A hypomorphic BMPR1B mutation causes du Pan acromesomelic dysplasia

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Abstract

Background: Grebe dysplasia, Hunter-Thompson dysplasia, and du Pan dysplasia constitute a spectrum of skeletal dysplasias inherited as an autosomal recessive trait characterized by short stature, severe acromesomelic shortening of the limbs, and normal axial skeleton. The majority of patients with these disorders have biallelic loss-of-function mutations of GDF5. In single instances, Grebe dysplasia and a Grebe dysplasia-like phenotype with genital anomalies have been shown to be caused by mutations in BMPR1B, encoding a GDF5 receptor.

Methods: We clinically and radiologically characterised an acromesomelic chondrodysplasia in an adult woman born to consanguineous parents. We sequenced GDF5 and BMPR1B on DNA of the proposita. We performed 3D structural analysis and luciferase reporter assays to functionally investigate the identified BMPR1B mutation.

Results: We extend the genotype-phenotype correlation in the acromesomelic chondrodysplasias by showing that the milder du Pan dysplasia can be caused by a hypomorphic BMPR1B mutation. We show that the homozygous c.91C>T, p.(Arg31Cys) mutation causing du Pan dysplasia leads to a significant loss of BMPR1B function, but to a lesser extent than the previously reported p.Cys53Arg mutation that results in the more severe Grebe dysplasia.

Conclusions: The phenotypic severity gradient of the clinically and radiologically related acromesomelic chondrodysplasia spectrum of skeletal disorders may be due to the extent of functional impairment of the ligand-receptor pair GDF5-BMPR1B.

Keywords: Acromesomelic dysplasias, Grebe dysplasia, du Pan dysplasia, BMPR1B

Background

The acromesomelic dysplasias (ACD) constitute a rare subgroup of osteochondrodysplasias that are characterised by short stature and shortened limbs with anomalies of the hands and feet [1]. According to the 2010 revision of the nosology and classification of genetic skeletal disorders, five ACD subgroups are recognised, namely the severe Grebe dysplasia (including Grebe (OMIM #200700) and Hunter-Thompson (OMIM #201250) types), the milder du Pan dysplasia (OMIM #228900), a Grebe dysplasia-like phenotype with genital anomalies (OMIM #609441), as well as the ACD types Maroteaux (OMIM #602875) and Osebold-Remondini (OMIM 112910) (the latter two types will not be considered further here) [2]. While Grebe dysplasia is characterised by severe distal limb anomalies with rudimentary fingers and toes, there is mild short stature and milder limb involvement in du Pan dysplasia, including fibular hypoplasia and complex brachydactyly.

Both Grebe dysplasia and du Pan dysplasia are autosomal recessive disorders and can be caused by biallelic loss-of-function mutations of GDF5 (previously known as CDMP1), encoding the growth and differentiation factor 5 [3–6]. GDF5 belongs to the bone morphogenetic protein (BMP) family and binds to the BMP receptors BMPR1A and BMPR1B, with a preference for BMPR1B [7]. Consistent with this ligand-receptor interaction, ACD cannot only be caused by GDF5 mutations, but in rare cases also by loss-of-function BMPR1B mutations. Indeed, Demirhan and colleagues identified a homozygous truncating mutation of BMPR1B underlying a Grebe dysplasia-like phenotype with genital anomalies (absent ovaries, hypoplastic uterus, hypergonadotropic

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hypogonadism and primary amenorrhea) [8]; and we previously reported a homozygous missense mutation (p.Cys53Arg) and a homozygous nonsense mutation (p.Trp219*) of BMPR1B in Grebe dysplasia [9]. Functional analysis of the p.Cys53Arg missense alteration indicated that the mutant receptor had reduced localisation at the cell membrane, reduced activation by GDF5 binding and no effect on cell differentiation upon overexpression in an in vitro chondrogenesis assay, consistent with loss of function [9]. Here we have identified and characterised a hypomorphic BMPR1B missense alteration that causes du Pan dysplasia.

Methods
Clinical investigation and molecular analyses
The clinical evaluation was performed at the Department of Medical Genetics, Hospital Erasme, Brussels, Belgium, and the Institut de Pathologie et de Génétique, Gosselies, Belgium, and research was performed following a protocol approved by the University of Ulm Ethics Committee. After obtaining written informed consent for participation in the study, including consent to report individual patient data, we sequenced the coding exons and splice sites of GDF5 (NM_000557.2) and BMPR1B (NM_001256794.1) on genomic DNA after PCR amplification. Primer sequences are available on request. PCR products were sequenced on an ABI 3730 DNA Analyzer with BigDye chemistry v3.1 (Applied Biosystems). Sequence traces were assembled, aligned, and analyzed with the Seqman software (DNASTAR LaserGene). The BMPR1B intragenic short tandem repeat (STR) markers D4S2278 and BMPR1B-STR2 (forward primer 5'-ctccggcccaag atctctaTgtgtaaatgccccaccc-3', reverse 5'-gtgttgccatttacac Ataggatcttggccag-3') were previously described [9]. missense alterations were analyzed by performing energy minimization runs in vacuo employing only geometrical energy terms.

Cloning of BMPR1B expression constructs
The expression constructs for HA-tagged Bmpr1b WT and Bmpr1b Cys53Arg were previously described [9]. HA-tagged Bmpr1b WT in pSLAX13 was used as a template for in vitro mutagenesis to introduce the mutation coding for Arg31Cys (Primer forward 5'-cccagctctacAttgtaaatgccaccac-3', reverse 5'-cgcccaagatcctacAttgtaaatgccaccac-3', reverse 5'-gtgttgccatttacacTtaggatcttggccag-3'). Inserts were subcloned into the expression vector pCS2+ after Clal restriction.

Luciferase reporter gene assay
NIH/3 T3 cells (ATCC) were seeded in a 96-well plate in growth medium (DMEM 4.5 g/l glucose (Lonza), 10 % FBS superior (Biochrom), 2 mM L-glutamine (Lonza)) and transfected 24 h later using Lipofectamine 2000 (Invitrogen, Life Technologies) following the manufacturer's instructions. Cells were transfected in growth medium with the control vector pCS2+ or one of the Bmpr1b variants in pCS2+ together with the BRE luciferase reporter construct BRE-pLG3ti [14] and the Renilla luciferase normalization vector pRL-TK (Promega). After 18 h cells were stimulated with 2 nM human recombinant GDF5 (Biopharm) in serum-reduced medium (DMEM 4.5 g/l glucose (Lonza), 1 % FBS superior (Biochrom), 2 mM L-glutamine (Lonza)).
40 h after transfection cells were lysed in potassium phosphate buffer (9 mM potassium di-hydrogen phosphate, 91 mM di-potassium phosphate, 0.2 % Triton-X-100) and dual luciferase activity was measured as described previously [15] using the Mithras LB 940 (Berthold Technologies). For statistical analysis GraphPad Prism 5 (GraphPad Software, Inc.) was used.

Confocal microscopy
NIH/3 T3 cells were seeded into a 24-well plate in growth medium on cover glasses (Marienfeld). 24 h later Bmpr1b expression vectors were transfected into the NIH/3 T3 cells using Lipofectamine 2000 (Invitrogen, Life Technologies) following the manufacturer’s instructions. Another 24 h later cells were incubated under serum free conditions (DMEM 4.5 g/l glucose (Lonza), 2 mM L-glutamine (Lonza)) for 1 h, subsequently fixed with 4 % paraformaldehyde in PBS and blocked in PBS containing 10 % FBS superior (Biochrom) overnight. Non-permeated cells were incubated with a rabbit anti-HA antibody (H6908, Sigma-Aldrich; diluted 1:100 in PBS containing 10 % FBS superior (Biochrom)) for 1 h. After washing with PBS the cells were incubated with
the secondary antibody anti-rabbit-Alexa Fluor 488 (A11008, Molecular Probes Life Technologies) and with DAPI (Invitrogen, Life Technologies) for 1 h. The cover glasses containing stained cells were mounted on microscope slides (‘SuperFrost Plus’, Menzel) using Fluoromount-G (Southern Biotech). Confocal microscopy was performed using Zeiss Axio Imager.M2 equipped with a LSM700 confocal module (Carl Zeiss, 63-fold magnification).

Results
Clinical evaluation
The proposita was a 38 year-old woman who presented to the genetics clinic because of short stature and limb anomalies. She is the 11th child (of 11) born to first cousin parents originating from Morocco (Fig. 1a). One of her sisters and two of her brothers are similarly affected; they live in a remote area of Morocco with very limited access to medical care and no detailed clinical information, skeletal radiographs or DNA were available from them. The parents were unaffected; maternal height was 160 cm and paternal height 180 cm.

The proposita had disproportionate short stature with a height of 148 cm (−2.7 SD) and mild acromesomelic limb shortening. The fibulae were not palpable. She had short fingers and abnormal finger joints with deviations (Fig. 1b). Fingers II and V were most severely affected. Finger nails were normal. The toes were hypoplastic, in particular toes III to V, with broad and short toe nails (Fig. 1b). Radiological examination for the hands revealed proximal symphalangism and dysplastic middle phalanges (absence of the middle phalanx in the index finger, hypoplasia of the middle phalanges with narrow proximal phalangeal joints in the middle and ring fingers, and absence of the middle phalanx and long proximal phalanx in the little finger), shortening of the first metacarpal and a single long phalanx in the thumb, short proximal phalanx of the index finger and narrow carpal joint spaces (Fig. 1c). Radiographs of the feet showed similar findings, including a single misshapen phalanx in the great toe, hypoplasia of the middle phalanx in the second toe, absence of the middle phalanges in the third, fourth, and fifth toes, and calcaneocuboid...
fusion (Fig. 1c). The ankle joints were mal-aligned. The fibula was totally missing (Fig. 1c).

The proposita reported normal puberty with menarche at 13 years and regular menstrual cycles. Abdominal ultrasound was normal with the presence of a uterus and ovaries of normal size and shape. There was no hypergonadotropic hypogonadism with normal LH, FSH, estradiol and progesterone.

**Mutation identification**

Because the clinical presentation was compatible with du Pan dysplasia we first sequenced the coding region and exon-intron boundaries of GDF5 and identified no mutation. Upon sequencing of BMPRIB we detected a homozygous c.91C>T (p.Arg31Cys) variant (Fig. 1d). No DNA from other affected and unaffected family members was available to test for co-segregation with du Pan dysplasia. Consistent with BMPRIB being located in an autozygous region, genotyping of two highly polymorphic BMPRIB intragenic STRs showed homozygosity and SNP array genotyping confirmed a large (20.2 Mb) homozygous region at the BMPRIB locus in the proposita, defined by the flanking SNPs rs2131361 and rs17278473 (data not shown). Residue Arg31 is moderately conserved in BMPRIB orthologs. It is conserved e.g., in mouse, rat, cat, dog, and platypus; it is replaced with a histidine in e.g., green monkey, panda and hedgehog, and with a glutamine in e.g., opossum and turtles. None of the >60 species included in the UCSC multiple species alignment has a cysteine at the corresponding position. While the ExAC browser (http://exac.broadinstitute.org/) lists a p.Arg31His substitution (rs200035802) with an allele frequency of 18/121.162, p.Arg31Cys was only identified in 1/121.150 alleles, compatible with ACD du Pan type in our patient (Fig. 3a). We compared its activity to the previously described BMPRIB mutation Cys53Arg, which is associated with ACD Grebe type and to Arg31His, another allele listed in genomic databases [9]. The WT receptor induced the luciferase expression approximately 8-fold compared to a control vector and was strongly stimulated by the presence of recombinant GDF5. BMPRIB Arg31Cys showed only moderate activity when no ligand was used for stimulation (less than 2-fold). Arg31His induced the SMAD signaling pathway approximately 5-fold, but remained significantly less active than the WT receptor. SMAD1/5/8 activation via the BMPRIB variants Arg31His and Arg31Cys was significantly increased upon ligand stimulation. However, BMPRIB Arg31Cys still could not accomplish the level of BMPRIB Arg31His or BMPRIB WT, pointing to a partial loss of function. In line with previous findings BMPRIB Cys53Arg exhibited no SMAD activity and could not be stimulated by GDF5 either. Confocal microscopy showed that all investigated BMPRIB variants are translocated to the cell surface (Fig. 3b).

**Functional analyses of mutant BMPRIB receptors**

We performed a luciferase reporter gene assay using the SMAD dependent BRE reporter to determine the signal transduction capacity of the BMPRIB variant Arg31Cys, which is associated with ACD du Pan type in our patient (Fig. 3a). We compared its activity to the previously described BMPRIB mutation Cys53Arg, which is associated with ACD Grebe type and to Arg31His, another allele listed in genomic databases [9]. The WT receptor induced the luciferase expression approximately 8-fold compared to a control vector and was strongly stimulated by the presence of recombinant GDF5. BMPRIB Arg31Cys showed only moderate activity when no ligand was used for stimulation (less than 2-fold). Arg31His induced the SMAD signaling pathway approximately 5-fold, but remained significantly less active than the WT receptor. SMAD1/5/8 activation via the BMPRIB variants Arg31His and Arg31Cys was significantly increased upon ligand stimulation. However, BMPRIB Arg31Cys still could not accomplish the level of BMPRIB Arg31His or BMPRIB WT, pointing to a partial loss of function. In line with previous findings BMPRIB Cys53Arg exhibited no SMAD activity and could not be stimulated by GDF5 either. Confocal microscopy showed that all investigated BMPRIB variants are translocated to the cell surface (Fig. 3b).

**Discussion**

We have identified the homozygous BMPRIB missense mutation p.Arg31Cys in a patient with ACD du Pan type. Previously, GDF5 mutations have been shown to constitute the major cause of ACD Grebe and du Pan types [3–6], and BMPRIB mutations had been identified in single families with Grebe syndrome and a Grebe dysplasia-like phenotype with genital anomalies [8, 9]. However, whether BMPRIB mutations, presumably mutations with milder functional effects, can also cause du Pan dysplasia was unknown. The following evidence supports a diagnosis of du Pan dysplasia in the proposita.
rather than another ACD or ACD-like skeletal dysplasia: normal appearing radius, ulna and femur including the corresponding joints; mal-shaped but present phalangeal bones of the hands; normal length of the second metacarpal; and milder involvement of the tarsals, metatarsals, and phalangeal bones of the toes. In Grebe and Hunter-Thompson ACD the clinical and radiological manifestations are more severe: the humerus, radius and ulna are short and bowed, the hand bones are more deformed or missing and the elbow and knee joints are affected. Thus,

![Fig. 2](image)

Fig. 2 3D models for Arg31 substitutions in human BMPR1B. a Structure of the extracellular domain of BMPR1B (green) bound to GDF5 (grey). Position 31 is indicated with the C-atoms colored in magenta. b Model of the BMPR1B variant Arg31Cys. The unpaired cysteine residue located in β-strand 1 is highlighted in magenta; the five disulfide bonds formed in BMPR1B WT are shown as yellow sticks and are marked accordingly. c As in (b) but with an alternative disulfide bond network. The close proximity of the introduced cysteine residue at position 31 to the native residues Cys32, Cys47, Cys53, and Cys71 potentially leads to an alternative connectivity, which could then alter the conformation of the loops. If Cys31 connects to Cys47 an alternative non-native disulfide bond between Cys34 and Cys71 might be formed, which alters the conformation of the β1β2-loop important in the recognition and binding of BMP ligands. d Model of the BMPR1B variant Arg31His. The shorter histidine side chain places the hydrogen bond donor and acceptor groups of the imidazole ring in close proximity to Ser48 and Asn100 allowing to form new hydrogen bonds with the latter two residues thereby possibly altering the conformation of β-strand 2 and the β1β2-loop.

![Fig. 3](image)

Fig. 3 The Arg31Cys substitution in BMPR1B causes a moderate loss of function. a NIH/3 T3 cells were co-transfected with the empty vector pCS2+ or the indicated HA-tagged variants of Bmpr1b with the luciferase constructs BRE-pLG3ti and pRL-TK and stimulated with 2 nM of human recombinant GDF5. BMPR1B WT strongly induced luciferase activity. The variant BMPR1B Arg31Cys showed nearly no activity without ligand stimulation. It was activated after GDF5 treatment but could not accomplish the level of BMPR1B WT. Arg31His induced signaling, but considerably lower compared to BMPR1B WT. BMPR1B Cys53Arg did not activate SMAD signaling at all. Data were tested for normal distribution (Kolmogorov-Smirnoff normality test) and analyzed using a One-Way-ANOVA with subsequent Bonferroni’s Multiple Comparison Test (n = 8; ns not significant, *** p < 0.001). b NIH/3 T3 cells were transfected with the indicated HA-tagged Bmpr1b variants. Using an anti-HA antibody the expression of BMPR1B could be visualized via confocal microscopy. DAPI was used for staining of cell nuclei. All BMPR1B variants were translocated to the cell membrane.
our results show that both Grebe and du Pan dysplasia can be caused by BMPR1B mutations in rare cases. While the number of affected individuals with ACD due to BMPR1B mutations is small, our previous [9] and present results indicate a possible genotype-phenotype correlation according to which BMPR1B mutations with a strong functional effect would cause Grebe syndrome, while milder mutations would result in the clinically and radiologically milder du Pan dysplasia.

Indeed, when the Arg31Cys mutation or the Arg31His variant are compared with other BMPR1B mutations, e.g., the Cys53Arg exchange [9], the assumed impact of a missense substitution at position 31 on structure and BMP binding seems rather moderate. The substitution of Cys53 not only disrupts the structure-stabilizing disulfide bond network, but also likely causes local unfolding in the center of the BMPR1B ligand-binding domain due to strong van der Waals overlaps from introducing a bulky, charged arginine side chain into the tightly packed hydrophobic core around Cys53. In contrast, the side chain of Arg31 faces the protein surface being surrounded by bulk solvent. Thus, exchanges against most other amino acid types will likely not alter the structure of the BMPR1B ligand-binding domain. Furthermore, the residue at position 31 does not contact GDF5, thus only amino acid substitutions that (indirectly) cause a conformational rearrangement of ligand-binding loops can affect BMP binding.

A luciferase reporter gene assay indeed revealed that both BMPR1B variants, Arg31Cys and Arg31His, can still be stimulated by GDF5 but signal with diminished biological activity compared to BMPR1B WT. This suggests that the exchanges at position 31 have for that matter only moderate functional effect and do not completely prevent ligand binding, as it is the case for Cys53Arg.

Conclusion
In conclusion, we provide evidence for a causal role of a hypomorphic BMPR1B missense mutation in du Pan dysplasia, thereby broadening the clinical and molecular overlap between ACD types. This finding also has obvious implications for molecular diagnostic strategies, as BMPR1B sequencing should be considered in patients with ACD harboring no mutations in GDF5.

Competing interests
The authors declare that they have no competing interests.

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