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A mutation in the heparin-binding site of noggin as a novel mechanism of proximal symphalangism and conductive hearing loss ☆

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ABSTRACT

The access of bone morphogenetic protein (BMP) to the BMP receptors on the cell surface is regulated by its antagonist noggin, which binds to heparan-sulfate proteoglycans on the cell surface. Noggin is encoded by *NOG* and mutations in the gene are associated with aberrant skeletal formation, such as in the autosomal dominant disorders proximal symphalangism (SYM1), multiple synostoses syndrome, Teunissen–Cremers syndrome, and tarsal–carpal coalition syndrome. *NOG* mutations affecting a specific function may produce a distinct phenotype. In this study, we investigated a Japanese pedigree with SYM1 and conductive hearing loss and found that it carried a novel heterozygous missense mutation of *NOG* (c.406C > T; p.R136C) affecting the heparin-binding site of noggin. As no mutations of the heparin-binding site of noggin have previously been reported, we investigated the crystal structure of wild-type noggin to investigate molecular mechanism of the p.R136C mutation. We found that the positively charged arginine at position 136 was predicted to be important for binding to the negatively charged heparan-sulfate proteoglycan (HSPG). An *in silico* docking analysis showed that one of the salt bridges between noggin and heparin disappeared following the replacement of the arginine with a non-charged cysteine. We propose that the decreased binding affinity of *NOG* with the p.R136C mutation to HSPG leads to an excess of BMP signaling and underlies the SYM1 and conductive hearing loss phenotype of carriers.

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1. Introduction

Proximal symphalangism (SYM1, OMIM 185800) is a hereditary disease showing ankylosis of the proximal interphalangeal (PIP) joints and is frequently accompanied by conductive hearing loss; the disease results from mutations of *NOG* (OMIM 602991) [1] or *GDF5* (OMIM 601146) [2]. The noggin binds to bone morphogenetic protein (BMP) family, a subtype of the TGF-β superfamily, and antagonizes binding of these proteins to the BMP receptors (BMPRs) in a dose-dependent manner [3,4]; this interaction controls a cascade of developmental processes including morphogenesis and body patterning [4,5], middle ear formation [6,7], as well as chondrogenesis of the digits and interdigital apoptosis [6,8,9]. Another important feature of noggin for BMP-mediated morphogenesis is that the noggin–BMP complex associates with heparan-sulfate proteoglycan (HSPG), a major proteoglycan on the cell surface and in extracellular matrices [10], and is regulated by sulfatases to control the local activity of BMPs [11].

Mutations in *NOG* are associated with various autosomal dominant syndromes that are characterized by a spectrum of skeletal defects, conductive hearing loss, and synostoses, such as multiple synostoses syndrome (SYNS1, OMIM 186500) [1], Teunissen–Cremers syndrome (TCS, OMIM 184460) [12,13], tarsal–carpal

Abbreviations: ABR, auditory brainstem response; BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor; CT, computed tomography; DIP, distal interphalangeal; DPOAE, distortion product otoacoutic emission; HSPG, heparan-sulfate proteoglycan; PIP, proximal interphalangeal; SYM1, proximal symphalangism; SYNS1, multiple synostoses syndrome; TCC, tarsal–carpal coalition syndrome; TCS, Teunissen–Cremers syndrome.

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coalition syndrome (TCC, OMIM 186570) [14,15], Fibrodysplasia ossificans progressive (FOP, OMIM 135100) [16], Brachydactyly type B (BDB, OMIM 113000) [17], and Stapes ankylosis with broad thumb and toes (OMIM 184460) [18]. According to a previous *in vitro* study, *NOG* with p.P35R mutation has a diminished affinity for BMP-7 due to reduced hydrophobic interaction [19]. Each noggin with mutation of p.G189C, p.W217G, or p.P223L is either poorly or not secreted in cultured mammalian cells [20], indicating a defect in protein folding and maturation.

Mutation-induced structural change of a protein and prediction of its pathogenicity can be explored through molecular modeling. This approach is effective especially when examining structure of molecules that are extremely difficult to be crystallized, for instance, proteins associated with structurally mobile sugar chain such as heparan-sulfate, whereas it can be investigated by docking simulations.

Here, we report a patient with a novel heterozygous missense mutation (c.406C > T) in *NOG*, which replaces the arginine residue at position 136 with a cysteine (p.R136C) in the heparin-binding site of noggin. A simulation of the binding mode of the mutated noggin to heparin successfully predicted its molecular dysfunction. We propose a novel molecular mechanism for the etiology of the SYM1 phenotype: *NOG* with the p.R136C mutation allows excess BMP signaling through a decreased binding affinity of the noggin–BMP complex to HSPG on cell surfaces and leads to aberrant bone formation in PIP joints and the middle ear.

2. Materials and methods

2.1. Ethics statements

All the procedures were approved by the Ethics Review Committee of National Mie Hospital and National Tokyo Medical Center, and were conducted only after written informed consent had been obtained from each individual or from the parents of the children.

2.2. Subjects

Medical history and results of clinical examination (physical, audiological, and radiological) were obtained in four members of a Japanese SYM1 family, three affected (proband, old sister, and father) and one unaffected (mother). Audiological examinations included pure tone audiometry, distortion product otoacoustic emission (DPOAE) test, tympanometry, and stapedial reflex test. Radiological examinations included X-rays of hand and feet and computed tomography (CT) of temporal bones. Genetic analysis was also conducted in the four family members. The normal controls comprised 183 subjects who had healthy hands, feet, and normal hearing level according to pure tone audiometry. A comprehensive family history was obtained by interviewing parents of the proband.

2.3. Genetic analysis

Genomic DNA was extracted from blood samples using the Gentra Puregene Blood kit (QIAGEN, Hamburg, Germany), and primers specific for *NOG* (GenBank NG_011958.1) were designed. For PCR amplification of *NOG* exon, the primer sets 5′-tgtaaaacgacggccagtgTGCGGCCAACTTGTGTGCCTTTCT-3′ and 5′-caggaaacagctatgaccACAGCCACATCTGTAACTTCCTCC-3′, and 5′-tgtaaaacgacggccagtgGCCATGCCGAGCGAGATCAAAGG-3′ and 5′-caggaaacagctatgaccAGAGGGTGGTGGAACTGGTTGGAGGC-3 were used with the PC-818 Program Temp Control System (ASTEC, Shizuoka, Japan). Each primer is specific for a given genomic sequence (upper case) and was used in combination with either a universal forward M13 (lower case) or reverse M13pUC (lower case) primer. The following PCR program was used: 98 °C for 10 min; 37 cycles of 98 °C for 10 s, 62 °C for 10 s, and 72 °C for 1 min; and then 72 °C for 3 min. PrimeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan) was used for the PCR. The amplicons were sequenced using the ABI 3730 DNA sequence analyzer with the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). Characterization of the sequences was undertaken using SeqScape software v2.6 (Applied Biosystems) and DNASIS Pro (Hitachisoft, Tokyo, Japan) with the *NOG* sequence (NG_011958.1, NCBI Build37.1) as the reference. Control DNA was obtained from 96 Japanese subjects with normal hearing. To study amino acid conservation among different species, noggin orthologs from the chimpanzee (XP_523802), mouse (NP_032737), rat (XP_343955), cow (XP_582573), chicken (NP_989454), African clawed frog (AAI69670), and zebrafish (NP_571058) were compared by ClustalW [21].

2.4. Docking study of noggin and heparin

The crystal structure of wild-type noggin (PDB: 1M4U) [19] was used as the input data for the receptor molecule in the docking study and as a template for modeling the structure of the p.R136C mutant. The p.R136C mutant and the missing residues in the PDB file of wild-type noggin were modeled using the Swiss-Model modeling server [22]. The pentasaccharide (fondaparinux) in the crystal structure of the antithrombin-S195A factor Xa-pentasaccharide complex (PDB: 2GD4) [23] is very similar to naturally-occurring heparin. Hence, this pentasaccharide structure was used here as a substitute for naturally-occurring heparin. Rigid body docking of heparin (ligand) to noggin (receptor protein) was performed using AutoDock4.2 [24]. The noggin–heparin complex conformation that scored highly in terms of van der Waals and electrostatic interactions was selected. Energy optimization was then performed for the conformation using Discovery Studio 3.1 (Accelrys Inc., San Diego, Ca). All structural figures were generated with Chimera [25]. Electric surface potentials were rendered with Pov-Ray [26].

3. Results

3.1. Clinical features of the family

The family included five individuals with SYM1 (Fig. 1A). The proband (IV:6) was an 10-year-old daughter of non-consanguineous Japanese parents. She had limited mobility of the second to fifth fingers and toes bilaterally at birth. Photographs and radiographs of her hands and feet indicating ankylosis of the PIP joints and the distal interphalangeal (DIP) joints are shown (Fig. 1B–F). She did not have hyperopia or strabismus, and her face was not dysmorphic. From age 8 to 10, bilateral, low-frequency, progressive hearing loss was identified (Fig. 1G and Supplementary Fig. S1A). DPOAE tests showed abnormal response in both ears (Supplementary Fig. S1B). The stapedial reflex was absent in both ears (Fig. 1H). CT of the temporal bones revealed no abnormalities (data not shown). These audiological and radiological findings suggested stapes ankylosis. Her father (III:5) and elder sister (IV:5) also had ankylosis of the PIP joints and conductive hearing loss (Supplementary Fig. S1C–G).

3.2. Results of genetic analysis

The constellation of clinical features in this family was consistent with a diagnosis of SYM1, and prompted us to carry out a screen for mutations of *NOG*. Sequence analysis of the *NOG*

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