A Novel *GDF6* Mutation in a Family with Multiple Synostoses Syndrome without Hearing Loss

Ragnhild Drage Berentsen^a Bjørn I. Haukanes^a Pétur B. Júlíusson^{b, d}
Karen Rosendahl^c Gunnar Houge^a

Departments of ^aMedical Genetics, ^bPediatrics, and ^cRadiology, Haukeland University Hospital, and
^dDepartment of Clinical Science, University of Bergen, Bergen, Norway

Keywords

GDF6 · Hearing loss · Multiple synostoses syndrome

Abstract

A 4-generation family with multiple synostoses syndrome type 4 (SYNS4) is reported, the third family identified so far. The phenotype segregated with a previously undescribed Asn399Lys (c.1197C>A) substitution in *GDF6*. N399 is part of a hydrophobic pocket critical for binding the BMP/GDF antagonist noggin. The N399K substitution renders GDF6 more similar to noggin-resistant members of the BMP family, namely GDF2 and BMP10, both of which contain lysine in the corresponding position. To further define the SYNS4 phenotype, we examined 6 of 9 affected family members. The phenotype was carpal and tarsal synostoses with painful feet after walking, but the condition could also be asymptomatic. Interestingly, unlike the previous SYNS4 families, the family presented here has no history of hearing loss, and a 73-year-old mutation carrier had normal audiometry for his age. Based on structure modelling, BMPR2 binding should not be affected by the GDF6-N399K substitution, unlike the S429R and Y444N mutations found in the 2 other families. Hypothetically, this difference may be related to lack of hearing loss.

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Multiple synostoses syndromes are a group of autosomal dominant disorders characterized by multiple joint fusions in hands, feet and/or fingers, and sometimes also joint fusions affecting larger joints such as elbows, hips, and vertebrae. Other findings can be brachydactyly, conductive hearing loss, and dysmorphic facial features [Potti et al., 2011]. Mutations in *NOG* (noggin, causing multiple synostoses syndrome type 1 – SYNS1; OMIM 602991), *GDF5* (growth/differentiation factor 5, causing SYNS2; OMIM 610017), and *FGF9* (fibroblast growth factor 9, causing SYNS3; OMIM 612961) have been identified in multiple synostoses syndromes with overlapping skeletal findings. *NOG* and *GDF5* are genes involved in bone morphogenetic protein (BMP) signaling. All BMPs share a common signaling mechanism: dimeric growth factors (BMPs or GDFs) bind to type I and type II BMP receptors (BMPR1 and BMPR2) resulting in a heterotetrameric receptor complex assembly, which further activates downstream targets [Yadin et al., 2016]. The homodimer noggin, encoded by *NOG*, is a BMP/GDF antagonist that acts as a ligand trap by binding to BMP/GDFs and thereby blocking their BMP-receptor interac-

R.D.B. and B.I.H. share first authorship.

Fig. 1. Family pedigree with cosegregation results of the *GDF6* c.1197C>A heterozygous variant.

tion sites [Groppe et al., 2002]. Increased BMP signaling may be caused by loss-of-function mutations in *NOG* or gain-of-function mutations in the BMP-receptor ligand *GDF5*. Different *NOG* mutations are associated with multiple synostoses syndrome type 1, proximal symphalangism type 1A, tarsal-carpal coalition syndrome, and stapes ankylosis with broad thumbs and toes [Gong et al., 1999; Dixon et al., 2001; Brown et al., 2002; Groppe et al., 2002]. They all constitute a group of overlapping syndromes, suggested to be called *NOG*-related symphalangism spectrum disorders [Potti et al., 2011]. *GDF5* missense mutations associated with noggin resistance cause the dominant multiple synostoses syndrome type 2 and proximal symphalangism type 1B [Dawson et al., 2006; Seemann et al., 2009; Schwaerzer et al., 2012].

A novel type of multiple synostoses syndrome was recently described numbered type 4 (SYNS4). SYNS4 is caused by gain-of-function mutations in *GDF6*, encoding growth/differentiation factor 6 [Wang et al., 2016; Terhal et al., 2018]. The SYNS4 phenotype comprises variable carpal and tarsal synostoses, and some patients have otosclerosis and conductive hearing loss. Just as *GDF5*, *GDF6* is a secreted growth factor of the BMP family that plays an important role in joint formation [Settle et al., 2003]. Since increased BMP signaling of various causes is associated with other multiple synostoses syndromes, noggin resistance of mutant *GDF6*s is postulated to be the major cause of SYNS4 [Wang et al., 2016; Terhal et al., 2018]. Other missense mutations in *GDF6* have been re-

ported in Klippel-Feil syndrome type 1, Leber congenital amaurosis type 17, and isolated microphthalmia [Tassa-behji et al., 2008; Asai-Coakwell et al., 2009], but since several of these missense mutations are frequently found in normal control populations, their contribution to the etiology of these conditions is doubtful [Terhal et al., 2018].

We have identified a third family with SYNS4, caused by a novel *GDF6* missense mutation Asn399Lys (= N399K) in a residue predicted to interact with noggin and BMPRI1, but not BMPRI2. The skeletal phenotype of symmetrical carpal and tarsal synostoses is similar to the skeletal phenotype found in the 2 previous families [Wang et al., 2016; Terhal et al., 2018]. However, unlike these families, none of the 9 affected individuals in our family have mechanical hearing loss, and we speculate that this may be related to *GDF6*-N399K having no effect on receptor dimerization.

Clinical Report

In a 4-generation family, 6 of 9 affected individuals with similar and bilateral fusions of carpal and tarsal bones were examined physically and radiologically (Fig. 1). Apparently healthy individuals were not examined. The proband (IV-3 in Fig. 1), a 10-year-old girl, was referred to our skeletal dysplasia clinic at age 8 years because of painful feet after walking longer distances and tarsal bone fusion on X-ray. Her mother, older brother, maternal grandfather, maternal uncle and cousin, as well as more distant relatives, had similar radiological findings, indicating fully penetrant autosomal

Fig. 2. Clinical pictures and radiographs of the patients. **a** Normal hand of patient IV-4 at the age of 14 years. **b** Brachydactyly in adult patient III-4. **c** Broad forefeet, overlapping toes, and pes planus in adult patient III-3. **d** Cutaneous syndactyly between the 2nd and 3rd toes (bilateral) and pes planus in patient IV-2 at the age of 13 years. **e** Bilateral broad forefeet and overlapping toes in adult patient III-4. **f–h** Radiograph of the right hand in patient IV-4 at the age of 14 years (**f**) and patient IV-2 at the age of 13 years and 2 months (**g**), and of both hands in patient II-3 at the age of 73 years (**h**). There is fusion of the trapezoid, capitate, and scaphoid bones, and fusion of the lunate and triquetrum in patients IV-2 and II-3 (**g, h**). **i** Radiograph of the left foot in proband IV-3 at the age of 7 years and 5 months. The talus and the navicular bones are fused and also in part fused with the intermediate and the lateral cuneiforms. The 3rd tarsometatarsal joint is fused, and the 2nd and 4th tarsometatarsal joints are partially fused; the middle and distal phalanges of the 5th toe are also fused. Note the cone epiphyses to the proximal phalanges of the 2nd to 4th toes and the hypoplastic middle and distal phalanges. **j** Radiograph of the left foot in patient

IV-2 at the age of 6 years and 7 months with fusion of the talus, navicular, intermediate and lateral cuneiform bones. Note the narrowed 4th tarsometatarsal joint space, the fused middle and distal phalanges of the 5th toe, the hypoplastic mid- and distal phalanges, and the cone epiphysis in the proximal phalanges of the 2nd–4th toes. **k** Radiograph of the left foot in the same patient IV-2 at the age of 12 years. Note that the 4th tarsometatarsal joint is now completely fused. **l** Radiograph of the left foot in patient III-3 at the age of 49 years with fusion between the talus, navicular, intermediate, and lateral cuneiforms as well as the cuboid bones. The 2nd–4th tarsometatarsal joints are fused, and there are fused middle and distal phalanges in the 5th toe and hypoplastic mid- and distal phalanges. Also note the rather short proximal phalanges of the 2nd–4th toes, most likely due to premature fusion of cone epiphyses. **m** Radiograph of the left foot in asymptomatic patient II-3 at the age of 73 years with fusion between the talus, navicular, calcaneus, intermediate, and lateral cuneiforms as well as the cuboid bones. Note fused middle and distal phalanges of the 5th toe, hypoplastic mid- and distal phalanges and rather short proximal phalanges of the 2nd–4th toes.

Fig. 3. a Interaction with inhibitor BMP7-NOG (PDB ID: 1M4U) [Groppe et al., 2002] and **b** receptor GDF5-BMPR1B (PDB ID: 3EVS) [Kotzsch et al., 2009]. Aspargines equivalent to GDF6 Asn399 are colored red, BMPR1B (type I BMP receptor B) Glu(E)105 is colored blue, and Noggin (NOG) Pro(P)35 is colored magenta. Protein structures were visualized and interpreted using PyMOL v2.0.3. **c** Protein multiple sequence alignment was generated using Clustal Omega v1.2.4. Equivalent Asn(N)-residues in red and amino acids identical to the GDF6 sequence on a yellow background. Reference sequences: *GDF6* (NM_001001557.3), protein NP_001001557.1 (455 AA); *GDF5* (NM_000557.4), protein NP_000548.2 (501 AA); *BMP7* (NM_001719.2), protein NP_001710.1 (431 AA); *GDF2* (NM_016204.3), protein NP_057288.1 (429 AA); *BMP10* (NM_014482.2), protein NP_055297.1 (424 AA).

dominant inheritance (Fig. 1). Physical examination of the proband showed bilateral pes planus, a mild valgus deformity of her ankles, and slightly reduced mobility of her feet. The mobility of other joints, including the wrists and fingers, appeared normal. Her stature, head circumference, facial features, vision, hearing, and intelligence were normal. Radiographs of the feet showed bilateral fusions between the talus and navicular, which was partially fused to the intermediate and the lateral cuneiforms, fused 3rd and partially fused 2nd and 4th tarsometatarsal joints, fused middle and distal phalanges of the 5th toe, cone epiphysis in the proximal phalanges of the 2nd to 4th toes, and hypoplastic middle and distal phalanges (Fig. 2i). Radiographs of the hands revealed bilateral fusions between the capitate and trapezoid bones (with a small adjacent scaphoid) and between the lunate and triquetrum. Radiographs of spine, pelvic bones, elbows, shoulders, ribs, and skull were normal.

Other affected family members had similar symmetrical fusions of carpal and tarsal bones (Fig. 2f–h, j–m) (online suppl. Table 1; see www.karger.com/doi/10.1159/000492418 for all online suppl. material). All but one presented with mildly diminished ankle and foot mobility and limited ability to walk long distances because of pain. In the neonatal period, individual III-3 (Fig. 2c, l) was diagnosed with pes equinovarus, and the left foot was treated with braces and a cast for a year. At the age of 15, she underwent an osteotomy of the right talonavicular joint. Individual II-3 (Fig. 2h, m) was asymptomatic, i.e., without foot pain or reduced walking ability, and he also had an ophthalmology examination that was normal. All patients had broad forefeet with overlapping toes. Their hands appeared normal, except for mild brachydactyly in individual III-4 (Fig. 2b). Individual IV-2 had bilateral cutaneous syndactyly between the 2nd and 3rd toes (Fig. 2d). No dysmorphic facial features were noted, statures were normal, and joint mobility was only restricted in the feet. None of the patients had any functional disability or pain in the wrists. A complete skeletal

survey in individual IV-2 was normal, apart from the fusions of carpal and tarsal bones. Similarly, radiographs of the spine in individual III-3 were normal. Fusions of finger joints were never observed or reported.

Audiometric testing in individual II-3 at the age of 73 years revealed a sensorineural hearing loss with an average pure-tone hearing threshold of 15 dB in the right ear and 13 dB in the left ear, with a symmetrical downward slope in the high-frequency range typical for presbycusis, i.e., a normal finding in a 73-year-old male. Audiometric testing was not indicative of otosclerosis or stapes fixation. Since none of the other patients complained of hearing loss, audiometry was not performed.

Methods and Results

Whole-exome sequencing was performed for individual IV-3 (proband) using the SeqCap EZ MedExome Target Enrichment System (Roche Life Science) followed by sequencing on the Illumina NextSeq 500 platform with NextSeq 500/550 High Output v2 kit (Illumina, San Diego, CA, USA). Variants were bioinformatically filtered in the NGS module of Cartagenia Bench (Agilent Technologies, CA, USA) using a gene panel consisting of 196 genes related to skeletal dysplasias, including *NOG*, *GDF5*, *FGF9*, and *GDF6*. A novel heterozygous missense variant, c.1197C>A (p.As399Lys), was detected in *GDF6* (NM_001001557.2). Results were verified by Sanger sequencing, and subsequent DNA-analyses in 5 of 9 affected family members showed that the variant cosegregat-

Fig. 4. Illustration of GDF/BMP-receptor type 1 (BMPR1) and 2 (BMPR2/ACVR2) interactions based on PDB ID 2H64 [Weber et al., 2007]. **a** BMPR2 (marked Rt 2: cyan) and BMPR1 (Rt 1: blue) with GDF/BMP chain **a** (green) “fingers” in between. Dorsally on “fingers” towards BMPR2: GDF6-S429/GDF5-S475 (orange spheres), and ventrally on “fingers” towards BMPR1: GDF6-Y444/GDF5-Y490 (wheat-colored spheres). GDF6-N399/GDF5-N445/BMP7-N375 (red spheres) are located in the “wrist” domain. **b** As in **a**, but illustrating GDF/BMP chain **b** [Groppe et al., 2002] instead of chain **a**. As seen in **a** and **b**, GDF/BMP “fingers” are interacting with both receptor types. Amino acids substitutions destabilizing the “fingers” may affect the ability to dimerize BMPR1 with BMPR2. **c** Assembly of **a** and **b** (rotated). GDF/BMP chain **a** “fingers” are in close proximity with the chain **b** “wrist” interacting with the same receptor type 1 subunit (Rt-1, blue). Protein structures were visualized and interpreted using PyMOL v2.0.3.

ed with the multiple synostoses syndrome phenotype (Fig. 1). Asparagine at codon 399 is conserved from *Caenorhabditis elegans* to humans and is located in the conserved C-terminal TGF β domain. GDF6-N399K is not reported in the gnomAD variant database (<http://gnomad.broadinstitute.org/>) and is predicted to be pathogenic by the computer programs Align GVGD (class C65), SIFT (deleterious, score: 0), MutationTaster (disease causing, *p* value: 1), and PolyPhen-2 (probably damaging, HumVar score: 0.999).

The GDF6 (BMP13) structure has not yet been reported, but since GDF6 is a paralogue of GDF5 and BMP7 (i.e., have virtually identical tertiary structures) [Hinck et al., 2016] with high sequence identity in the C-terminal TGF β domain, structural data of the BMP7-NOG complex (Fig. 3a, PDB ID:1M4U) and the GDF5-BMPR1B complex (Fig. 3b, PDB ID:3EVS) were used to visualize and interpret the interaction with inhibitor and receptor, respectively (PyMOL Molecular Graphics System, v2.0.3). GDF6-N399 (equivalent to GDF5-N445 and BMP7-N375) is predicted to interact with both BMP receptor type-1 and noggin (Fig. 3). In the BMP7-NOG complex (Fig. 3a), the equivalent BMP7-N375 is predicted to interact directly with the key noggin residue proline-35 in a hydrophobic pocket critical for noggin binding [Groppe et al., 2002]. Pro35-noggin missense mutations have been

found to cause tarsal-carpal coalition syndrome (P35R) [Dixon et al., 2001], proximal symphalangism type 1A (P35S) [Mangino et al., 2002], and brachydactyly type B2 (P35S and P35A) [Lehmann et al., 2007]. In the GDF5-BMPR1B complex (Fig. 3b), the equivalent GDF5-N445 is predicted to interact with BMPR1B-G106 and the negatively charged E105 [Kotzsch et al., 2009].

Structure and interpretation of GDF/BMP interactions with both type 1 (Rt-1/BMPR1) and type 2 receptor (Rt-2/BMPR2) is illustrated in Figure 4 (PDB ID 2H64) [Weber et al., 2007]. Both GDF6-S429/GDF5-S475 and GDF6-Y444/GDF5-Y490 are located in “fingers,” while GDF6-N399/GDF5-N445/BMP7-N375 is located in the “wrist” region interacting only with BMPR1 (Rt-1). Data from paralogous BMP ligand-inhibitor and receptor-ligand complexes thus suggest that GDF6-N399K inhibits noggin binding and affects BMPR1 binding, but not BMPR2 binding.

Discussion

We report 6 patients from a 4-generation family with a novel variant Asn399Lys in the *GDF6* gene causing carpal and tarsal synostoses without hearing loss. We consider this N399K missense variant to be pathogenic based

on amino acid conservation, results of prediction programs, absence in variant frequency databases like gnomAD, phenotypic similarities with the 2 other described families with SYNS4, and perfect cosegregation with disease in our family (Fig. 1). In addition, structural prediction strongly suggests that GDF6-N399K may affect noggin binding (Fig. 3a), in line with the notion that SYNS4 is caused by gain-of-function mutations making GDF6 noggin resistant [Wang et al., 2016; Terhal et al., 2018].

When comparing the carpal and tarsal synostoses in our patients to the 2 previously reported families, 1 Dutch and 1 Chinese [Wang et al., 2016; Terhal et al., 2018], the skeletal phenotype is highly similar. Synostoses in fingers, knees, elbows, or vertebrae have not been reported, which makes SYNS4 different from SYNS1, SYNS2, and SYNS3. In our family, the bony fusion of the carpal bones tended to occur in a sequential order (online suppl. Fig. 1), starting with the capitate and the trapezoid, both subsequently fusing with the scaphoid to form 1 of 2 major wrist bones. A fusion of the hamate, lunate, and triquetrum formed the second major wrist bone. The pisiform and the trapezium were not involved in any of the patients. One of the adult family members (individual II-3 in Fig. 1) was asymptomatic besides having broad forefeet and overlapping toes. This indicates that some patients with SYNS4 may be unaware of their carpal and tarsal synostoses because they are not painful or limit their walking distance.

Contrary to what has previously been observed, mechanical hearing loss has not been reported in our family. In the Chinese family [Wang et al., 2016], some of the patients had progressive conductive hearing loss after age 40, and in the Dutch family [Terhal et al., 2018], at least 6 of 10 patients had hearing loss, and severe stapes fixation was detected as early as age 6 years. Although we cannot completely rule out late-onset otosclerosis in our family, this seems unlikely as audiometric testing in individual II-3 at 73 years of age was not indicative of otosclerosis, and other affected family members (including II-1 and III-1) are reported to hear well.

Based on studies of the GDF6 paralogs GDF5 and BMP7, the N399K variant is predicted to interface either with noggin or the type-1 BMP receptor (Fig. 3), as previously reported for the GDF6-Y444N mutation [Seemann et al., 2009; Wang et al., 2016]. In the BMP7-NOG complex, the introduction of a positively charged lysine in the homologous BMP7-N375 position disrupts a hydrophobic pocket accommodating the key noggin residue Pro35 (Fig. 3a), i.e., providing a likely molecular explanation of noggin resistance in our family. GDF6-Y444N, found in

the Chinese SYNS4 family, is located in the same noggin-binding region of GDF6 and verified to be noggin resistant in functional studies [Wang et al., 2016]. Furthermore, GDF5-N445K, homologous to GDF6-N399K (Fig. 3c), was detected in a family with multiple synostoses syndrome and found insensitive to noggin inhibition in vitro [Seemann et al., 2009]. Finally, 2 other BMPs with high chondrogenic activity, GDF2 and BMP10, are naturally resistant to noggin [Seemann et al., 2009], and both have lysine (K) instead of asparagine (N) at the site corresponding to GDF6-N399 (Fig. 3c). Taken together, it seems clear that GDF6-N399K is noggin resistant, and this feature explains the carpal and tarsal synostoses.

When members of the BMP family of ligands bring BMP type 1 and 2 receptors together, the serine/threonine kinase of BMP receptor-2 (BMPR2) phosphorylates and activates BMP receptor-1 (BMPR1), and the serine/threonine kinase of BMPR1 in turns phosphorylates downstream targets like SMAD1/5/8, p38-mitogen-activated protein kinase (MAPK14), and phosphatidylinositol-3-kinase subunits [Katagiri and Tsukamoto, 2013]. Concerning the “alternative” binding of GDF6 to BMP receptor-1 instead of noggin, this is probably also affected by the N399K change. In the GDF5-BMPR1B complex, an N to K transition at the homologous GDF5-N445 position introduces a positive charge (K) that could affect interaction with the negatively charged BMPR1B-E105 (Fig. 3b) [Kotzsch et al., 2009]. Studies of alkaline phosphatase activity in C2C12 mouse cells expressing N445T mutant GDF5 suggested an altered signaling effect, but Biacore analysis did not reveal changes in BMPR1A or BMPR1B binding affinity [Seemann et al., 2009]. If GDF6-N399K affects BMPR-mediated signaling not only due to noggin resistance but also because of altered BMPR1 binding, is so far unknown, but noggin resistance is an adequate explanation of the phenotype.

The Dutch family with GDF6-S429R (corresponds to S475 in GDF5) had more severe hearing loss (stapes fixation at early age) than the Chinese GDF6-Y444N family. Both GDF6 mutations were all in the “fingers” important for both BMPR1 and BMPR2 interactions (Fig. 4a, b). GDF5-S475/GDF6-S429 are located at the “top” of the “fingers” and predicted to contribute to the BMPR2 interface, while the hydrophobic GDF6-Y444 is buried and located in the longer β -strand towards BMPR1. The increase in hydrophilicity caused by a Tyr to Asn substitution in GDF6-Y444N could destabilize the GDF6 “fingers” with effect on both BMPR1 and BMPR2 interactions. The GDF6-N399K substitution in our family is located in the “wrist” and has no obvious effect on the

dual function of the “fingers,” but contact receptor type-1 upon ligand dimerization (Fig. 4c).

Thus, mutations that destabilize the “fingers” have a greater potential for affecting not only noggin inhibition but also GDF6 ability to be a ligand for BMPR activation, and such a loss-of-function-like feature could be a major cause of hearing loss. Under such a hypothesis, missense *GDF6* mutations can be gain of function due to noggin resistance and thereby increased BMPR signaling, and loss of function due to diminished ability to dimerize BMPR1 with BMPR2 [Schwaerzer et al., 2012]. In our family, it may be that the mutation is noggin-resistant only.

In conclusion, we report the third family with SYNS4. This was due to a novel missense variant in a noggin-binding pocket of GDF6. We show that SYNS4 is a skeletal dysplasia with a consistent and highly penetrant tendency to form carpal and tarsal synostoses, and that such

synostoses can be asymptomatic. Our family suggests that mechanical hearing loss is not a compulsory part of SYNS4 and maybe related to affection of BMPR2 binding.

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Statement of Ethics

The authors have no ethical conflicts to declare.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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