TNXB Mutations Can Cause Vesicoureteral Reflux

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ABSTRACT

Primary vesicoureteral reflux (VUR) is the most common congenital anomaly of the kidney and the urinary tract, and it is a major risk factor for pyelonephritic scarring and CKD in children. Although twin studies support the heritability of VUR, specific genetic causes remain elusive. We performed a sequential genome-wide linkage study and whole-exome sequencing in a family with hereditary VUR. We obtained a significant multipoint parametric logarithm of odds score of 3.3 on chromosome 6p, and whole-exome sequencing identified a deleterious heterozygous mutation (T3257I) in the gene encoding tenascin XB (TNXB in 6p21.3). This mutation segregated with disease in the affected family as well as with a pathogenic G1331R change in another family. Fibroblast cell lines carrying the T3257I mutation exhibited a reduction in both cell motility and phosphorylated focal adhesion kinase expression, suggesting a defect in the focal adhesions that link the cell cytoplasm to the extracellular matrix. Immunohistochemical studies revealed that the human uroepithelial lining of the ureterovesical junction expresses TNXB, suggesting that TNXB may be important for generating tensile forces that close the ureterovesical junction during voiding. Taken together, these results suggest that mutations in TNXB can cause hereditary VUR.

developmental abnormalities of the genitourinary tract. Recent murine distal ureter genetic and developmental modeling studies implicated a significant contribution of disordered bladder trigone and common nephric duct interactions in PVUR development. Although these theories provide explanations for early events during embryogenesis that are likely to give rise to renal dysplasia and VUR, they do not completely explain the pathogenesis of PVUR. VUR is highly heritable, and although many loci have been found, the specific genes encoded by these loci have remained elusive. This is most likely due to a paucity of large pedigrees with adequate statistical power for genome-wide linkage study (GWLS).

We recently identified a 97-member kindred from the United States with at least 16 affected individuals dating back five generations. We performed sequential GWLSs and whole-exome sequencing (WES) on this family and identified mutations in tenascin XB (TNXB) as a cause of VUR. TNXB mutations have been associated with the joint hypermobility variant of Ehlers-Danlos syndrome. Studies have revealed that patients with VUR frequently show structural changes within the ureterovesical junction (UVJ) including replacement of the smooth muscle layer by collagenous matrix, suggesting that some patients with VUR may have defects in the extracellular matrix that supports the UVJ. Fibroblast cell lines carrying the T3257I mutation identified in the index family demonstrated reduced cell motility and reduced expression of phosphorylated focal adhesion kinase, signifying a defect in focal adhesion disassembly. We also identified a G1331R mutation in the same gene in another kindred with VUR. These findings bring the role of the extracellular matrix and cell adhesion in the development and function of the UVJ into the forefront.

RESULTS

We identified family 6606, a 97-member kindred from the United States dating back at least five generations (Figure 1A). Nine individuals in this family have radiologically confirmed VUR and/or duplex collecting systems. The voiding cystourethrogram (VCUG) and nuclear dimercaptosuccinic acid (DMSA) renal scan from one of the affected individuals is shown in Figure 1B. This individual has bilateral VUR (grade 4 on the left side) and evidence of reflux nephropathy on DMSA scan based on the appearance of the left kidney that shows a diffuse reduction in uptake and scars. In addition, we identified seven family members with recurrent urinary tract infection (UTI) who did not undergo VCUG testing. The clinical characteristics of the affected individuals are summarized in Table 1. We evaluated the statistical power to detect linkage in this family and a power calculation showed that this single pedigree is capable of generating a maximum attainable estimated logarithm of odds score of 4.88 assuming a dominant genetic model.

GWLSs

We obtained a whole-genome multipoint parametric LOD score of 3.3 on chromosome 6p (Figure 1C). This score is in accord for the different penetrance assumptions. This region did not overlap with other loci that have been reported for VUR in the literature (Supplemental Table 1). Haplotype analysis, assuming a dominant model revealed clear evidence that a single disease carrying chromosome segregated with affected individuals in family 6606; the affected haplotype is shown as a black column in Figure 1D.

WES Identifies a Mutation in TNXB

The DNA samples from the proband and affected relative were subjected to WES. We used the following filtering parameters to reduce the number of variants: (1) removal of all variants with minor allele frequency >1%; (2) removal of all variants that are in the dbSNP and 1000 Genome Project; (3) removal of all variants that are found in normal controls; (4) removal of all synonymous variants; and (5) removal of all intronic variants except those with alternate annotations. After applying these parameters genome-wide, 348 potential disease-causing variants were present in at least one individual; however, only 29 of these were present in both individuals. In the linkage region, three variants were present in at least one individual, and all three variants were confirmed by Sanger sequencing. However, only one variant (heterozygous change exon 29. 9770 C>T3257I in the gene tenascin XB: TNXB) was present in both individuals (Table 2). This variant is present in all affected individuals and all obligate carriers as well as some individuals with recurrent UTI who were classified as unknown for the purpose of linkage analysis. The variant is absent in all unaffected individuals in the family, >800 control chromosomes and also from the publicly available 1000 Genome Project dataset (therefore, >2800 control chromosomes). The exons and protein domains of TNXB are shown in Figure 2A. The T3257 amino acid is conserved in evolution (Figure 2, B and C). We directly sequenced all of the coding exons of TNXB and flanking introns in 11 other families with VUR using exon primers (list of primers in Supplemental Table 2). We identified another heterozygous mutation, exon 10. 3991G>A G1331R in a second family, the G1331 amino acid is also conserved in evolution (Figure 2, D and E). We did not find the G1331R mutation in ethnically matched 178 control chromosomes and the change was also absent from the 1000 Genomes Project database (therefore, 2178 control chromosomes). The second kindred is a Caucasian family, and the proband presented with a UTI in the first decade of life and grade 2 reflux on the right side was demonstrated by a VCUG. In addition, her sister also has history of recurrent UTI; however, DNA samples and imaging studies were not available in this individual. The pedigree for the second family and the segregation data are shown in Supplemental Figure 1. Other variants found in the 12 families are listed in Supplemental Table 3.
In Silico Modeling Reveals Deleterious Missense Mutations

In silico modeling with Polyphen 2 software showed that both variants are damaging with deleterious score of 0.999 of a maximum of 1.20 The T3257I mutation is predicted to be in the linker region between the 23rd and 24th fibronectin type III (FnIII) domains of TNXB (Figure 2A).21,22 Modeling the T3257I mutation in the three-dimensional structure of the protein results in some structural changes in FnIII 24 (Supplemental Figure 2A). The G1331 residue is located in the fifth FnIII domain of TNXB. Modeling the G1331R mutation results in a perturbation of the secondary structure of FnIII domain as predicted by I-TASSER software (Supplemental Figure 2B).21,22

Joint Examination for Hypermobility and Skin Biopsy

Although homozygous TNXB deficiency has been associated with a recessive form of Ehlers-Danlos syndrome that includes large joint dislocations, degenerative joint disease, mitral valve prolapse, and rectal and uterine prolapse, about 50% of patients with heterozygous TNXB mutations have been found to have isolated joint hypermobility.23–25 We therefore screened some of the affected and unaffected individuals in the index family for joint hypermobility. A rheumatologist (C.E.R.) blinded to participants’ affection status and genotype, performed joint hypermobility tests on two affected and one unaffected members of the family using the Beighton hypermobility score.26 The two affected individuals have significant joint hypermobility with scores of 7 of 9 and 5 of 9,
respectively, whereas the unaffected individual has normal joint mobility with a score of 0 of 9. There were no symptoms of skin hyperelasticity or easy bruising and no differences in skin biopsy findings between affected and unaffected individuals as determined by light microscopy and electron microscopy. However, fragmentation of elastic tissue was found in both individuals but was subjectively worse in the affected individual (data not shown).

**In Vitro Studies Reveal Reduced Cell Motility and Expression of Phosphorylated Focal Adhesion Kinase in Fibroblast Cell Line with the T3257I Mutation**

Because TNXB is composed of multiple fibronectin domains, we hypothesized that the T3257I mutation would alter cell adhesion and cell motility. To test this hypothesis, we performed a standard wound healing assay. In Figure 3A, fibroblasts from an affected patient expressing the mutant TNXB (Mut T3257I) migrated significantly less in response to 50 ng/ml of platelet-derived growth factor (PDGF) compared with the wild-type (WT) cell line. The results of these experiments are quantified in Figure 3B, which shows the mean from three different experiments.42 In Figure 3C, immunoblotting of unstimulated and PDGF-treated WT and mutant fibroblasts stained for TNXB, pY397-FAK and total FAK are shown. In WT fibroblasts, pY397-FAK staining in response to PDGF is significantly upregulated relative to baseline. Conversely, in fibroblasts expressing the mutant TNXB, pY397-FAK staining is relatively unchanged from baseline in response to PDGF. These findings suggest that the TNXB mutation may attenuate PDGF-induced fibroblast motility via a mechanism involving impaired transmission of extracellular-to-intracellular signals and decreased FAK activation.

**TNXB Is Expressed in the UVJs of Normal and Refluxing Ureters**

Immunohistochemical staining and immunofluorescence of normal human UVJs obtained from autopsy samples and UVJs obtained from individuals with radiologically demonstrated VUR undergoing re-implantation was carried out using rabbit anti-human TNXB polyclonal antibody. The patients with VUR who consented to these samples did not undergo mutational screening for TNXB, so their status is unknown. TNXB is expressed predominantly in the transitional epithelial cells of the bladder in both refluxing and nonrefluxing units (Figure 4), and appears to be more strongly expressed in the uroepithelial layer of patients with VUR, consistent with another report that showed upregulation of tenascin C (TNC) in the UVJ of patients with VUR.19 On the basis of gene expression data from the GenitoUrinary Development Molecular Anatomy Project (GUDMAP; http://www.gudmap.org), TNXB is expressed in mice during development and in the postnatal period.31,32 Its relative expression in the ureter is more pronounced compared with that of nephrin a key podocyte gene but similar to uroplakin II, a gene that is enriched in developing lower genitourinary tract (Supplemental Figure 3).

**DISCUSSION**

PVUR remains the most common CAKUT seen in children. Despite the fact that many loci have been reported in the last decade, the genes for PVUR remain elusive. We performed sequential GWLS and WES in a large kindred with VUR and identified heterozygous mutations in TNXB as a cause of
dominantly inherited VUR and joint hypermobility. We found another mutation in a family with a history of recurrent UTI and VUR. The two mutations are novel and have never been reported in patients with hypermobility syndrome. Greater than 95% of the TNXB gene encodes multiple fibronectin III domains, thus far, all reported missense mutations in the literature as well as the mutations in this study are in fibronectin III domains of TNXB.25

Tenascins (TNXB, TNC, and TNR) are a family of large extracellular matrix proteins.33,34 They possess a similar structure that is characterized by N-terminal assembly domains, EGF-like repeats, multiple fibronectin III domains and a C-terminal fibrinogen–like domain. In contrast to other fibronectins, the fibronectin domains of most tenascins have anti-adhesive properties; thus, during development and cell proliferation they appear to control cell adhesion and migration.33,34 TNXB is expressed throughout development and its expression in the mouse fetus is detected at E15,31,32 coinciding with the time when the UVJ is being formed.35

The role of TNXB mutations in the pathogenesis of VUR is unknown. However, autosomal recessive (AR) TNXB mutations have been associated with Ehlers-Danlos syndrome type

Figure 2. Missense mutations in TNXB as a cause of VUR. (A) Exons and protein domains of TNXB. (B and C) Missense heterozygous mutation exon 29. 9770 C>T T3257I found in the index family with hereditary VUR; this mutation is conserved in evolution. (D and E) Another kindred with VUR was found to have G1331R mutation in exon 10; this mutation is also conserved in evolution.
III, a condition that is characterized by joint hypermobility, hyperelastic skin, and easy bruising. On the other hand, the phenotype associated with heterozygous mutation in the TNXB gene is variable, with one study reporting that only about 50% of individuals with one single mutation will have joint hypermobility and cutaneous stigmata seen in the AR form of the disease is uniformly absent in these individuals. We are not aware that any of the members of the family have symptomatic joint hypermobility, but examination of two affected individuals in the family revealed asymptomatic joint hypermobility. Therefore, the finding of T3257I mutation that is segregating with VUR in this study will suggest that VUR/duplex kidney may be the predominant phenotype in individuals with heterozygous mutations in TNXB. Searches of the literature revealed that VUR/duplex kidney have not been previously reported to be associated with either classic AR EDS type III or in individuals with haploinsufficiency or heterozygous missense mutation in TNXB. Interestingly, a recent study showed that 24% of participants with VUR had generalized joint hypermobility compared with 6.7% of normal controls. Other investigators have reported a 3-fold increase in the prevalence of VUR in children with isolated hypermobility compared with those without hypermobility.

In this study, we identified two missense mutations from two families with VUR and we showed that two affected individuals in the index family have asymptomatic joint hypermobility. Our findings, combined with existing epidemiologic data suggest that mutations in TNXB represent a hitherto unrecognized cause of VUR. In this study, we showed through immunohistochemical and confocal microscopy of UVJ from refluxing and nonrefluxing units in humans that TNXB is abundantly expressed at the UVJ. Furthermore, a review of the publicly available database, GUDMAP, showed that TNXB is expressed in the ureter and bladder of mice on days E15, a period that coincides with the time when the UVJ is being formed. Taken together, these data suggest that TNXB is important in the ontology of the UVJ and development of VUR. The mechanisms by which mutations in TNXB are likely to cause VUR remain unknown. It is possible that the TNXB mutations lead to VUR because of dysfunctional fibronectin domains. Fibroblast cell lines isolated from an individual with the T3257I mutation displayed reduced cell motility in response to PDGF and reduced expression of phosphorylated FAK, suggesting persistent and enhanced cell adhesion. On the basis of these findings, we posit that this may be a gain-of-function mutation. In addition, our results suggest that the mutation...
may alter integrin-mediated adhesion and dysregulate the extracellular matrix and tensile forces needed to close the vesicoureteric junction as part of the antireflux mechanism. Because TNXB appears to regulate collagen synthesis or deposition, an alternative mechanism by which mutations in TNXB may cause VUR is by altering the microenvironment in which smooth muscle cells develop or by affecting the tensile strength of the extracellular matrix that they anchor into, thereby affecting the antireflux mechanisms of the UVJ. One study reported that TNC was present in the musculature and connective tissue of the UVJ from individuals with VUR but was absent from individuals without reflux. Future detailed studies of the morphology of the UVJ in individuals with TNXB mutations will shed light on the mechanisms by which defects in TNXB cause VUR. In addition, large-scale screening of patients with VUR for TNXB mutations and joint hypermobility will reveal the role of TNXB in the etiology of primary VUR.

In conclusion, we identified mutations in TNXB as a new cause of PVUR in a large kindred using a sequential linkage analysis and WES approach. These findings emphasize the role of tenascins and other extracellular matrix proteins during genitourinary development.

CONCISE METHODS

Clinical Ascertainment
Institutional review board approval was obtained from Duke University Medical Center (Durham, NC). Study participants were classified as affected, unaffected, and unknown. Family members were considered affected if they had VUR on a VCUG. Supportive evidence included history of recurrent UTI and abnormal findings on renal ultrasonography. Individuals were considered unaffected if there was no detectable VUR on screening VCUG performed as part of routine clinical care or if they were unrelated married individuals in the family. Finally, individuals were considered unknown if they were asymptomatic or if they had a history of UTI but with no radiologic investigations.

Power Analyses
Power analysis for the index family 6606 was performed using SIMLINK to determine statistical power using a rare dominant affecteds-only model with disease allele frequency of 0.01 and markers with 2–4 alleles with frequencies of 0.4, 0.3, 0.2, and 0.1. GWLSs and Fine Mapping
A GWLS was performed using the 10,000 single nucleotide polymorphism linkage panel obtained from the Illumina Infinium 2.5 million genotyping beadchip assay (Illumina Inc., San Diego, California). We used an autosomal dominant affecteds-only model and a disease-causing allele frequency of 0.01. We ran simulations assuming the parametric linkage analysis for a dominant model under assumptions of both complete and reduced penetrance. Two-point and multipoint LOD scores were calculated for all single nucleotide polymorphisms using the VITESSE statistical program. The region of linkage was confirmed by genotyping informative microsatellites in the identified disease locus and haplotype analysis was carried out as previously described.

WES
WES was performed on the proband and an affected relative (separated from the proband by two generations) using standard protocols provided by the vendor (Agilent Technologies, Santa Clara, CA). We used the Agilent All Exon 50 MB kit using one lane of a HiSequence 2000 sequencer. The average depth of coverage of the exomes for the two individuals sequenced was 90× with 96% of the targeted capture regions having at least 5× coverage. Reads were aligned to the Human Reference Genome (HG 18) using Burrows-Wheeler Alignment software. Single
nucleotide variants were called using SAMtools. The variants were annotated to Ensembl 50 using the SequenceVariantAnalyzer and were analyzed using ATAV software (http://www.duke.edu/~minhe/atav/).

**Sanger Sequencing**
All of the potential disease-causing variants and exons of TNXB were sequenced by the Sanger method. Primer sequences are listed in Supplemental Table 2. All sequences were analyzed with the Sequencher software (Gene Codes Corp, Ann Arbor, MI).

**In Silico Prediction of Effect of Amino Acid Substitution**
The variants identified in the TNXB gene were scored using Polyphen-2 to examine the predicted damaging effect of the amino acid substitution to the function of TNXB. PolyPhen-2 calculates a naïve Bayes posterior probability that any mutation is damaging and this is represented with a score ranging from 0 to 1. The effect of amino acid change on secondary structure of the protein was assessed by the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/).

**Joint Examination for Hypermobility**
Joint hypermobility variant of Ehlers-Danlos syndrome has previously been associated with TNXB mutations; therefore, joint examinations for hypermobility were carried out in two affected and one unaffected family members using the Beighton hypermobility score. The examiner (C.E.R.) was blinded to the renal phenotype and type and whole-exome findings.

**Skin Biopsy and Staining for Elastic Tissue**
Skin biopsies were obtained from one individual with the T3257I mutation and from an unaffected age-matched control. Samples were fixed and stained by routine hematoxylin and eosin stain and Fontana-Masson method for elastic tissue staining. The sections were examined with a Zeiss microscope and by transmission electron microscopy.

**Scratch Wound Healing Assay**
Fibroblast cell lines were established by culturing skin biopsy samples in DMEM. Cells were cultured on collagen I-coated plates and allowed to grow to confluence before scratch wound creation. Scratch wounds were created and cells were treated with PDGF (Cell Signaling Inc., Beverly, MA). Fibroblast wound healing images were obtained at 0 and 15 hours using an EVOS microscope and wound healing was quantified as the percent wound closure. Immunoblotting was performed using standard methods. Detailed methods are available in the Supplemental Methods.

**Immunohistochemical Staining and Immunofluorescence of the UVJ**
Human tissue samples with radiographically confirmed PVUR in paraffin were obtained from the pathology department and Pediatric Renal Biobank at the University of Iowa under approval of institutional board review protocol #20091770. Normal controls were obtained from autopsy specimens. Immunohistochemistry and immunofluorescence were performed on slide sections using standard methods. Slides were incubated with rabbit anti-tenascin-XB antibody (Proteintech Inc, Chicago, IL) overnight, rinsed in PBS and incubated with biotinylated anti-rabbit IgG 1:200 (BA-1000; Vector Labs, Burlingame, CA) and rinsed with PBS. Detailed methods are available in the Supplemental Methods.

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