Report

GDF5 Is a Second Locus for Multiple-Synostosis Syndrome

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Multiple-synostosis syndrome is an autosomal dominant disorder characterized by progressive symphalangism, carpal/tarsal fusions, deafness, and mild facial dysmorphism. Heterozygosity for functional null mutations in the *NOGGIN* gene has been shown to be responsible for the disorder. However, in a cohort of six probands with multiple-synostosis syndrome, only one was found to be heterozygous for a *NOGGIN* mutation (W205X). Linkage studies involving the four-generation family of one of the mutation-negative patients excluded the *NOGGIN* locus, providing genetic evidence of locus heterogeneity. In this family, polymorphic markers flanking the *GDF5* locus were found to cosegregate with the disease, and sequence analysis demonstrated that affected individuals in the family were heterozygous for a novel missense mutation that predicts an R438L substitution in the GDF5 protein. Unlike mutations that lead to haploinsufficiency for GDF5 and produce brachydactyly C, the protein encoded by the multiple-synostosis–syndrome allele was secreted as a mature GDF5 dimer. These data establish locus heterogeneity in multiple-synostosis syndrome and demonstrate that the disorder can result from mutations in either the *NOGGIN* or the *GDF5* gene.

Multiple-synostosis syndrome (SYNS1 [MIM 186500]) is an autosomal dominant condition characterized by progressive joint fusions of the fingers, wrists, ankles, and cervical spine; characteristic facies; and progressive conductive deafness. Two additional syndromes have very similar phenotypes: proximal symphalangism (SYM1 [MIM 185800]) and tarsal-carpal coalition syndrome (TCC [MIM 186570]). Heterozygosity for *NOGGIN* (GenBank accession number NM_005450) mutations has been identified in all three disorders (Gong et al. 1999; Dixon et al. 2001). In addition, heterozygosity for mutations in *NOGGIN* has been identified in stapes ankylosis syndrome without symphalangism (MIM 184460) (Brown et al. 2002). To date, 14 distinct *NOGGIN* mutations have been reported (Gong et al. 1999; Dixon et al. 2001; Takahashi et al. 2001; Brown et al. 2002; Mangino et al. 2002). The majority (10 of 14) are missense mutations, and the 4 nonsense mutations are predicted to result in premature translation termination codons. Noggin was initially identified in *Xenopus* as a secreted signal released by the Spemann organizer and is involved in developmental processes, including induction of neural tissue from the ectoderm and dorsalization of the ventral mesoderm (Zimmerman et al. 1996). It also participates in the regulation of chondrogenesis in somites and limb buds, in which it acts as an antagonist to the bone morphogenetic proteins (Zimmerman et al. 1996; Brunet et al. 1998; McMahon et al. 1998).

GDF5 (growth differentiation factor 5, also known as CDMP1 or BMP14), a member of the bone morphogenetic protein and TGF-β families, is a secreted growth factor expressed during several steps in skeletal development, including the formation of the cartilage anlagen (chondrogenesis), chondrocyte differentiation, and joint morphogenesis (Chang et al. 1994; Storm and Kingsley 1999). Several skeletal dysplasias are known to result from mutations in *GDF5* (GenBank accession number NM_000557). Brachydactyly C (BDC [MIM 113100]), a disorder characterized by shortened middle phalanges of the second, third, and fifth digits and by hyperphalangia, results from heterozygosity for functional null

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GDF5 mutations (Polinkovsky et al. 1997). Recently, in the disorder brachydactyly A2 (BDA2 [MIM 112600]), heterozygosity for a GDF5 mutation (L441P) within the active-signaling domain of the molecule was identified (Kjaer et al. 2005). Homozygosity for functional null mutations in *GDF5* have been identified in autosomal recessive Hunter-Thompson (MIM 201250), Grebe (MIM 200700), and Du Pan (MIM 228900) chondrodysplasias, as well as in a rare form of BDC in the brachypodism mouse (Storm et al. 1994; Thomas et al. 1996, 1997; Faiyaz-Ul-Haque et al. 2002; Schwabe et al. 2004). Individuals with these disorders have severe abnormalities in the bones and joints of the mesomelic and acromelic limb segments. Interestingly, carriers of the mutations of the recessive forms of *GDF5* disorders manifest mild abnormal metacarpohalangeal profiles, which suggests gene-dose sensitivity in the developing phalanges (Schwabe et al. 2004).

After obtaining institutional approval for human subjects study, we studied a cohort of six genetically independent individuals with SYNS1 diagnosed on the basis of clinical and radiographic evaluations. Five cases occurred sporadically, and one individual was a member of a large four-generation Ashkenazi Jewish family (fig. 1*A*). In this family, phenotypic findings included a broad hemicyclindrical nose, progressive symphalangism, and carpal, tarsal, and vertebral fusions. Within this family, there was phenotypic variability in the extent of joint fusions and in the presence or absence of equinovarus. To test the hypothesis that heterozygosity for *NOGGIN* mutations would lead to SYNS1 in this cohort, we determined the sequence of the single exon encoding *noggin,* using published methods (Gong et al. 1999). One patient (R02-360) was heterozygous for a point mutation (1425G→A) predicted to lead to a premature translation termination codon, W205X (human *NOGGIN*). This mutation created an *Afe1* restriction endonuclease cleavage site, and the mutation was further confirmed by cleavage of a PCR-generated DNA fragment with the enzyme (data not shown). No mutations were identified in the remaining five probands. For two of the patients, normal dosage of the *NOGGIN* gene was demonstrated by Southern analysis (data not shown).

To confirm the exclusion of *NOGGIN* as the disease gene in the familial case of SYNS1, linkage analysis using polymorphic markers from chromosome 17q21-22 was performed. The data demonstrated exclusion of linkage between the phenotype and the markers *D17S787* (maximum LOD score of 0 [$\theta = 0$]) and *D17S957* (maximum LOD score of 1.0 [$\theta = 0$]), which flank the *NOGGIN* gene, thus excluding *NOGGIN* as the disease gene. To define the second locus for SYNS1, we considered other genes known to be involved in joint morphogenesis, including the gene encoding GDF5, which is a direct antagonist of noggin. We tested linkage to the marker *D20S195,* which is located 2.2 Mb centromeric to

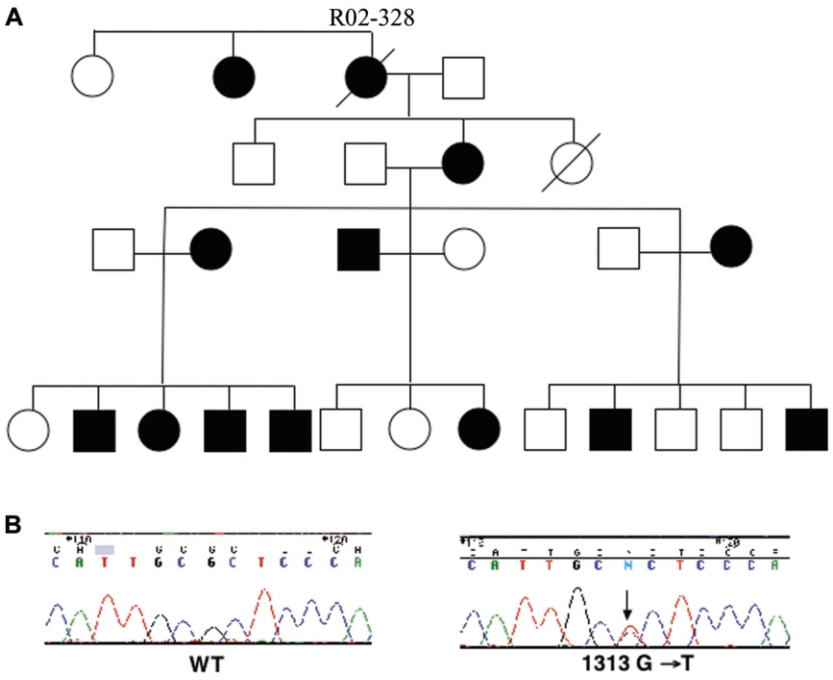

Figure 1 *A*, Pedigree of family R02-328 with SYNS1. *B*, Chromatograms showing the *GDF5* wild-type (WT) sequence and the nucleotide substitution, 1313G→T, predicted to lead to the amino acid substitution R438L.

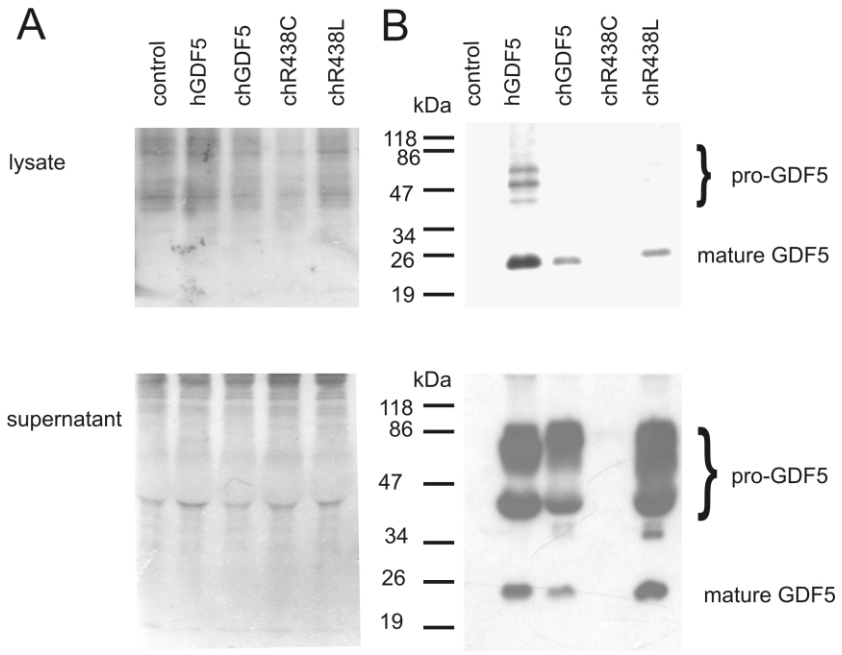

Figure 2 Synthesis and secretion of normal and mutant GDF5 proteins. DF-1 cells transfected were control vector, human GDF5, chicken (ch) GDF5, ch R438C-GDF5, and ch R438L-GDF5. *A,* Ponceau red-stained western blot demonstrating protein expression and uniform loading in samples derived from the cell lysate and supernatant. *B,* Western blot analysis of lysate from ch GDF5 transfected cells showing pro-GDF5 in the human control lane and mature GDF5 in wild-type ch GDF5 and in ch R438L-GDF5 but not in ch R438C-GDF5 (*top*); western blot analysis of supernatant from ch GDF5–transfected cells showing pro-GDF5 in the human control lane and mature GDF5 in wild-type ch GDF5 and in ch R438L-GDF5 but not in ch R438C-GDF5 (*bottom*).

GDF5. In the family, a single allele cosegregated with disease without exception (maximum LOD score of 2.2 [$\theta = 0$]). Since these data supported the candidacy of the *GDF5* gene, the sequences of the two exons of *GDF5* were determined as described elsewhere (Polinkovsky et al. 1997) for the five *NOGGIN* mutation–negative probands. Heterozygosity for a nucleotide substitution (1313G→T) (human *GDF5*) was found in the proband—the index case from the large family—predicting an R438L amino acid change in the protein (fig. 1*B*). The sequence change was found in all affected members of the family but not in the unaffected individuals, and it is predicted to substitute a hydrophilic arginine with a hydrophobic leucine in a highly conserved residue within the active-signaling domain of the mature protein. However, in four probands, neither a *NOGGIN* mutation nor a *GDF5* mutation was identified, which suggests that other possible loci could lead to SYNS1.

To determine the consequences of the substitution on dimerization and secretion of GDF5, we used an *RCAS* viral construct (Lehmann et al. 2003) to transiently transfect and express a chicken *GDF5* cDNA carrying the R438L sequence in DF-1 cells. The data were compared with control constructs carrying either the wild-type sequence or a cDNA with an R438C mutation, which causes BDC (Everman et al. 2002). The BDC mutation is known to lead to inefficient dimerization and secretion of the mature protein, which leads to functional haploinsufficiency for GDF5 (Everman et al. 2002). Proteins secreted into the medium and within the infected cells were each separated by PAGE under nonreducing conditions, were transferred to membranes, and were incubated with a monoclonal antibody to the aminoterminal portion of GDF5 (kind gift from Biopharm GmbH). The antibody recognizes the disulfide-linked full dimer (pro-GDF5) and the mature, active-domain dimer (GDF5) (Wang et al. 2004). The R438L protein was assembled into mature GDF5 dimers and was secreted in a similar way as wild-type GDF5 (fig. 2*B*). In contrast, but consistent with prior studies (Everman et al. 2002), the R438C construct did not lead to efficient formation or secretion of either pro-GDF5 or mature GDF5 dimers. Thus, although the same amino acid residue is altered in both BDC and SYNS1, the different substitutions lead to distinct fates for the mutant protein. It is likely that altered GDF5 activity due to the R438L substitution, rather than due to haploinsufficiency, produces SYNS1, revealing an alternative pathway to disease resulting from a GDF5 mutation (Seeman et al. 2005)

To date, all reported mutations causing SYNS1 have been heterozygous *NOGGIN* mutations (Marcelino et

al. 2001). This report identifies *GDF5* as a second locus for the SYNS1 phenotype. A role for *GDF5* in joint formation is further supported by the report of a large family with features similar to SYNS1, described in abstract form only (Akarsu et al. 1999). In the family, an S475N substitution, also in the highly conserved active-signaling domain of GDF5, was found to segregate with the phenotype. The effects of the substitution on the protein are unknown.

One of the questions raised by the identification of a second locus for SYNS1 is whether there are phenotypic differences between the individuals in whom *NOGGIN* versus *GDF5* mutations have been identified. Detailed review of the phenotypes of the individuals in the family described here show characteristic clinical and radiographic findings for the disorder, including a broad hemicyclindrical nose, progressive symphalangism, and carpal, tarsal, and vertebral fusions. Analysis of hand radiographs of affected individuals showed no findings characteristic of BDC, indicating that the substitution does not result in an overlapping SYNS1/BDC phenotype. Detailed evaluation of this family did not reveal any distinguishing clinical features between SYNS1 patients with *NOGGIN* mutations and patients with *GDF5* defects.

NOGGIN and *GDF5* are both required for proper joint morphogenesis. In the absence of noggin, BMP growth factors are unregulated, resulting in chondrocyte hyperplasia instead of apoptosis in the developing joint and, thus, leading to lack of normal joint formation. *Noggin*$^{-/-}$ mice had a lethal skeletal phenotype with a very abnormal skeleton, characterized by joint fusions with a complete absence of limb joints, costovertebral defects, and cartilage spurs, and with up-regulation of *GDF5* in the areas of the presumptive joints (Brunet et al. 1998). Human *NOGGIN* mutations that lead to SYNS1 are due to functional haploinsufficiency and a presumed lack of appropriate antagonism of GDF5. Since the GDF5 R438L protein is able to form mature secreted dimers, we suggest the hypothesis that the mutation leads to increased *GDF5* activity and to SYNS1 (Seeman et al. 2005). This is mechanistically similar to the consequences of haploinsufficiency for *NOGGIN*, in that the resultant effect is the up-regulation of GDF5 in the mesenchyme surrounding the developing joint. This study identifies *GDF5* as a locus for SYNS1. In addition, mutations were not identified in either *NOGGIN* or *GDF5* in the remaining four patients in the cohort, suggesting the possibility of further locus heterogeneity.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *NOGGIN* [accession number NM_005450] and *GDF5* [accession number NM_000557])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SYNS1, SYM1, TCC, stapes ankylosis syndrome without symphalangism, BDC, BDA2, and Hunter-Thompson, Grebe, and Du Pan chondrodysplasias)

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