

***IHH* Gene Mutations Causing Short Stature With Nonspecific Skeletal Abnormalities and Response to Growth Hormone Therapy**

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Context: Genetic evaluation has been recognized as an important tool to elucidate the causes of growth disorders.

Objective: To investigate the cause of short stature and to determine the phenotype of patients with *IHH* mutations, including the response to recombinant human growth hormone (rhGH) therapy.

Patients and Methods: We studied 17 families with autosomal-dominant short stature by using whole exome sequencing and screened *IHH* defects in 290 patients with growth disorders. Molecular analyses were performed to evaluate the potential impact of *N*-terminal *IHH* variants.

Results: We identified 10 pathogenic or possibly pathogenic variants in *IHH*, an important regulator of endochondral ossification. Molecular analyses revealed a smaller potential energy of mutated *IHH* molecules. The allele frequency of rare, predicted to be deleterious *IHH* variants found in short-stature samples (1.6%) was higher than that observed in two control cohorts (0.017% and 0.08%; $P < 0.001$). Identified *IHH* variants segregate with short stature in a dominant inheritance pattern. Affected individuals typically manifest mild disproportional short stature with a frequent finding of shortening of the middle phalanx of the fifth finger. None of them have classic features of brachydactyly type A1, which was previously associated with *IHH* mutations. Five patients heterozygous for *IHH* variants had a good response to rhGH therapy. The mean change in height standard deviation score in 1 year was 0.6.

Conclusion: Our study demonstrated the association of pathogenic variants in *IHH* with short stature with nonspecific skeletal abnormalities and established a frequent cause of growth disorder, with a preliminary good response to rhGH. (*J Clin Endocrinol Metab* 103: 604–614, 2018)

Most apparently healthy children with short stature are classified as having idiopathic short stature (ISS). The conventional evaluation based on clinical findings complemented by laboratory and image examinations is unable to identify the cause of growth impairment observed in these children. Variation in human height is mostly explained by heritable factors; thus, genetic evaluation could elucidate the causes for growth disorders (1). Common polymorphisms are associated with height in healthy individuals, with a small effect size per locus (2). Alternatively, there is evidence that rare genetic variants are involved with larger effects on stature (3–5). The candidate gene approach has identified defects in a few genes associated with growth impairment observed in children classified as ISS (6). Recently, several whole-exome sequencing (WES) studies have reported gene defects responsible for distinct forms of short stature, including genes expressed in growth plate (7). Heterozygous mutations in *ACAN* have been identified in families with ISS with accelerated bone maturation (8, 9). An *FBN1* mutation was identified in two patients with severe short stature and mild form of acromicric dysplasia (10). Finally, heterozygous mutations in *NPPC* were recently associated with autosomal-dominant short stature and small hands phenotype (11). Hence, WES is a potent tool to screen for candidate genes, identify associations between genes and the phenotype, and broaden the phenotypic spectrum of growth disorders associated with a known gene.

In the current study, we first investigated families with autosomal-dominant short stature through WES. This approach allowed us to identify three families with pathogenic variants in the Indian hedgehog gene [*IHH*, Mendelian Inheritance in Man (MIM) 600726]. This gene codifies an important paracrine regulator of endochondral ossification. Heterozygous mutations in *IHH* are recognized as the cause of brachydactyly type A1 (BDA1; MIM 112500) (12). Because our patients with heterozygous *IHH* variants did not present classic features of this skeletal dysplasia, we have expanded the investigation of *IHH* defects in a large cohort of patients with ISS and patients with mild skeletal dysplasia. Our results have extended the known phenotype of *IHH* defects and revealed that heterozygous *IHH* mutations are a fairly frequent cause of short stature with nonspecific skeletal abnormalities (MIM 616255). Additionally, we have observed a good short response to recombinant human growth hormone (rhGH) in affected patients under therapy.

Patients and Methods

Patients

The respective local ethics committees approved the studies, and the patients or guardians gave their written informed consent. WES was performed on 17 independent Brazilian families with autosomal-dominant ISS as part of an exome sequencing project of patients with growth disorders. All probands fulfilled the following diagnostic criteria: normal birth weight for gestational age, height standard deviation score (SDS) ≤ -2 , unremarkable medical history, and absence of abnormal findings on clinical examination or in laboratory tests that could account for short stature. A second Brazilian cohort of children with short stature of unknown cause [111 with ISS and 19 born small for gestational age (SGA) but with family history of short stature] had *IHH* screened by Sanger sequencing (n = 81) or targeted gene sequencing panel (n = 49). Clinical characteristics of the Brazilian cohort are shown in Supplemental Table 1. Additionally, a Spanish cohort of short-stature patients referred for unspecific brachydactyly (n = 60) or mild skeletal dysplasia (n = 100) was evaluated with a skeletal dysplasia–targeted gene sequencing panel that included *IHH*. In all enrolled patients and their relatives, genomic DNA was obtained from peripheral blood leukocytes by using standard techniques. A cohort of 609 Brazilian elderly adults (<http://abraom.ib.usp.br/>), representative of the healthy population, was analyzed with WES and was used to compare the frequency of *IHH* variants in the Brazilian healthy population.

Exome sequencing

We performed WES in the probands and their first-degree relatives according to previously published protocols (10, 13). Briefly, the libraries were constructed with the SureSelect Target Enrichment System (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. The sequences were generated in the HiSeq 2500 platform (Illumina, San Diego, CA) running on paired-end mode. Reads were aligned to the GRCh37/hg19 assembly of the human genome with the Burrows-Wheeler Alignment (BWA-mem) aligner. Variant calling included single-nucleotide variants and small insertions and deletions and was performed with FreeBayes. The resulting data (in variant call format) were annotated with ANNOVAR. The median coverage of the target bases was 114 \times , with 97% of the target bases having $\geq 10\times$ coverage.

Targeted sequencing

Patients were analyzed by a customized panel of targeted sequencing, which included *IHH* [for Brazilian patients: Agilent SureSelect assay (Agilent Technologies); for Spanish patients: NimbleGen SeqCap EZ capture (Roche, Pleasanton, CA)]. Sequencing was performed on an Illumina MiSeq or NextSeq platform in paired-end mode. Inhouse bioinformatic analysis was performed. The sequences were aligned to the reference human assembly (GRCh37/hg19). The median coverage of the target bases was 409 \times and 215 \times , with 95% and 94.1% of the target bases being covered $\geq 20\times$ for Brazilian and Spanish patients, respectively.

Data analysis

The exome and the targeted panel sequencing data were screened for rare variants (minor allele frequency < 0.1% in public and in-house databases) located in exonic regions and consensus splice site sequences. Subsequently, our variant filtration prioritized genes on the basis of their potential to be pathogenic: loss-of-function variants and variants predicted to be pathogenic by multiple *in silico* programs. For variants identified by WES, we selected variants that fitted an autosomal-dominant model in the selected families (Supplemental Figs. 1–3). The sequencing reads carrying candidate variants were inspected visually using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA).

Sanger sequencing

All putative pathogenic variants observed by exome and targeted panel sequencing as well as segregation analysis in the families were validated and genotyped by Sanger sequencing. The primers for *IHH* candidate gene screening were designed to amplify all exons and exon-intron boundaries of the NM_002181.3 transcript (primer sequences and amplification protocols are available upon request). Polymerase chain reaction products were bidirectionally sequenced and analyzed in an ABI 3100/3700XL sequencer (Applied Biosystems, Foster City, CA).

Pathogenicity analysis of identified variants

The pathogenicity of the variants identified during genetic analysis was classified according to the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines (13). To support the interpretation of familial cosegregation, a mathematical quantitative criterion was added to the ACMG/AMP guidelines to allow a statistical quantification of familial aggregation data (14). Population data criteria were evaluated by using two public genomic databases: Genome Aggregation Database (gnomAD: <http://gnomad.broadinstitute.org/>) and Brazilian genomic variants (ABraOM: <http://abraom.ib.usp.br/>). Several

different computational tools were used to obtain predictive data of each variant (Supplemental Table 2). Because the crystal structure of the N-terminal domain of human *IHH* had been resolved, the potential impact of the mutations located in this region (Table 1) was further evaluated by molecular simulations carried out with the human *IHH* molecular model and its mutated versions (Supplemental Methods).

To provide additional genetic evidence of the association of *IHH* with short-stature phenotype with nonspecific skeletal findings (15), we performed aggregate variant analyses comparing allele frequencies between our cohorts of patients and public databases (gnomAD and ABraOM). In these databases, we selected variants with similar characteristics of the *IHH* variants observed in our cohort of short stature patients: rare variants (minor allele frequency < 0.01) with loss-of-function effect (stop gain, frame-shift, or consensus splicing site variants) or rare nonsynonymous variants predicted to be pathogenic by at least four *in silico* tools (PROVEAN, SIFT, PolyPhen-2, and Mutation Assessor). Allele frequency differences between groups were analyzed by χ^2 test, and statistical significance was set at $P < 0.05$. Statistical analyses were performed by using the SIGMAstat statistical software package (Windows version 4.0; SPSS Inc., San Rafael, CA).

Results

Genetic analysis

Three different heterozygous *IHH* variants were identified in 3 of 17 families with ISS individuals analyzed through WES (Fig. 1A–1C; Supplemental Figs. 1–3). Because the affected individuals did not present specific signs of a skeletal dysplasia (including BDA1), we screened 130 children with short stature of unknown cause for rare variants in *IHH*. In this group, another two heterozygous *IHH* variants were identified (Fig. 1D and 1E). Among a cohort of Spanish patients with short stature and

Table 1. *In Silico* Analysis and ACMG/AMP Classification of *IHH* Identified Variants

<i>IHH</i> cDNA Variant ^a (Protein)	Domain	SIFT	Polyphen	Mutation Assessor	PROVEAN	CADD V1.3	GERP ++	ACMG-AMP Classification
Identified in Brazilian patients								
c.172G>A (p.Glu58Lys)	N-terminal signaling domain	0/D	1.0/D	3.085/M	−3.03/D	33.0	4.22	Pathogenic
c.446G>A (p.Arg149His)	N-terminal signaling domain	0.01/D	1.0/D	3.085/M	−4.63/D	35.0	5.78	Likely pathogenic
c.532G>A (p.Val178Met)	N-terminal signaling domain	0.02/D	0.99/D	3.085/M	−2.77/D	32.0	5.68	Likely pathogenic
c.532G>C (p.Val178Leu)	N-terminal signaling domain	0/D	0.699/P	3.085/M	−2.77/D	24.7	5.68	Pathogenic
c.1139G>T (p.Gly380Val)	C-terminal domain	0.04/D	1.0/D	3.235/M	−5.87/D	29.0	4.64	Likely pathogenic
Identified in Spanish patients								
c.319delT (p.Cys107Alafs*5)	N-terminal signaling domain	NA	NA	NA	NA	NA	NA	Pathogenic
c.531G>C (p.Trp177Cys)	N-terminal signaling domain	0/D	1.0/D	3.075/M	−11.96/D	25.9	5.68	Likely pathogenic
c.797dupC (p.Arg267Thrfs*15)	C-terminal domain	NA	NA	NA	NA	NA	NA	Pathogenic
c.949G>A (p.Val317Met)	C-terminal domain	0.01/D	0.998/D	3.145/M	−2.12/N	26.3	5.16	Likely pathogenic
c.1202T>C (p.Phe401Ser)	C-terminal domain	0.009/D	0.99/D	2.470/M	−2.78/D	28.9	4.64	Likely pathogenic

ACMG/AMP classification (13, 14).

Abbreviations: cDNA, complementary DNA; NA, not available.

^aGene transcript ID: NM_002181.3, Gene:ENSG00000163501, transcript: ENST00000295731.

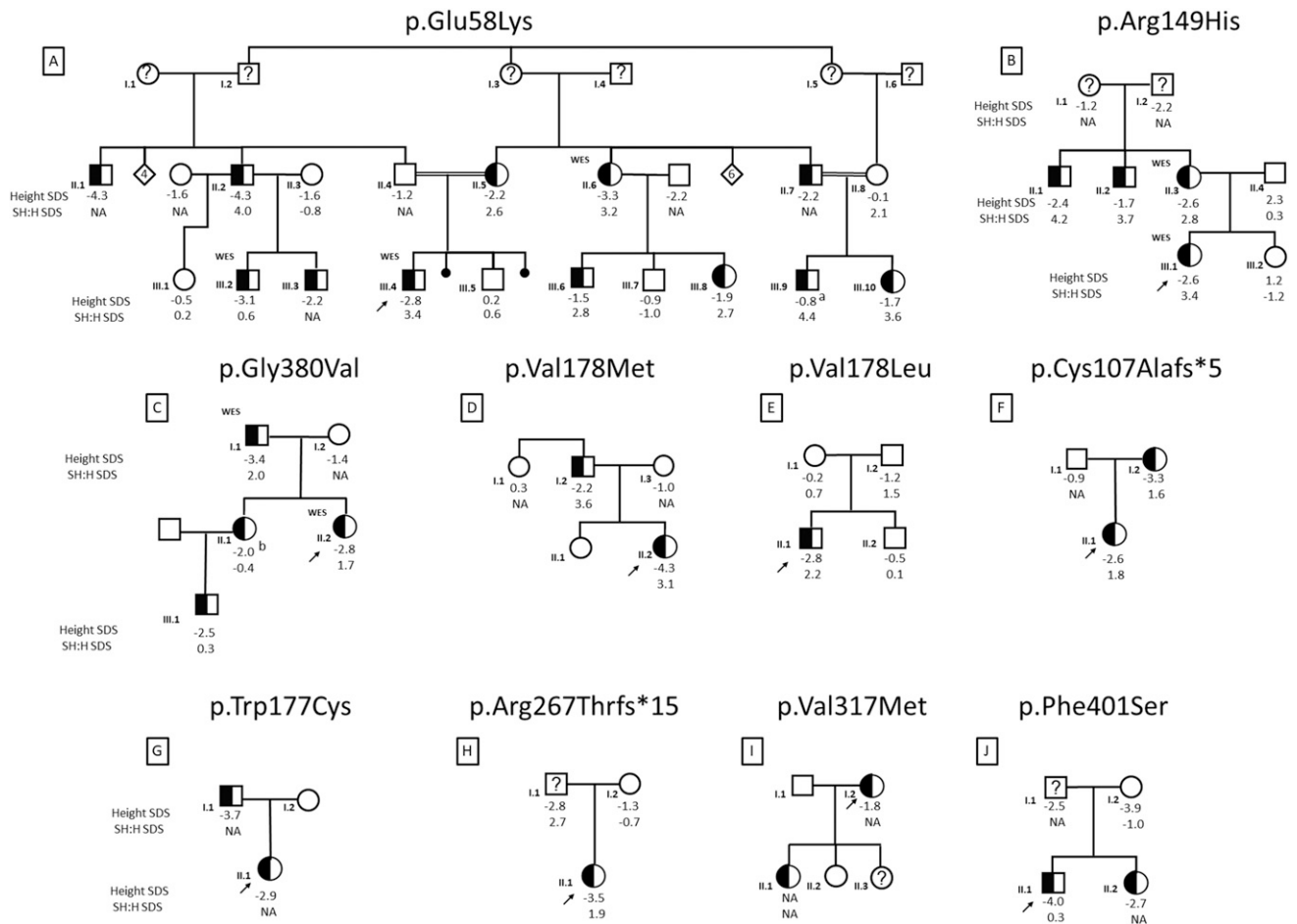


Figure 1. Pedigrees of the affected families carrying heterozygous *IHH* mutations. (A–E) Brazilian families with ISS. (F–J) Spanish families with mild skeletal dysplasia or brachydactyly. The arrows indicate the probands, and WES indicates individuals who underwent WES. Individuals with heterozygous *IHH* mutations are indicated by half-filled symbols, whereas individuals homozygous for *IHH* wild-type allele are indicated as open symbols. Question marks indicate an unknown genotype. Height SDS and SH:H SDS at first medical visit are shown below the symbols. Abbreviation: NA, no data available. ^aAdult height SDS of -1.9 ; ^bprevious treatment with rhGH.

mild skeletal dysplasia or brachydactyly ($n = 160$), five additional heterozygous *IHH* variants were identified by targeted sequencing (Fig. 1F–1J). Thus, a total of 10 heterozygous *IHH* variants were identified, 2 frameshift mutations resulting in the premature termination of the protein, and 8 missense variants (Table 1). All of them are predicted to be pathogenic by multiple *in silico* programs. Six of the variants are located in the N-terminal domain [p.Glu58Lys (c.172G>A), p.Arg149His (c.446G>A), p.Cys107Alafs*5 (c.319delT), p.Trp177Cys (c.531G>C), p.Val178Leu (c.532G>C), p.Val178Met (c.532G>A)], and four are located in the C-terminal domain [p.Arg267Thrfs*15 (c.797dupC), p.Val317Met (c.949G>A), p.Gly380Val (c.1139G>T), p.Phe401Ser (c.1202T>C)]. Nine of the variants are absent in public databases, whereas the p.Val317Met variant is present at an extremely low allelic frequency (1/241,680) in gnomAD. Eight of the variants were confirmed to segregate with the autosomal-dominant short-stature phenotype (Fig. 1). One variant was *de novo* (paternity and maternity confirmed). Thus, after

applying the ACMG-AMP guidelines, four variants were classified as pathogenic and six as likely pathogenic (Table 1).

Primary sequence analyses showed that all *IHH*-mutated amino acid residues are extremely conserved among non-redundant homologous and related sequences. Although some hydrogen bonds were locally disrupted near the mutated amino acid residues, in general, the presence of the mutations provided more molecular contacts for the whole molecules (Supplemental Fig. 4). In addition, results of the *IHH* molecular simulations revealed that *IHH* models bearing N-terminal mutations presented a smaller mean potential energy compared with that of the native molecules (Supplemental Table 3; Supplemental Fig. 5). Conversely, except for the *IHH* Glu58Lys and Arg149His molecules, the binding energies of the atoms Zn412, Ca413 and Ca414 to all others mutated molecules are greater than that of the wild type one (Supplemental Table 3).

The allele frequency of rare *IHH* variants predicted to be deleterious found in both cohort of patients with short

stature (1.7% and 1.56% for Brazilian and Spanish cohorts, respectively) was expressively higher than that observed in individuals from gnomAD (0.017%; $P < 0.001$) (Table 2). Considering only the Brazilian cohort, we also observed a significantly higher frequency of rare *IHH* variants predicted to be deleterious in short-stature individuals (1.7%) in comparison with the ABraOM database [1/1218 alleles (0.08%); $P < 0.001$] (Table 2; Supplemental Fig. 6). Furthermore, the allele frequency of *IHH* variants was similar in Brazilian children with [4/214 alleles (1.9%)] or without [1/80 alleles (1.3%)] familial short stature.

Clinical phenotypes

The phenotypic characteristics of the probands and other children with heterozygous *IHH* variants are shown in Table 3. Four of the 12 Brazilian children were born SGA regarding birth length, and the other 8 had a birth length SDS in the lower part of the normal range (mean birth length, -1.4 ± 0.09 SDS; range, -2.7 to -0.1 SDS). The mean height SDS of the Brazilian children was -2.6 ± 0.9 (range, -4.3 to -0.8 SDS). The affected patient (AIII.9) with a height SDS of -0.8 (Fig. 1A) was a 14.6-year-old boy with advanced bone age (16.5 years), and his adult height SDS is -1.9 . Most affected Brazilian children ($n = 8/12$) presented mild disproportionate short stature [mean sitting height to height ratio (SH:H) SDS, 2.4 ± 1.2 ; range, 0.3 to 3.6]. Most affected children had mild delayed bone age, but we observed two of them with advanced bone age (Table 3 individuals AIII.6 and AIII.9). Abnormal hand radiographs were observed in 6 of 12 Brazilian children. Two children presented only shortening of the middle phalanx of the fifth finger. Other radiological hand findings include varying degrees of shortening of the middle phalanx of the second and fifth fingers with cone-shaped epiphyses (Fig. 2A; Supplemental Fig. 7). Foot radiographs of patients with heterozygous *IHH* mutations showed a recurrent shortening of the distal phalanx of the hallux. Other radiological findings observed in feet

were cone-shaped epiphyses of proximal phalanges of the second, third, fourth, and fifth toes (Fig. 2B; Supplemental Fig. 8).

All five Spanish cases were detected in a cohort of patients with mild skeletal dysplasia (3/100) or suspected to have brachydactyly (2/60). Low birth length (-2.9 SDS) was observed in one patient (GII.1). The height SDS of Spanish children ranged from -4.0 to -2.6 SDS (mean, -3.2 ± 0.6 SDS). SH:H SDS values were in the upper part of the normal range (1.8 and 1.9 SDS) in two of three available probands. Patient JII.1 had a severe delay (-3.8 years) in bone age with respect to chronological age. All five patients had mild abnormal skeletal surveys, typically shortening of middle phalanx of the second and fifth fingers (Supplemental Fig. 7).

A total of 14 adult individuals heterozygous for *IHH* variants could be assessed (10 Brazilian and 4 Spanish). Their mean height SDS was -3.0 ± 1.1 (range, -4.3 to -1.7). Eight of nine affected adult individuals available have altered body proportion (SH:H SDS ranged from 2.0 to 4.2) (Supplemental Table 4).

Growth response to rhGH treatment

Five patients, all from the Brazilian cohort, with heterozygous *IHH* variants have been treated with rhGH therapy ($50 \mu\text{g}/\text{kg}/\text{d}$) (Table 4). The onset of treatment was prepuberty in all of them. One girl had started puberty during the first year of rhGH treatment (patient CII.2). After exclusion of this patient from first-year growth response, the four remaining patients had a mean improvement in height SDS of 0.6 (range, 0.46 to 0.79) and their height velocity ranged from 8.0 to 10.8 cm/y.

Discussion

Longitudinal bone growth is essential to determine adult body height and depends on the growth plate chondrogenesis, a complex process regulated by endocrine and paracrine factors. Over the past years, mutations in different genes involved in the growth plate have been

Table 2. Comparison of Allele Frequency for Rare and Potential Deleterious Alleles Between Patients Selected for Short Stature and Public Databases

Variable	Potential Deleterious Alleles ^a	Total No. of Analyzed Alleles	Allele Frequency (%)
gnomAD	40	236,524	0.017
ABraOM	1	1218	0.082
Brazilian cohort of children with short stature of unknown cause	5	294	1.70 ^b
Spanish cohort of patients with mild skeletal dysplasia	5	320	1.56 ^a

^aRare variants (MAF of 0.01) with loss-of-function effect (stop gain, frameshift, or consensus splicing site variants) or rare nonsynonymous variants predicted to be pathogenic by at least four *in silico* tools (PROVEAN, SIFT, PolyPhen-2 and Mutation Assessor).

^b $P < 0.001$ for comparison with gnomAD or ABraOM.

Table 3. Clinical Phenotypes of Proband and Their Child Family Members With Heterozygous *IHH* Mutations

Patient ID	<i>IHH</i> Mutations	Sex	Birth Length SDS	Age (y)	Bone Age (y)	Height SDS	SH:H SDS	Hand Radiographic Findings
Brazilian children								
AIII.2	p.Glu58Lys	M	-2.1	5.6	5.0	-3.1	0.6	Cone-shaped epiphyses of middle phalanx of fifth finger and of distal phalanx of thumb
AIII.3	p.Glu58Lys	M	-0.8	1.5	NA	-2.2	NA	NA
AIII.4	p.Glu58Lys	M	-2.7	3.4	2.7	-2.8	3.4	Cone-shaped epiphyses of middle phalanx of second and fifth fingers and of distal phalanx of thumb
AIII.6	p.Glu58Lys	M	-1.6	8.9	9.4	-1.5	2.8	No abnormal findings
AIII.8	p.Glu58Lys	F	-1.9	3.5	2.7	-1.9	2.7	Shortening of middle phalanx of fifth finger
AIII.9	p.Glu58Lys	M	-0.1	14.6	16.5	-0.8 ^a	4.4	No abnormal findings (adult bone age)
AIII.10	p.Glu58Lys	F	-0.7	7.4	6.8	-1.7	3.6	Cone-shaped epiphysis of middle phalanx of fifth finger
BIII.1	p.Arg149His	F	-2.4	8.0	6.8	-2.6	3.4	Shortening of middle phalanx of second and fifth fingers with cone-shaped epiphyses
CII.2	p.Gly380Val	F	-0.2	9.2	7.9	-2.8	1.7	No abnormal findings
CIII.1	p.Gly380Val	M	-1.1	1.5	NA	-2.5	0.3	No abnormal findings
DII.2	p.Val178Met	F	-2.3	9.4	5.0	-4.3	3.1	Shortening of middle phalanx of fifth finger
EII.1	p.Val178Leu	M	-1.0	7.1	5.8	-2.8	2.2	No abnormal findings
Spanish individuals								
FII.1	p.Cys107Alafs*5	F	NA	13	13	-2.6	1.8	Braquimetacarpal with shortening of middle phalanx of second and fifth fingers
GII.1	p.Trp177Cys	F	-2.9	16	NA	-2.9	NA	Severe shortening of middle phalanx of second and fifth fingers with cone-shaped epiphyses
HII.1	p.Arg267Thrfs*15	F	NA	7	6.0	-3.5	1.9	Severe shortening of middle phalanx of second and fifth fingers
II.2	p.Val317Met	F	NA	Adult	NA	-1.8	NA	Severe shortening of middle phalanx of fifth finger and slight shortening of middle phalanx of second finger
JII.1	p.Phe401Ser	M	-0.4	7.8	4.0	-4.0	0.3	No abnormal findings

Birth length and height SDS are according to population curves.

Abbreviations: F, female; M, male; NA, data not available.

^aDuring follow-up, the patient had a growth velocity of 0.6 cm/y, and his height SDS for adult age is -1.9.

implicated in the etiology of several cases of skeletal dysplasia, a condition frequently presented with marked growth impairment (6, 16). Furthermore, a limited number of genes implicated in severe skeletal dysplasia [*SHOX* (17), *NPR2* (18), *ACAN* (8), *FBN1* (10), *NPPC* (11)] have been associated with nonsyndromic short stature, which is commonly classified as ISS in clinical practice.

In the current study, we identified heterozygous *IHH* mutations in 5 of 147 children initially classified as ISS. Additionally, another 5 heterozygous *IHH* mutations were identified among short stature patients with subtle signs of a skeletal dysplasia (3/100) or with mild brachydactyly (2/60). According to ACMG-AMP criteria (13), the identified *IHH* variants were classified as pathogenic or likely pathogenic (Table 1).

Along with *IHH*, Hedgehog (Hh) family proteins include other two Hh homologs with high level of sequence conservation: Sonic Hh (SHH) and Desert Hh, essential for the nervous system and testis development, respectively (19). Hh proteins are synthesized in the endoplasmic reticulum as a precursor that undergoes an autocatalytic process to yield the N-terminal domain, which has biological signaling activities (20). Hh action is mediated through Patched and Smoothed receptors

and is positively regulated by cell adhesion molecule-related, downregulated by oncogenes (CDO) and brother of CDO in a calcium-dependent binding mode (19). *IHH* is expressed in the prehypertrophic chondrocytes of cartilage and coordinates proliferation and differentiation of chondrocytes during endochondral bone development (21). Homozygous *IHH* mutations cause acrocapitofemoral dysplasia (MIM 607778), a disorder characterized by severe disproportionate short stature with cone-shaped epiphyses in hands and hips (22). In addition, heterozygous mutations in *IHH* cause BDA1 (MIM 112500). BDA1 is characterized by a marked shortening of the middle phalanges, which are frequently rudimentary or fused with the terminal phalanges. The proximal phalanges of the thumbs and big toes are also short (12). Interestingly, short stature was not consistently demonstrated in patients with BDA1 (12, 23).

The variants identified in the current study were located throughout the *IHH*, including two frameshift mutations and eight missense mutations. These findings are in contrast with previous variants associated with BDA1, which were all missense and restricted to the central region of the N-terminal active fragment of *IHH*

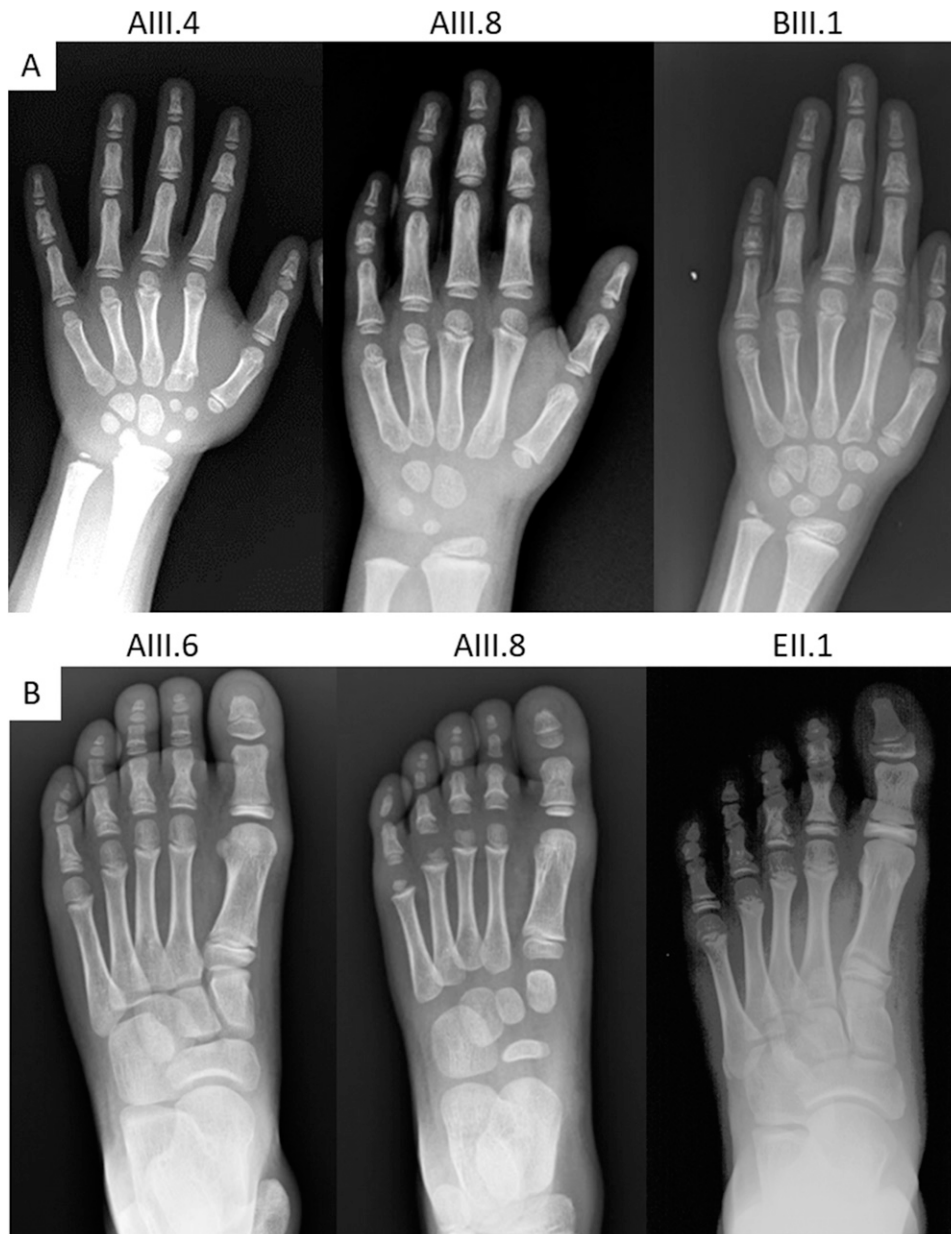


Figure 2. Hand and feet radiographs of children carrying heterozygous *IHH* mutations. (A) Hand radiographs show varying degrees of shortening of the middle phalanx of the second and fifth fingers with cone-shaped epiphyses. (B) Foot radiographs show shortening of distal phalanx of the hallux (individuals AIII.6 and AIII.8) and cone-shaped epiphyses of proximal phalanges.

(24) (Supplemental Fig. 9). Similar to our findings, variants in *SHH* associated to holoprosencephaly (missense, nonsense, or frameshift) are located throughout the gene (25). In addition, after single–amino acid code alignment

of *IHH* and *SHH*, we observed that two of the *IHH* mutations identified in our patients (p.Glu58Lys and p.Arg149His) are located in the identical residues of pathogenic *SHH* mutations (p.Glu53Lys and

Table 4. Response to rhGH Therapy in Patients With Heterozygous *IHH* Variants

Patient ID	<i>IHH</i> Mutations	Sex	Age at Initiation of rhGH (y)	Height SDS Before rhGH	Height SDS at 1 y of rhGH	Height Velocity after 1 y of rhGH (cm/y)	Duration of rhGH Therapy (y)	Last Available Height SDS	Total Height SDS Change
AIII.2	p.Glu58Lys	M	6.6	−3.0	−2.6	8.0	1.0	−2.6	0.4
AIII.6	p.Glu58Lys	M	10	−1.5	−1.0	8.0	1.0	−1.0	0.5
BIII.1	p.Arg149His	F	8	−2.6	−1.9	8.1	3.0	−1.8	0.8
EII.1	p.Val178Leu	M	7.1	−2.8	−2.0	10.8	4.7	−0.4	2.4
CII.2	p.Gly380Val	F	10.4	−2.6	−1.7	11.2	4.0	−1.5	1.1

Abbreviations: F, female; M, male.

p.Arg144Pro, respectively) (26). The results of the structural analyses and molecular mechanics simulations experiments based on the *IHH* molecular structure showed that all mutations located at N-terminal domain could affect the structure and, therefore, the biological function of the molecule. The presence of these mutations determines a smaller total mean potential energy that is associated with a more stable molecule, affecting the calcium and zinc cofactor-binding energy (Supplemental Table 3). A greater calcium-binding energies observed in four mutated *IHH* molecules (p.Glu58Lys, p.Val178Leu, p.Val178Met, and p.Trp177Cys) could affect the interaction to binding partners CDO and brother of CDO, a mechanism already described to *IHH* mutations associated to BDA1 (19).

It is a challenge to determine the functional impact of variants located in the C-terminal domain of Hh proteins. Nevertheless, one study found no difference in phenotype severity between individuals with holoprosencephaly caused by *SHH* mutations located in the N- or C-terminal domains (27). It is postulated that C-terminal variants could impair the preprotein maturation and autocleavage process (26). Further studies are necessary to elucidate molecular mechanisms of *IHH* variants responsible for nonsyndromic short stature.

We provide several lines of genetic evidence that the identified *IHH* variants are associated with short stature with nonspecific skeletal abnormalities (15). First, at each case level, we demonstrated in several families a segregation with growth impairment phenotype in a dominant inheritance pattern (Fig. 1). Additionally, a *de novo* variant (both paternity and maternity confirmed) was identified in a patient with short stature and normal stature parents (Fig. 1E). Second, we provide statistical evidence that rare *IHH* variants are enriched in the two analyzed cohorts of short-stature patients in comparison with public databases that involve a large number of individuals not selected by stature (Table 2; Supplemental Fig. 6).

Patients described in the current study typically manifest mild disproportional short stature. It seems that disproportionality has become more pronounced over the years during childhood. Four probands were born SGA regarding birth length. Intrauterine growth impairment with normal birth weight, especially in children with familial short stature, may suggest defects in genes that play a role in bone elongation. There is a variable phenotype considering the severity of short stature and radiological findings, even among individuals within the same family. The most recurrent radiological finding observed in our patients was varying degrees of shortening of the middle phalanx of the fifth finger, a defining feature of brachymesophalangia V (BMP-V) (28). BMP-V is the most common skeletal anomaly of the hand and

can be observed alone (clinically known as brachydactyly type A3) or in association to other skeletal abnormalities and developmental syndromes. Epiphyses of the fifth finger may be associated with a susceptibility to a chondrogenesis disorder because they are among the last to be ossified in the hand. BMP-V was observed in 64.3% (9/14) hand radiographs from individuals heterozygous from *IHH* variants in our cohort, whereas the frequency of BMP-V observed in a population study was 12.1% (165/1360; $P < 0.001$) (28).

The response to rhGH therapy could be assessed in five patients heterozygous for *IHH* variants (Table 4). We considered the short-term response to rhGH therapy presented by this small group of children to be good. The improvement in height SDS at 1 year of rhGH therapy was similar to or better than that previously reported in two larger studies that evaluated responsiveness to rhGH in children with ISS (0.60 SDS and 0.49 SDS, respectively) (29, 30). A different study developed criteria based on height velocity targets for first-year responses in ISS children. Those researchers proposed that height velocity below the mean -1 SD on their plots should be considered a “poor” response. None of our patients had a first-year height velocity below the mean -1 standard deviation; two were plotted above the mean and can be considered as good (31).

In conclusion, our data demonstrate the association of rare pathogenic variants in *IHH* with short stature with nonspecific skeletal abnormalities (MIM 616255). Given the subtle and low specific clinical and radiological findings observed in our patients, only a broad molecular study allowed this association. Thus, we have expanded the phenotype associated with heterozygous *IHH* mutations and established a frequent cause of growth disorder, with a preliminary good response to rhGH.

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