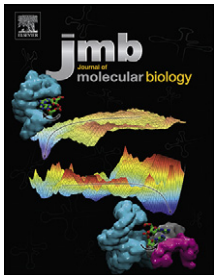

Molecular Analysis of Two Novel Missense Mutations in the GDF5 Proregion That Reduce Protein Activity and Are Associated with Brachydactyly Type C

Katja Stange[1,2], Tino Thieme[3], Karen Hertel[3], Silke Kuhfahl[3], Andreas R. Janecke[4,5], Hildegunde Piza-Katzer[6], Maila Penttinen[7], Marja Hietala[7], Katarina Dathe[8], Stefan Mundlos[1,8,9], Elisabeth Schwarz[3] and Petra Seemann[1,2]

*1 - **Berlin Brandenburg Center for Regenerative Therapies,** Charité–Universitätsmedizin Berlin, 13353 Berlin, Germany*
*2 - **Berlin Brandenburg School for Regenerative Therapies,** Charité–Universitätsmedizin Berlin, 13353 Berlin, Germany*
*3 - **Martin-Luther-University Halle-Wittenberg,** Technical Biochemistry, 06120 Halle, Germany*
*4 - **Department of Pediatrics I,** Innsbruck Medical University, 6020 Innsbruck, Austria*
*5 - **Division of Human Genetics,** Innsbruck Medical University, 6020 Innsbruck, Austria*
*6 - **Department of Plastic,** Reconstructive and Aesthetic Surgery, Innsbruck Medical University, 6020 Innsbruck, Austria*
*7 - **Department of Clinical Genetics,** Turku University Hospital, 20014 Turku, Finland*
*8 - **Institute for Medical Genetics and Human Genetics,** Charité–Universitätsmedizin Berlin, 13353 Berlin, Germany*
*9 - **Research Group Development and Disease,** Max-Planck-Institut für Molekulare Genetik, 14195 Berlin, Germany*

***Correspondence to Petra Seemann:** Berlin Brandenburg Center for Regenerative Therapies, Charité–Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany. petra.seemann@charite.de*
http://dx.doi.org/10.1016/j.jmb.2014.07.029
Edited by M. Yaniv

Abstract

Growth and differentiation factor 5 (GDF5) plays a central role in bone and cartilage development by regulating the proliferation and differentiation of chondrogenic tissue. GDF5 is synthesized as a preproprotein. The biological function of the proregion comprising 354 residues is undefined. We identified two families with a heterozygosity for the novel missense mutations p.T201P or p.L263P located in the proregion of GDF5. The patients presented with dominant brachydactyly type C characterized by the shortening of skeletal elements in the distal extremities. Both mutations gave rise to decreased biological activity in *in vitro* analyses. The variants reduced the GDF5-induced activation of SMAD signaling by the GDF5 receptors BMPR1A and BMPR1B. Ectopic expression in micromass cultures yielded relatively low protein levels of the variants and showed diminished chondrogenic activity as compared to wild-type GDF5. Interestingly, stimulation of micromass cells with recombinant human proGDF5^{T201P} and proGDF5^{L263P} revealed their reduced chondrogenic potential compared to the wild-type protein. Limited proteolysis of the mutant recombinant proproteins resulted in a fragment pattern profoundly different from wild-type proGDF5. Modeling of a part of the GDF5 proregion into the known three-dimensional structure of TGFβ1 latency-associated peptide revealed that the homologous positions of both mutations are conserved regions that may be important for the folding of the mature protein or the assembly of dimeric protein complexes. We hypothesize that the missense mutations p.T201P and p.L263P interfere with the protein structure and thereby reduce the amount of fully processed, biologically active GDF5, finally causing the clinical loss of function phenotype.

Introduction

Growth and differentiation factor 5 (GDF5) acts as a key player during skeletal development due to its ability to regulate cartilage formation. *GDF5* is expressed in prechondrogenic aggregates and is an early marker for the joint forming regions. The promotion of cell assembly increases the size of these condensations. In later stages of cartilage formation, GDF5 also stimulates chondrocyte

proliferation. Besides chondrogenesis, the growth factor plays a crucial role in the formation of tendons and ligaments [1,2].

GDF5 belongs to the TGFβ superfamily and shares a conserved structure and signal transduction mechanism with its family members. Full length (FL) GDF5 is translated as an inactive preproprotein. After the signal peptide is removed by processing in the Golgi apparatus, the proprotein is supposed to form a homodimer. Subsequently, the proregion is removed by prohormone convertases of the furin type at the processing site [RRKRR] N-terminal to position 382 presenting the start of the mature domain [3]. Cleavage is assumed to take part to a large extent in intracellular compartments. However, tissue-type-dependent extracellular cleavage has been reported, and the proprotein and the mature growth factor can be secreted [4,5]. Mature GDF5 binds to the bone morphogenetic protein (BMP) type I receptor BMPR1A or BMPR1B, whereas the growth factor has a higher affinity to the latter one. The type I receptor dimer, in complex with BMP type II receptors, induces downstream signaling cascades, mainly via SMAD-dependent signal transduction (reviewed in Ref. [6]).

Although the GDF5 proregion comprises approximately 70% of the whole protein, its function is unknown. For the related proteins Activin A and TGFβ1, it has been shown that secretion of the mature growth factors is impaired in the absence of the proregions [7]. The TGFβ1 proregion and the mature protein stay in a non-covalent complex that remains inactive since the latent TGFβ1 binding proteins are covalently bound to the proregion [8,9]. The proregions of the TGFβ superfamily are not highly conserved and functions differ between the individual family members.

The importance of the GDF5 proregion becomes apparent by missense mutations in this region leading to skeletal malformations. Examples are brachydactyly type A2 (p.R380Q [5]), brachydactyly type C (BDC) (p.M173V [10], p.L176P [11], p.S204R [12], p.R301stop [13]), Du Pan syndrome (p.R378Q [14]), chondrodysplasia Grebe type (p.Q100fs [15], p.L176P [11]) or proximal symphalangism (p.L373R [16]). Although some mutations in the proregion of GDF5 have been known for many years, only two reports elucidated potential disease-associated molecular mechanisms. One of these is p.R380Q, a missense mutation that causes brachydactyly type A2 and leads to impaired processing by prohormone convertases [5]. The second is the recently reported missense mutation p.L176P, which was found to cause BDC and chondrodysplasia Grebe type due to impaired GDF5 secretion [11].

Here, we present a cell biological and biochemical analysis of two missense mutations in the proregion of GDF5 causing BDC. We provide evidence that both mutations lead to a loss of function by reducing GDF5 protein amount, possibly due to structural changes in the protein.

Results

Heterozygosity for GDF5 mutations p.T201P and p.L263P causes a classical BDC phenotype

We analyzed two families that were diagnosed with BDC [Online Mendelian Inheritance in Man® (OMIM) #113100]. Additionally observed skeletal manifestations in one of the families as described in Fig. 1a are most likely not associated features of BDC. Genetic analysis of the father and the two children of family 1 (Fig. 1a) revealed a heterozygosity for a missense mutation in the *GDF5* gene at position c.601A > C (RefSeq: NM_000557.3), which is predicted to lead to the amino acid exchange of threonine to proline at position 201 (p.T201P). The mother was clinically inconspicuous. Analysis of the father and the child of family 2 (Fig. 1b) disclosed a heterozygosity for a missense mutation in the *GDF5* gene at position c.788T > C (RefSeq: NM_000557.3) predicted to result in the amino acid exchange of leucine to proline at position 263 (p.L263P). The child (patient V) committed suicide at the age of 21 years. The mother was unaffected. Both identified mutation sites are located in the core domain of GDF5 (Fig. 2a) [17]. A sequence alignment of GDF5 between human, mouse, chicken, cow and zebrafish revealed that p.T201 is conserved among these species and that p.L263 is conserved among all shown species except for chicken, where leucine is replaced by valine, which is also a hydrophobic amino acid. A segment of the GDF5 proregion including the core domain was modeled into the known proTGFβ1 structure (Fig. 2b). The core domain of the GDF5 proregion corresponds to a part of the homologous arm domain of the proTGFβ1 protein, which together with the straitjacket segment encircles the mature TGFβ1 [18]. The homologous positions for T201 and L263 can be found in the β1 and the β4 strand of the TGFβ1 proregion, respectively.

FL GDF5^{T201P} and FL GDF5^{L263P} lead to reduced BMPR1A and BMPR1B activation

Mature GDF5 is known to bind to the receptor BMPR1A and its high-affinity receptor BMPR1B and thereby inducing SMAD-dependent signal transduction. A luciferase assay using the SMAD binding element (SBE) in the fibroblast cell line NIH/3T3 was performed to observe SMAD signaling stimulated by FL GDF5^{WT}, FL GDF5^{T201P} or FL GDF5^{L263P} (Fig. 3). In comparison to cells transfected with a control vector, FL GDF5^{WT} led to an induction of luciferase activity when cotransfected with *Bmpr1a* or *Bmpr1b*