, showing no or mild improvements on height (23–25). An initial good response observed in proband 8 (Supplemental Figure 2) suggests that rhGH treatment may be beneficial for patients with heterozygous *NPR2* mutations if the treatment is started at an early age. Nevertheless, larger prospective studies will be necessary to confirm this.

In summary, in our study 6 of 173 suspected LWD referrals (~3%) were found to have a heterozygous mutation in *NPR2*, whereas no mutation was found in the ISS cohort (n = 95). Functional analysis of the identified pathogenic mutations demonstrated a dominant-negative effect on the CNP-dependent guanylate cyclase activity, resulting in a decreased cGMP synthesis, as the likely mo-

lecular mechanism implicated in the etiology of disproportionate short stature. Hence, *NPR2* mutation screening is recommended in LWD-suspected individuals without Madelung deformity in whom no alteration was detected in *SHOX* or its enhancers.

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