A Mutation in *TGFB3* Associated With a Syndrome of Low Muscle Mass, Growth Retardation, Distal Arthrogryposis and Clinical Features Overlapping With Marfan and Loeys–Dietz Syndrome

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The transforming growth factor β (TGF-β) family of growth factors are key regulators of mammalian development and their dysregulation is implicated in human disease, notably, heritable vasculopathies including Marfan (MFS, OMIM #154700) and Loeys–Dietz syndromes (LDS, OMIM #609192). We described a syndrome presenting at birth with distal arthrogryposis, hypotonia, bifid uvula, a failure of normal post-natal muscle development but no evidence of vascular disease; some of these features overlap with MFS and LDS. A de novo mutation in TGF3 was identified by exome sequencing. Several lines of evidence indicate the mutation is hypomorphic suggesting that decreased TGF-β signaling from a loss of TGFB3 activity is likely responsible for the clinical phenotype. This is the first example of a mutation in the coding portion of TGFB3 implicated in a clinical syndrome suggesting TGFB3 is essential for both human palatogenesis and normal muscle growth. © 2013 Wiley Periodicals, Inc.

Key words: transforming growth factor beta; Marfan syndrome; Loeys–Dietz syndrome; distal arthrogryposis; low muscle mass; bifid uvula; exome sequencing; de novo mutation; hyomyoplasia

INTRODUCTION

The transforming growth factor β (TGF) family of growth factors are key regulators of mammalian development and their dysregulation is implicated in human disease, notably, heritable vasculopathies including Marfan (MFS, OMIM #154700) and Loeys–Dietz syndrome (LDS, OMIM #609192). We describe a syndrome

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presenting at birth with distal arthrogryposis, hypotonia, bifid uvula, a failure of normal post-natal muscle development without evidence of vascular disease; some features overlap with MFS and LDS. A de novo mutation in TGFβ3 was identified by exome sequencing. Several lines of evidence indicate the mutation is hypomorphic, suggesting that decreased TGFβ signaling from a loss of TGFβ3 activity is likely responsible for the clinical phenotype. This is the first example of a mutation in the coding portion of TGFβ3 implicated in a clinical syndrome, suggesting TGFβ3 is essential for both human palatogenesis and normal muscle growth.

CLINICAL REPORT

The proband is a 9-year-old European-American female born to nonconsanguineous, healthy parents with two older healthy children and no previous miscarriages. Mother was 34 years old and father was 42 at delivery. No cytogenetic abnormality was observed on chromosome samples obtained at 19 weeks gestation by amniocentesis. The pregnancy was unremarkable and the mother reported that the fetus moved in utero similarly to older siblings. The child was born at full term by repeat caesarian; Apgars were 9 and 9 at 1 and 5 min, respectively. The birth weight was 2.9 kg (5th centile), length was 51 cm (50th centile), and OFC was 35.75 (>50th centile). The physical exam showed contractures in the right hand, most severe in the 3rd and 4th fingers (see Fig. S1 in Supplementary online material) and all toes. The digits on the left hand and foot showed minimal contractures. Range of motion of knees, hips, elbows, jaws, and back were considered normal. Hands and feet appeared long and narrow although no measurements were taken. There was a midline facial nevus flammeus and mild hypotonia.

At age 3 months, the patient was evaluated for failure-to-thrive because of low weight (<5th centile) and a delay in gross motor function. An evaluation at 17 months showed that she did not crawl or roll, but could stand with support. She weighed 7.5 kg (<1st centile) and was 76 cm in height (5th centile) (Fig. 1, left picture). Also noted was bilateral pes planus, mild pectus excavatum, hyperextensibility of multiple large joints, and mild retrognathia. Eyes appeared prominent and hypertelorism was present with an outer canthal distance of 7.8 cm and an inner canthal distance of 2.8 cm (>97th centile). Her skin had normal texture, tension, and wound healing. Head circumference was 45 cm (25th centile) with normally placed and well-formed ears. A bifid uvula with intact hard palate, normal arch, and normal voice quality were present. A small metopic ridge and normal teeth were observed. The sclerae were blue. She had marked contractures at the proximal phalangeal joints of the right second and third digits and toes bilaterally (R > L). Her motor examination revealed decreased bulk in all appendicular and axial muscles, strength 1/5, low tone, and diminished reflexes throughout. Markedly reduced subcutaneous fat was noted.

The patient could stand independently with a positive Gower sign at 20 months, and walked at 24 months in a “hip waddle” fashion. Her neurocognitive development was appropriate for age. Slit lamp examination of both eyes was normal. Radiographic studies of the hands and pelvis showed age-appropriate ossification and bilateral coxa valga deformity, respectively. At age 3 years, a 3-year trial of losartan at a dose of up to 2.0 mg/kg/day produced no change in muscle strength or mass (Figs. 1, middle picture and 2). At the age seven, she weighed 15.5 kg (<1st centile) and was 115 cm (5th centile) in height; the physical exam was otherwise unchanged. The skin had normal texture, subcutaneous fat was minimal, and marked hyperextensibility was present in elbows and knees. No abnormal spinal curvature was evident. A right quadriceps muscle biopsy taken at age 7 years showed normal a checkerboard pattern with Type 1 fiber predominance but mild focal Type 1 fiber disproportion consistent with disuse or decreased usage (type 1: 47 μ vs. Type 2: 50 μ) (see Figs. S4–S7 in Supplementary online material).

Yearly echocardiograms beginning at 18 months showed no cardiac defect or dysfunction. An echocardiographic examination at the age 6.5 years measured the aortic annulus and aortic root at 1.34 cm (z score 0.59) and 1.72 cm (z score 0.39), respectively; the pulmonary artery dimensions were also consistently within the normal range. Visual acuity remained normal. The proband had physical and occupational therapy since the age 6 months, and has worn orthotic foot appliances since age 2. Although digit contractures persist, at age 9 she has very functional hands.
METHODS

Genomic DNA was extracted from peripheral mononuclear blood cells from the proband, two unaffected sibs, and parents. The details of the exome and Sanger sequencing and the Xenopus and cultured cell methods are described in Supplementary online material. The focus of the sequence data filtering was for novel non-synonymous variants that were unique. These included heterozygous novel missense and nonsense substitutions and frame-shifting insertions and deletions (in/dels) not detected in other family members. In addition, we looked for homozygous non-synonymous variants (missense or nonsense substitutions or frame-shifting in/dels) where she was the only family member homozygous for the damaging allele, and where both parents were heterozygous. Finally, we looked compound heterozygosity of deleterious alleles in genes associated with heritable disorders of connective tissue.

RESULTS

The proband shared the clinical feature of low muscle mass with hypotonia with three related conditions: Marfan, Loeys–Dietz, and Beals–Hecht syndrome (BHS, OMIM # 121050). She was hypertelorism and had a bifid uvula, the two non-vascular findings that define LDS; the skeletal findings typical of MFS included arachnodactyly, pectus excavatum, pes planus, and hyperextensible large joints. The proposita did not meet the diagnostic criteria established for MFS, BHS or LDS. Among the inconsistencies with these diagnoses were significant growth retardation, the absence of cardiovascular findings, and the distinct muscle histopathology. These three syndromes are allied in their pathophysiology. TGF-β signaling is dysregulated in the first two conditions and analogous pathophysiology is suspected in BHS, given the similar functions of FBN1 and FBN2. The clinical overlap with these autosomal dominant syndromes and the unaffected status of the parents suggested a de novo mutation acting as a dominant trait in a gene affecting TGF-β signaling. Analysis of the six genes (TGFBR2, SMAD3, FBNI, FBN2) associated with MFS, LDS, or BHS identified no mutation. We sought to identify de novo mutations through exome sequencing; consistent with expectations [Neale et al., 2012], we found two nucleotide changes unequivocally unique to the proband, in CDH2 and TGFBR3.

A nonsense mutation was found at codon 70 in CDH2 (c.208C>T, pQ70X), encoding the 907 amino acid protein cadherin-2 or N-cadherin. Seventeen CDH2 SNVs predicted to be damaging by PolyPhen are reported in the NHLBI exome variant server (EVS) database. In dermal fibroblasts isolated from the proband, the concentration of N-cadherin by Western blot was not statistically different from six age-matched, passage-matched controls (see Supplementary online material, Fig. S8). A second de novo mutation was found in TGFBR3 (c.1226G>A; pC409Y). Validation by Sanger sequencing confirmed that this mutation was de novo in the proband. No ligand-coding sequence variants have been reported in TGFBR3 including dbSNP130, EVS, or the 1000 Genomes dataset.

To determine the effect of the cysteine-to-tyrosine substitution on TGFBR3 function, we co-transfected an epitope-tagged wild-type (w-t) TGFBR3ΔDNA or a similarly tagged TGFBR3G1226AΔDNA under the control of a CMV promoter into 293T cells (see Supplementary online material). Conditioned medium (CM) was collected, the tagged TGFBR3 species purified by immunoprecipitation and the relative TGF-β signaling activities compared using HeLa cells transfected with the reporter plasmid, p3TPLux, a Smad2-responsive reporter. Figure 3 shows that the wild-type (w-t) TGFBR3 gene generated a transcriptional TGF-β signal, whereas the TGFBR3G1226A gene construct did not. We conclude that the mutant allele encodes a TGFBR3 ligand that is not functional.

Xenopus embryos provide a sensitive assay system for assessing effects of ectopically expressed proteins on TGF-β (SMAD2

![FIG. 2. Picture of the proband at age 30 months with V.A. McKusick, M.D. Evident in the proband are the blue sclera, the well-placed and normal ear, tubular nose with metopic ridge, mild hypertelorism, retrognathia, and hypomalar eminences.](image)

![FIG. 3. TGFBR3ΔC409Y does not activate TGF-β signaling. Constructs containing FLAG-tagged TGFBR3ΔWT and TGFBR3ΔC409Y were expressed in HEK293T cells and secreted FLAG-tagged proteins were immunoprecipitated under native conditions and normalized to anti-FLAG reactivity. Serial dilutions of these proteins were applied to HEK293T cells transfected with p3TPL-Lux [TGF-β reporter] and pRL-CMV [normalizer]. Error bars are standard error of the mean (N = 3).](image)
and ERK1/2) and BMP (SMAD1) signaling. To examine how TGFB3G1226A altered TGF-β signaling in Xenopus embryos, RNA from the w-t human TGFB3 allele and the TGFB3G1226A allele were injected into fertilized Xenopus eggs either separately or together using a constant amount of RNA from the w-t allele with an increasing amount of RNA from the mutant allele. Prospective ectodermal tissue from late blastula embryos was examined by Western blot for phosphorylation of SMAD2 and ERK1/2 (pSMAD2 and pERK1/2, respectively). Figure 4 shows that RNA from the w-t human TGFB3 allele results in a significant increase in pSMAD2 and pERK1/2 signals by Western blot as expected (see Supplementary online material Fig. S9 for controls). The TGFB3-dependent pSMAD2 and pERK1/2 signals are significantly diminished, however, with increasing amounts of the mutant RNA relative to wild-type. A 1:1 ratio of mutant-to-wild-type TGFB3 RNA diminished the pSMAD2 and pERK1/2 signals to approximately 40% and 60%, respectively, of that generated by the w-t TGFB3 RNA alone. Gastrula stage embryos require activation of SMAD1 by endogenous BMP2/4/7 for normal dorsal-ventral patterning. Ectopic expression of TGFB3G1226A had no effect on either SMAD1 phosphorylation (pSMAD1) or dorsal ventral patterning in Xenopus gastrulae (Fig. 5). We conclude that in the frog system the mutant TGFB3G1226A allele, when co-expressed with w-t TGFB3, has a dominant negative effect on TGF-β signaling measured by pSMAD2 and pERK1/2, but does not affect signaling by BMP2/4/7 ligands measured by pSMAD1.

**DISCUSSION**

The principal clinical concerns for this patient have been growth retardation, weakness related to decreased muscle mass and uncertainty about her risk for vascular disease. Distal arthrogryposis...
indicative of reduced fetal movement suggested an inborn error of development affecting muscle mass and other developing mesenchymal tissues, including the soft palate.

A muscle biopsy showed essentially normal fiber size and architecture. There was no evidence of chronic dystrophic or inflammatory changes presenting a strikingly different histologic picture compared to the myopathic findings in congenital or classical MFS in which endomysial thickening, fat deposition, split fibers, fibrosis, and marked fiber size disproportion are consistent histopathological observations. The phenotypic discordance in the proband—significant underdevelopment of muscle mass or hypomyoplasia with the absence of abnormal cardiac or aortic findings by age 8 years—suggests a pathophysiology distinct from that caused by excess TGF-β signaling observed with FBN1 mutations in MFS. Furthermore, in one mouse model of MFS, muscle mass was normal and there was complete histologic restoration of muscle architecture after treatment with pan-anti-TGFB antibodies or losartan, suggesting that excess TGF-β signaling in MFS has its major pathological effects on muscle during the post-natal growth phase of muscle development [Cohn et al., 2007]. The proband had no change in strength or muscle mass after a significant therapeutic trial of losartan, further suggesting a pathophysiology distinct from mutations enhancing TGF-β signaling.

The proband was heterozygous for two de novo mutations. The CDH2 levels were not statistically significantly different from control fibroblasts (see Supplementary online material). Mice heterozygous for a null mutation in CDH2 were phenotypically normal at 2 years; specifically, muscle mass was not affected [Garcia-Castro et al., 2000]. We conclude that the proband’s normal at 2 years; specifically, muscle mass was not affected indicates of reduced fetal movement suggested an inborn error of development affecting muscle mass and other developing mesenchymal tissues, including the soft palate.

Based on these biochemical data in the context of the clinical findings, we conclude that the proband’s normal at 2 years; specifically, muscle mass was not affected indicates an inborn error of development affecting muscle mass and other developing mesenchymal tissues, including the soft palate.

In summary, we describe a clinical syndrome characterized by abnormal development of several mesenchymal-derived tissues including muscle and cranio–palato–facial structures accompanied by low muscle mass, growth retardation, distal arthrogryposis and other secondary changes. Though the proband shares some clinical features with known syndromes that enhance TGF-β signaling, such as MFS and LDS, her findings are clinically distinct [Holm et al., 2011]. We identified a mutation in TGFβ3 and demonstrate in model systems that the altered TGFβ3 ligand results in a decrease in canonical and non-canonical TGF-β signaling, suggesting that the phenotype is a consequence of a hypomorphic allele. The full phenotypic spectrum and natural history of TGFβ3 mutations must await other cases; we can, however, assert from these observations that the development of normal muscle mass appears to require a minimum of TGFβ3 signaling, as does complete palatal fusion. While loss-of-function mutations in the TGFβ2–TGFBβ–SMAD3 axis appear to enhance TGFβ1 or TGFβ2 expression in vascular tissue with at times catastrophic consequences, mutations in other components of the pathway, such as TGFβ3 may not have the same effects on vascular tissue or developing muscle. Clearly, ligand and tissue-specific factors contribute to the distinct clinical findings that render each syndrome unique.
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Ingenuity Variant Analysis software (www.ingenuity.com/variants) was used in this study. An interactive online supplement is available at https://variants.ingenuity.com/Rienhoff2013 which provides direct access to the dataset discussed in this manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

**FIG. S1.** The hands of the proband, age 8.

**FIG. S2.** The lower extremities of the proband, age 8.

**FIG. S3.** The face of the proband, age 4.5. Notable are the blue sclera, hypertelorism, malar hypoplasia, and tubular nose.

**FIG. S4.** Right quadricep biopsy (40×) stained with hematoxylin and eosin showing normal muscle fiber architecture.

**FIG. S5.** Right quadricep biopsy (40×) treated with ATPase, pH 9.4, showing the size and distribution of myofibers. Type 1 fibers are light; Type 2 fibers are dark.

**FIG. S6.** Right quadricep biopsy (40×) with trichrome staining showing normal intramyofibril membranes and interstitial collagen.

**FIG. S7.** Distribution of size in Type 1 and Type 2 fibers in representative sections of the proband’s muscle.

**FIG. S8.** N-cadherin expression levels. Protein expression levels of N-cadherin in whole cell lysate from human skin fibroblasts analyzed by Western blot. β-Tubulin was used as a loading control. Values expressed are normalized to loading controls.

**FIG. S9.** TGFβ3 signaling in Xenopus embryos. Indicated amount of synthetic mRNAs were microinjected into Xenopus embryos after fertilization, and embryos harvested at Stage 9 for Western blot analysis. This representative Western blot is shown to indicate that any of the injected synthetic mRNAs are not sufficient alone to trigger phosphorylation of Smad2. As seen in Figure in the text, human TGFβ3 and the TGFBR2 are required together to trigger the phosphorylation of Smad2.