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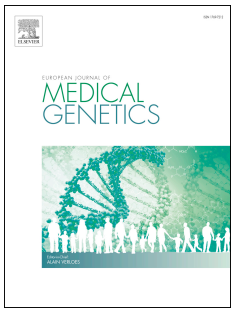

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Novel variants in Natriuretic Peptide Receptor 2 in unrelated patients with Acromesomelic Dysplasia type Maroteaux

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Abstract

Acromesomelic dysplasia are a heterogeneous group of disorders with variable spectrum and severity of skeletal anomalies in the affected individuals. Acromesomelic dysplasia type Maroteaux (AMDM) is characterized by extreme shortening of the forelimbs and disproportionate short stature. Several homozygous inactivating mutations in *NPR2* have been identified in different AMDM patients. We report five novel variants in affected individuals in four different families. These include two nonsense and three missense variants. This study broadens the genotypic spectrum of *NPR2* mutations in individuals with AMDM and also describes the intra- and inter-familial phenotypic variability due to *NPR2* variants.

Keywords: Acromesomelic dysplasia; AMDM; NPR2; Skeletal dysplasia; short stature

Introduction

Acromesomelic dysplasia are a heterogeneous group of autosomal recessively inherited disorders, categorized on the basis of their disease severity and the gene mutated in affected individuals. Disproportionate shortening of skeletal elements is the key feature of acromesomelic dysplasia which predominantly affects the forelimbs (forearms and legs) and distal segments (hands and feet) of the appendicular skeleton. In Acromesomelic dysplasia type Maroteaux (OMIM#602875) the anomalies of the appendicular skeleton may be accompanied with changes in the axial skeleton, including wedging of the vertebrae. Affected individuals have normal intellect and have no extra-skeletal phenotypes.

NPR2 (OMIM#602875) variants have been identified as the cause of AMDM (Bartels et al., 2004). *NPR2*, located on chromosome 9, encodes natriuretic peptide receptor B (NPR-B) (Lowe et al., 1990), a receptor homodimer which is present in chondrocytes and is responsible for producing cyclic GMP after binding C-type natriuretic peptide (CNP). NPR-B (NPR2) is one of three natriuretic peptide receptors (NPR-A, NPR-B, NPR-C) (Potter et al., 2005) which interacts with natriuretic peptides and regulates different physiological process including endochondral ossification, cardiac development and blood pressure (Langenickel et al., 2006; Tamura et al., 2004). NPR2 has four domains, an extracellular ligand binding domain, a transmembrane domain, an intracellular kinase homology domain (KHD) and a guanylyl cyclase (GC) domain at the C-terminal (Schulz, 2005).

In the present study, we describe the phenotypic and genetic findings of three Pakistani and one Finnish family with multiple affected individuals exhibiting typical features of Acromesomelic

dysplasia type Maroteaux. We have identified five novel variants in *NPR2* segregating with the disease phenotype in these families.

Material and methods

Samples

The study was approved by the institutional review board of School of Biological Sciences, University of the Punjab, Lahore, Pakistan and Research Ethics Committee of Helsinki University Hospital, Finland. Families MID-02, NAD-08 and NAD-09 were from Pakistan and were identified and recruited for study through personal contacts. Written informed consents were obtained from all participating individuals or from the parents for minor children. Family FD-01 was followed at Children's Hospital, Helsinki. Data were collected retrospectively from hospital records.

Heights of the participants were measured and some individuals were photographed. Blood samples of all available individuals were obtained for extraction of genomic DNA. The DNA was isolated by a standard protocol involving sucrose lysis and salting out. Radiographs of hands, feet and spine of affected individuals were obtained except for family MID-02 who did not consent to radiographic assessment. Amino-terminal proCNP (NT-proCNP) testing, a biomarker for AMDM due to NPR2 variants, (Wang et al. 2016), could not be performed due to lack of testing facility.

Mutational Screening

For family MID-02, whole-exome sequencing was performed on DNA sample from individual IV: 3 using Agilent V4 enrichment kit (Agilent Technologies, Santa Clara, CA). 50× coverage of

paired-end reads were obtained on an Illumina Hi-Seq 2000 sequencer (Otogenetics, Norcross, GA). Reads were mapped to UCSC hg19 reference human genome (http://genome.ucsc.edu/). The annotation of variants was completed using wANNOVAR (http://wannovar.usc.edu/). All heterozygous variants and variants with a minor allele frequency (MAF) equal to or greater than 0.01 in public databases (dbSNP database, GnomAD, Exome Aggregation Consortium (ExAC), 1000 Genomes and 6500 exon sequence project) were excluded. Exonic and splice site variants were considered for downstream analysis. Agilevariant mapper (http://dna.leeds.ac.uk/agile/AgileGenotyper/) was used for identification of homozygous chromosomal intervals in the exome data.

Genetic analysis for all members of family FD-01 was performed commercially (CTGT laboratory, USA) by targeted gene sequencing. Candidate gene screening was used for mutational analysis in families NAD-08 and NAD-09. Primers spanning all 22 exons and splice sites of *NPR2* were designed (sequences available upon request). PCR amplification with each primer set was carried out and followed by Sanger sequencing with BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). All variants were checked for segregation with the phenotype in the respective families by Sanger sequencing of the corresponding exons containing the mutation. The sequencing data were analyzed using Seq Scape software. The information for the identified variants was deposited in Leiden Open Variation Database 3.0-LOVD, (http://www.lovd.nl/3.0/home).

The pathogenicity of identified variants was assessed using online prediction tools including Polyphen 2 (http:// http://genetics.bwh.harvard.edu/pph2/) MutationTaster (http://www.mutationtaster.org/), Mutation Assessor (http://mutationassessor.org/r3/) and M.CAP (http://bejerano.stanford.edu/mcap/). Alignments of orthologous NPR2 protein

sequences were generated using (1) Clustal Omega after retrieving sequences from Ensembl (2) and (2) MUSCLE in Geneious after obtaining orthologues from NCBI's Whole Genome Shotgun contig database via BLASTing (discontiguous megablast) a human reference sequence (Ensembl).

Results

Clinical Features

Family MID-02 is comprised of four affected individuals (Supplementary Figure 1) with typical features of Acromesomelic Dysplasia, type Marotaeux including extremely short forearms and forelegs. One of the parents was of normal height, while the other was reported to have below average height. Family NAD-08 had five individuals with skeletal anomalies (Supplementary Figure 1). All affected individuals had typical features of AMDM with short stature, extreme shortening of forearms and forelegs, brachydactyly (Fig. 1a) and restricted movement of elbow (Table 1). The heights of the obligate carriers I: 1 and: I: 2 were 167 cm (Z-score -1.2) and 155 cm (Z-score-1.2) respectively, which are slightly below average normal heights.

In family NAD-09 there are three affected individuals from three different consanguineous marriages (Supplementary Figure 1). Samples from only one affected individual, his parents and unaffected brother were available for genetic analysis. The affected individual presented with very short height and extreme shortening of forelimbs. He had brachydactyly of fingers and macrodactyly of toes. He has severe back pain for the last few years which is currently being treated. Both his parents had short heights, 162 cm (Z-score -3.2) (father) and 142 cm (Z-score -2.0) (mother) who also had short hands (Table 1).

Family FD-01 included two daughters, born to healthy and unrelated Finnish parents (Supplementary Figure 1). Heights of parents were 172 cm (Z-score -0.7) (father) and 160 cm (Z-score -0.5) (mother). Both girls had significant short stature (Table 1).

Radiographs of family NAD-08 (not shown), NAD-09 (Fig. 1b) and FD-01 (Fig. 1c) showed marked shortening of radius and ulna, and short fingers and toes, with premature fusion of the growth plates. Spinal radiographs, available for the older child in family FD-01 at 10 years, showed lack of widening of the interpedicular distance in the lumbar spine (Fig. 1c). Longitudinal data of heights was available for the two affected individuals in family FD-01.Their heights deviated from normal values during the first year and again during puberty (Fig. 1d).

Genetic Analysis

Analysis of whole-exome sequencing data revealed a missense variant c.872A>G; p.(Gln291Arg) (LOVD ID: 00179514) in *NPR2* (NM_ 003995.3) which segregated with the disease phenotype in family MID-02 (Fig. 2a, Table 2). This variant was absent from public databases including gnomAD (http://gnomad.broadinstitute.org/) as well as in ethnically matched 200 control chromosomes. The amino acid Gln291 is completely conserved among 133 species of mammals examined (Supplementary Figure 2) as well as birds but not in lower vertebrates which include lizard, frog and fish (Fig. 2b).

Analysis of sequencing data of family NAD-08 revealed a homozygous missense mutation c.368G>T; p.(Gly123Val) (LOVD ID: 00179511) in exon 1 of *NPR2* (Table 2). The variant segregated with the phenotype as all affected individuals were homozygous and obligate carriers were heterozygous (Fig. 2c). The variant was predicted to be pathogenic or deleterious by

various online tools (Table 2). The amino acid Gly123 is conserved among all species examined (Fig. 2d).

In family NAD-09, a homozygous stop-gain mutation c.1185G>A; p.(Trp395Ter) (LOVD ID: 00179513) was identified in exon 5 of *NPR2* (Table 2). The parents and the unaffected brother were heterozygous for the mutation (Fig. 2e).

In family FD-01 compound heterozygous variants in *NPR2* at positions c.2965C>T; p.(Arg989Ter) and c.2966G>T; p.(Arg989Leu) (LOVD ID: 00179516) were identified in both affected individuals (Table 2, and data not shown). The c.2965C>T variant was inherited from the father while the c.2966G>T variant was inherited from the mother (Supplementary Figure 1). The residue Arg989 is conserved among all species examined (Fig. 2f).

All five variants were absent from the public Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/, accessed May, 2018) and also from recently published research. The p.(Gln291Arg), p.(Trp395Ter), p.(Arg989Ter), and p.(Arg989Leu) variants were absent in public databases whereas only one heterozygous allele for variant p.(Gly123Val) was present in ExAC database with an allele frequency of 0.000008239 (http://exac.broadinstitute.org/).

Discussion

Acromesomelic dysplasia type Maroteaux is characterized by short stature, extreme shortening of forearms and forelegs including shortening of hands and feet bones and also involvement of appendicular skeleton with broadening of vertebrae. The birth lengths of limbs may be normal and decreased growth becomes evident from the second year of life. Pathogenic variants in *NPR2* cause skeletal dysplasia in patients with AMDM (Bartels et al., 2004). We identified five novel variants in *NPR2* in unrelated affected individuals. The phenotypes of all affected

individuals were typical of AMDM. To date, homozygous or compound heterozygous mutations have been identified in AMDM patients of different ethnic origins. Heterozygous variants have been identified in individuals with idiopathic short stature (mutations with dominant-negative effect) (Olney et al., 2006; Vasques et al., 2013; Wang et al., 2015) or tall stature (gain-of-function mutations) (Hannema et al., 2013; Miura et al., 2014; Miura et al., 2012). In the present study, biallelic variants in *NPR2* account for the phenotype of AMDM in the patients and mild short stature in the heterozygous carriers. Our finding that heterozygous carriers of variant p.(Trp395Ter) also have short stature (Table 1), indicates that loss-of-function *NPR2* alleles can also result in idiopathic short stature in humans.

NPR2 interacts with C-type natriuretic (CNP) peptide which acts in an autocrine or paracrine manner. CNP is highly expressed in the hypertrophic zone of growth plate and plays an important role in endochondral ossification and cartilage matrix synthesis (Nakao et al., 2015). The CNP/NPR2 interaction is thought to play an important role in the development of longitudinal growth of bones and any disruption in this signaling can lead to defective ossification of bones(Tamura et al., 2004). Several animal studies have provided evidence that homozygous or compound heterozygous variants in *Npr2* can lead to AMDM in animal models (Sogawa et al., 2007; Tsuji and Kunieda, 2005).

All reported variants in *NPR2* are hypothesized to cause disease by either altering its ligand binding affinity or by diminishing guanylyl cyclase activity (Wang et al., 2016). The p.(Gly123Val), p.(Gln291Arg), and p.(Trp395Ter) variants identified in the present study are located in the ligand binding domain (Fig. 2g). We hypothesize that these variants cause the disease by altering the binding of CNP to NPR2. The variants identified in family FD-01

p.(Arg989Leu) and p.(Arg989Ter) are located in guanylyl cyclase domain of NPR2 and likely cause the disease by diminishing the activity of NPR2 protein.

The nonsense mutation identified in NAD-09 is located in exon 5 of *NPR2* which has 22 exons in total. Therefore, it is likely to cause nonsense mediated decay of *NPR2* mRNA and will probably result in absence of NPR2 in these patients.

The Glutamine 291 codon mutated in family MID-02 is conserved in all mammals examined but is not conserved in lizards, amphibians and fish. This could be due to the skeletal differences or epistatic effects among these classes. The retention of Gln291 in the 133 mammal species we examined suggests that natural selection has strictly maintained this residue for approximately 160 million years, underscoring its probable functional importance in humans. The NPR2 pathogenic missense variants p.(Thr297Met), p.(Tyr338Cys) and p.(Ala409Thr) previously reported in different AMDM individuals, also affect residues which are only conserved in mammals, though this fact was not stated in that study (Bartels et al., 2004).

The affected individuals in families NAD-08 and NAD-09 had long faces as described in a few earlier studies (Khan et al., 2012; Srivastava et al., 2016). The affected individual in family NAD-09 has a severe backache complaint for the last two years. Spinal radiographs of one of the subjects in family FD-01 showed narrow spinal canal in the lumbar region indicating a risk for later development of spinal stenosis, which could be the cause of back pain also in the individual in NAD-09. The heterozygous carriers of the variants in all families had mild short stature. However, the heterozygous carriers in family NAD-09 with the nonsense allele were shorter (-3.2 and -2.0 SD) as compared to those who were heterozygous for the missense variant in family MID-02 and NAD-08 (-1.2 and -1.2 SD). Moreover, the heterozygous variant carriers in family

NAD-09 had short hands. Disproportionate body, short stature and nonspecific skeletal anomalies have been previously reported in individuals with idiopathic short stature due to heterozygous variants in *NPR2* (Vasques et al., 2013). A dominant negative effect of mutant alleles has been identified which impairs NPR2 in these individuals. Interestingly, for family NAD-09, haploinsufficiency also results in a mild phenotype in the carriers of the variants.

The finding of three different variants of *NPR2* identified in three unrelated families from Pakistan in our study depicts the allelic heterogeneity of AMDM contrary to the identification of c.2720C>T; p.(Thr907Met) pathogenic variant in five unrelated Pakistani families. This was shown to be due to a founder effect (Khan et al., 2012). Although these five families were from the province of Punjab (Khan et al., 2012) as were the three Pakistani families that we present in this work, it is possible that the members participating in the previous study were from adjacent villages or cities of Punjab, which would explain the different mutational spectrum in these studies.

In conclusion we have identified five novel variants in *NPR2* in four different families. The mutations cause disease phenotype in the respective families. Moreover, the *NPR2* variant p.(Gln291Arg) is conserved only among mammals, which highlights the fact that some functional properties conferred by an amino acid to a protein may be important for proper biological function in mammals only.

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Web resources

Agilevariant mapper (http://dna.leeds.ac.uk/agile/AgileGenotyper/)

Fathmm, (http://fathmm.biocompute.org.uk/inherited.html)

gnomAD (http://gnomad.broadinstitute.org/)

Human gene mutation database (http://www.hgmd.cf.ac.uk/)

Leiden Open Variation Database 3.0- LOVD, (https://databases.lovd.nl/)

MutationTaster, http://www.mutationtaster.org/

Mutation Assessor, http://mutationassessor.org/r3/

Polyphen2, http:// http://genetics.bwh.harvard.edu/pph2/

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Legends

Figure 1. Clinical features of selected patients.

(a) Selected photos of Pakistani patients, their forearms/hands feet. **(b)** Radiographs of one Pakistani patient (NAD-09, IV:4), showing shortening of radius and ulna and broadened metacarpals; short and broadened tibio-fibula; and stubby toes. **(c)** Radiographs and photos of the Finnish patient showing shortened and broadened metacarpals and toes. **(d)**. Growth curves of individuals FD-01, II: 1 (left) and FD-01, II (right). (Colored figure can be observed in the online edition)

Figure 2. *NPR2* variants, conservation and protein structure

(a) Partial chromatograms of DNA sequence of *NPR2* of family MID-02. Arrow indicates the changed nucleotide. **(b)** Clustal Omega sequence alignment of NPR2 from diverse vertebrate species showing conservation of Glutamine at position 291 **(c)** Partial chromatograms of DNA sequence of *NPR2* of family NAD-08. The variant nucleotide is indicated with an arrow. **(d)** Clustal Omega sequence alignment of NPR2 showing conservation of Glycine at position 123 **(e)** Partial chromatograms of *NPR2* DNA sequence of family NAD-09. The variant nucleotide is indicated with an arrow**. (f)** Clustal Omega sequence alignment of NPR2 showing conservation of Arginine at position 989 among diverse orthologues. Arrows indicate the point of variant. **(g)** Schematic representation of NPR2 indicating position of variants identified in present study in NPR2. (Colored figure can be observed in the online edition)

Table 1. Phenotypes of individuals of AMDM families

Family	Individual	Sex	Age (years)	Height (cm)	SD	Zygosity	Clinical features
MID-02 c.872A>G	IV:3	F	18	92.7	-10.2	Homozygous	Markedly short forearms, short and broad fingers and toes, limited extension of elbows, motor milestones normal.
	IV:4	F	16	99	-9.7	Homozygous	Markedly short forearms, short and broad fingers and toes, limited extension of elbows, motor milestones normal.
	IV:5	F	12	96.5	-7.3	Homozygous	Markedly short forearms, short and broad fingers and toes, limited extension of elbows, motor milestones normal.
NAD-08 c.368G>T	II:1	F	58	155	-1.2	Heterozygous	Slightly short hands and feet.
	II:2	M	65	167.5	-1.2	Heterozygous	Slightly short hands and feet.
	III:3	M	20	119	-8.0	Homozygous	Markedly short forearms, short and broad fingers and toes and broad forehead and face, limited extension of elbows, motor milestones normal.
	III:4	M	27	122	-7.5	Homozygous	Markedly short forearms, short and broad fingers and toes and broad forehead and face, limited extension of elbows, motor milestones normal.

	III:6	F	30	116	-7.2	Homozygous	Markedly short forearms, short and broad fingers and toes and broad forehead and face, limited extension of elbows, motor milestones normal.
	III:8	F	23	167.6	+0.6	Wild type	Normal hands and feet.
	V:5	M	25	NA	NA	Homozygous	Markedly short forearms, short and broad fingers and toes broad forehead and face, limited extension of elbows, motor milestones normal.
NAD-09	III:1	F	55	142	-3.2	Heterozygous	Short hands and feet.
c.1185G>A	III:2	M	59	162	-2.0	Heterozygous	Short hands and feet.
	IV:2	M	28	167	-1.3	Heterozygous	Short hands and feet.
	IV:3	M	25	109	-9.3	Homozygous	Markedly short forearms, short and broad fingers and toes, limited extension of elbows, motor milestones normal. Severe pain in back for last few years.
FD-01	I:1	F	NA	160	-0.5	Heterozygous	No skeletal phenotype.
c.2965C>T	I:2	M	NA	172	-0.7	Heterozygous	No skeletal phenotype.
c.2966G>T	II:1	F	17	119.2	-8.2	Compound Heterozygous	Broad forehead, low nasal bridge, markedly short forearms short and broad fingers and toes, limited extension of elbows, motor milestones normal.

II:2	F	4	76.3	-6.9	Compound Heterozygous	Broad forehead, low nasal bridge, markedly short forearms short and broad fingers and toes, limited extension of elbows, motor milestones normal.

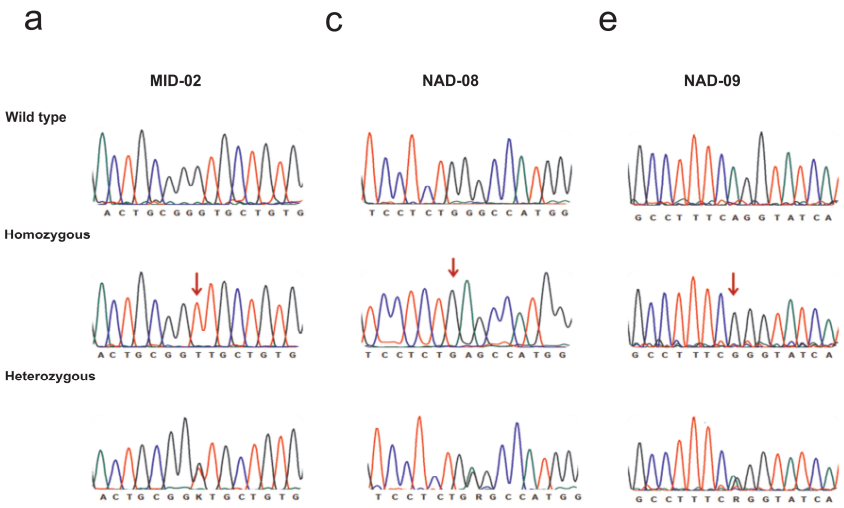
cm: centimeters, F: female, M: male.NA, not available

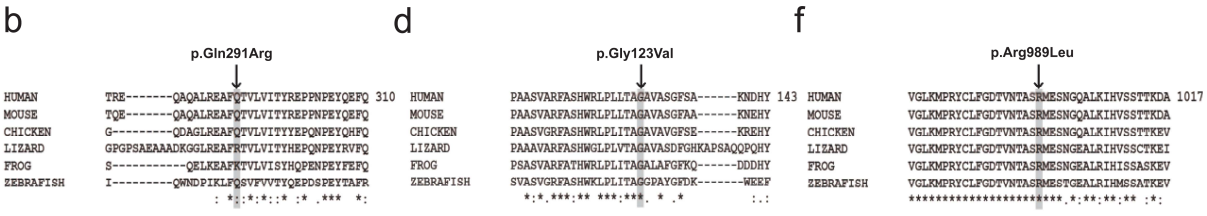
Table 2. *NPR2* variants identified in the present study

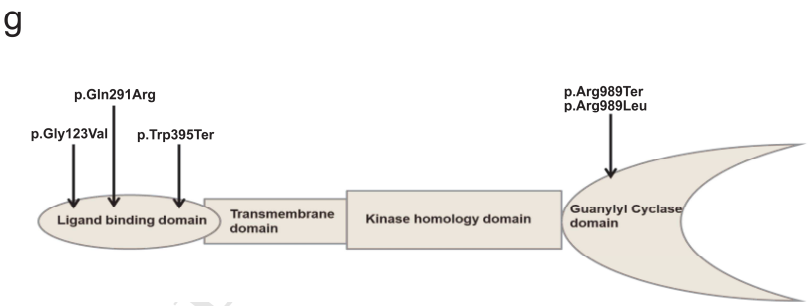
Family	Chr. Position*	cDNA change**	Amino Acid change	M-CAP	Mutation Assessor	Polyphen 2 Score /prediction	Mutation Taster
NAD-08	9:35,792,773	c.368G>T	p.(Gly123Val)	0.451	Medium impact	0.735/ Possibly damaging	Disease causing
NAD-09	9:35,800,447	c.1185G>A	p.(Trp395Ter)	NA	NA	NA	Disease causing
MID-02	9:35,794,099	c.872A>G	p.(Gln291Arg)	0.035	Neutral	0.084/Neutral	Disease causing
FD-01	9: 35,808,829	c.2965C>T	p.(Arg989Ter)	NA	NA	NA	Disease causing
	9:35,808,830	c.2966G>T	p.(Arg989Leu)	0.554	High	1.0/ Probably damaging	Disease causing

*Positions with reference to human genome build GRChg19/37

**cDNA change is with respect to transcript: NM_003995.3







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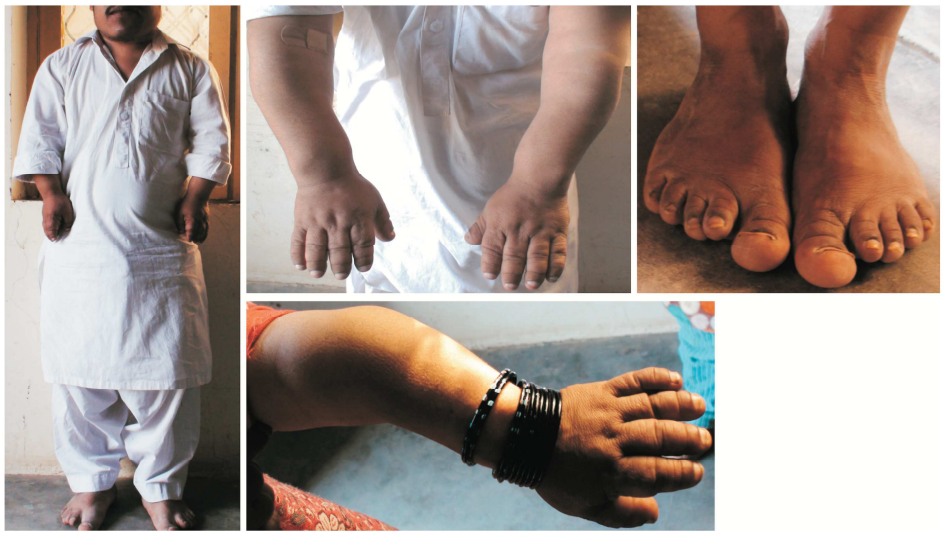

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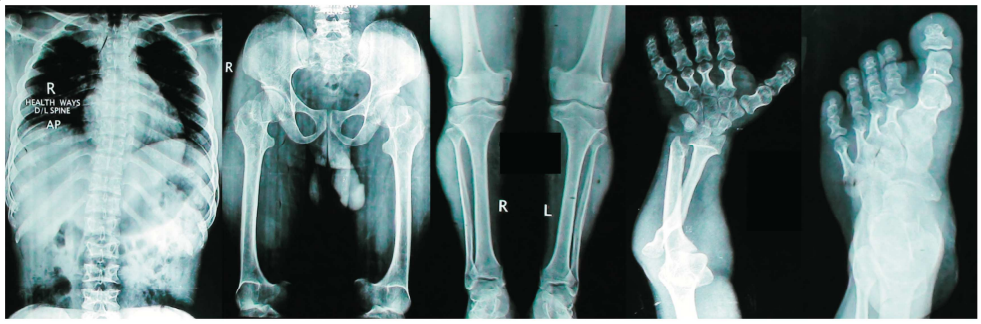
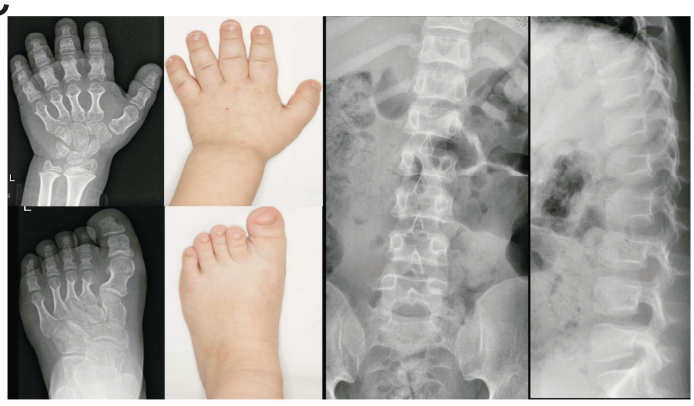

R
HEALTH WAYS
D/L SPINE
AP
R
R
L

c



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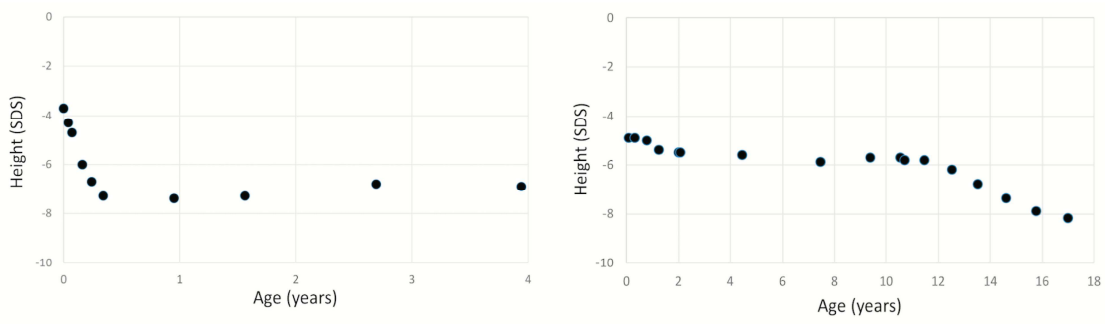
Height (SDS)
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-2
-4
-6
-8
-10
0
1
2
3
4
Age (years)
Height (SDS)
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-2
-4
-6
-8
-10
0
2
4
6
8
10
12
14
16
18
Age (years)

Highlights

- We describe five novel variants in *NPR2*
- Biallelic *NPR2* variants cause AMDM in four families
- We show that carriers of loss-of-function variants also had short stature