Broadening the spectrum of Ehlers Danlos syndrome in patients with congenital adrenal hyperplasia

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Context: The contiguous gene deletion syndrome, CAH-X, was described in a subset (7%) of congenital adrenal hyperplasia (CAH) patients with a \(\text{TNXA}/\text{TNXB}\) chimera resulting in deletions of \(\text{CYP21A2}\), encoding 21-hydroxylase necessary for cortisol biosynthesis, and \(\text{TNXB}\), encoding the extracellular matrix glycoprotein tenascin-X (TNX). This \(\text{TNXA}/\text{TNXB}\) chimera is characterized by a 120 bp deletion in exon 35 and results in \(\text{TNXB}\) haploinsufficiency, disrupted transforming growth factor-\(\beta\) (TGF-\(\beta\)) signaling, and an Ehlers Danlos syndrome (EDS) phenotype.

Objective: To determine the genetic status of \(\text{TNXB}\) and resulting protein defects in CAH patients with a CAH-X phenotype but not the previously described \(\text{TNXA}/\text{TNXB}\) chimera.

Design, Settings, Participants, Intervention: A total of 246 unrelated CAH patients were screened for \(\text{TNXB}\) defects. Genetic defects were investigated by Southern blotting, multiplex ligation-dependent probe amplification, Sanger, and next-generation sequencing. Dermal fibroblasts and tissue were used for immunoblotting, immunohistochemical, and co-immunoprecipitation experiments.

Main Outcome Measures: The genetic and protein status of tenascin-X in phenotypic CAH-X patients.

Results: Seven families harbor a novel \(\text{TNXB}\) missense variant c.12174C>G (p.C4058W) and a clinical phenotype consistent with hypermobility-type EDS. Fourteen CAH probands carry previously described \(\text{TNXA}/\text{TNXB}\) chimeras and 7 unrelated patients carry the novel \(\text{TNXB}\) variant resulting in a CAH-X prevalence of 8.5%. This highly conserved pseudogene-derived variant in the TNX fibrinogen-like domain is predicted to be deleterious and disulfide bonded, results in reduced dermal elastin and fibrillin-1 staining and altered TGF-\(\beta\)1 binding, and represents a novel \(\text{TNXA}/\text{TNXB}\) chimera. Tenascin-X protein expression was normal in dermal fibroblasts, suggesting a dominant negative effect.

Conclusions: CAH-X syndrome is commonly found in CAH due to 21-hydroxylase deficiency and may result from various etiological mechanisms.
A deficiency in 21-hydroxylase (encoded by CYP21A2) leads to congenital adrenal hyperplasia (CAH, OMIM 201 910), an autosomal recessive disorder of the adrenal cortex characterized by cortisol deficiency, with or without aldosterone deficiency, and androgen excess, the severity of which depends on the degree of 21-hydroxylase impairment (1). The severe or classic form is an orphan disease occurring in 1 in 16 000 live births, while the mild or nonclassic form is estimated to occur in 1 in 1000 people (2). Flanking CYP21A2 is the TNXB gene encoding tenascin-X (TNX), an extracellular matrix (ECM) glycoprotein that is highly expressed in connective tissue. TNX plays a role in collagen fibrillogenesis and matrix maturation, though it functions in collagen fibril deposition independent of collagen synthesis (3). Normal collagen fibril deposition in connective tissue is essential for the collagenous matrix integrity and defects lead to Ehlers Danlos syndrome (EDS), a hereditary connective tissue disorder (4). Complete TNX deficiency was first reported in a patient with CAH and EDS (5). While autosomal recessive complete TNX deficiency is a cause of classical EDS (OMIM 130 000), (6) TNX haploinsufficiency is associated with hypermobile EDS (OMIM 130 020), (7) and when present in patients with CAH, results in a contiguous gene deletion syndrome termed CAH-X (8).

The CYP21A2 (OMIM 201 910) and the TNXB (OMIM 600 985) genes reside in tandem on chromosome 6p23.1 within the human leukocyte antigen histocompatibility complex in a module characterized by highly homologous sequences between functional genes (CYP21A2 and TNXB) and their corresponding pseudogenes (CYP21A1P and TNXA), which leads to frequent homologous recombination. Chimeric genes generated by large gene deletion or gene conversion events, account for 20%–30% of the common CYP21A2 pathogenic variants in 21-hydroxylase-deficient patients (9–11). To date, 9 CYP21A2/CYP21A1P (CH-1 to CH-9) and one TNXA/TNXB chimera (termed CH-1 here) have been described (5, 11). The previously described TNXA/TNXB CH-1 results in a contiguous CYP21A2 and TNXB deletion found in 7% of CAH patients, and has been estimated to account for 13% of large CYP21A2 deletions (8). TNXA/TNXB CH-1 is characterized by a 120 bp deletion crossing exon 35 and intron 35 carried over from the TNXA pseudogene; this is the only well-documented discrepancy between TNXB and TNXA.

The TNX protein is divided into an N-terminal signal peptide, followed by TNX assembly, epidermal growth factor (EGF)-like, fibronectin type III, and C-terminal fibrinogen-like domains (12). All but one previously reported TNX genetic defects are in the fibronectin type III domains (13–15). Though the exact mechanism behind TNX’s ability to organize the ECM is unknown, the TNX fibrinogen-like domain activates the transforming growth factor-β (TGF-β) pathway, a regulator of collagen production important in connective tissue dysplasias (16). Additionally, we recently showed aberrant TGF-β signaling in patients with CAH-X syndrome (17).

In this study, we evaluated the TNXB gene and the TNX protein in CAH patients with a CAH-X phenotype who did not have the previously described TNXA/TNXB CH-1. Since the junction site of a chimeric TNXA/TNXB gene theoretically can be anywhere between exons 32 through 44 (the homologous region), we hypothesized that novel TNXA/TNXB chimeras might exist. Our study provides functional evidence of a novel TNXA/TNXB chimera resulting in CAH-X syndrome, thereby broadening the genetic and phenotypic spectrum of CAH due to 21-hydroxylase deficiency.

Materials and Methods

Study Subjects
In a large prospective cohort of 246 unrelated patients (146 females, aged 1–65 years) with CAH due to 21-hydroxylase deficiency, we studied patients with a phenotype suspicious for CAH-X syndrome. Patients were enrolled in an ongoing prospective natural history study at the National Institutes of Health Clinical Center in Bethesda, MD (Clinical Trials # NCT00250159) and approval was obtained from the Emunice Kennedy Shriver National Institute of Child Health & Human Development Institutional Review Board. Written informed consent was obtained from all participants and parents of participating children. All minors at least 8 years old gave assent. The diagnosis of 21-hydroxylase deficiency was determined by hormonal evaluation (17-hydroxyprogesterone > 1200 ng/dL) and CYP21A2 genotyping (18). Previously reported subjects with CAH-X syndrome due to TNXB haploinsufficiency (14 probands, 16 related patients) and CAH controls were used as comparison groups (8).

All patients were evaluated prior to genotyping for joint hypermobility using the Beighton 9-point scale and for major and minor criteria of classical and hypermobile EDS as previously described (8). Generalized hypermobility was determined by an individual’s age and Beighton score and defined as ≥ 5 for children and ≥ 4 for postpubertal adolescents and adults (19). Skin characteristics were evaluated, including extensibility, thinness, scar formation, and striae. Transthoracic 2-dimensional echocardiography and magnetic resonance imaging (MRI) of the heart (1.5 T scanner) were performed in patients with a CAH-X phenotype and their relatives when possible.

Genetic Analysis
Sanger sequencing (CYP21A2 and TNXB genes) and next-generation sequencing (EDS panel: COL3A1, COL5A1, COL5A2 genes) were performed in a commercial diagnostics laboratory (PreventionGenetics, Marshfield, WI, USA). The
amino acid substitution prediction programs PolyPhen-2 and SIFT were used to predict missense variant pathogenicity (12, 13). Multiplex ligation-dependent probe amplification (MLPA) and Southern blotting were done as previously described (8, 20). Two hundred healthy subjects (Coriell Repository, http://ccr.coriell.org) served as controls for evaluating allele frequency in the general population. All new TNXB variants described were submitted to the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/).

Tenascin-X Modeling and Disulfide Prediction

The primary sequence of the tenascin-X C-terminal fibrinogen-like domain, corresponding to residues 4023–4233, was submitted to the Robetta server (http://www.robbeta.org) for domain (Ginzu) (21) and tertiary structure predictions (Rosetta) (22, 23). The disulfide bonding state of C4058 was analyzed by submitting the C-terminal fibrinogen-like domain to several university-based online disulfide bonding state predictors (http://bioserv.rpbs.jussieu.fr/cgi-bin/CysState) (24, 25).

Fibroblast Cell Culture

Primary skin fibroblasts were initiated from 3 mm-punch biopsy explants from 7 CAH patients with the novel TNXB variant and 10 age- and sex-matched CAH controls with a normal TNXB genotype. Fibroblasts from second through third passages were cultured to confluence in high glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen) at 37°C in 5% CO2.

Western Blot Analysis

Protein expression was analyzed by SDS-PAGE and western blot as previously described (17) with the following changes: 10 μg protein was loaded onto a 3%–8% NuPAGE Novex Tris-acetate precast gel (Invitrogen) and immunoblotting was done using a rabbit polyclonal antihuman tenascin-X (H-90) antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal antihuman β-tubulin antibody (1:2000; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Secondary antibody incubation, blot visualization, and quantification were done as previously described (17). All data were normalized to the loading control. Experiments were performed in triplicate.

Elastin and Fibrillin-1 Staining in Dermal Biopsies

Five-micrometer sections of paraffin-embedded skin biopsies were mounted on Superfrost Plus slides (Erie Scientific, Portsmouth, NH, USA) and sections were stained with hematoxylin and eosin (H&E) using a Leica CV5030 Autostainer (Leica, Buffalo Grove, IL, USA).

Elastin-Van Gieson (EVG) staining of elastic fibers was done using an Artisan Multistainer (DakoCytomation, Carpinteria, CA, USA) and Dako Artisan staining reagents (Artisan EVG Kit, DakoCytomation). The epidermal layer served as an internal negative control for each skin biopsy.

Mouse antifibrillin-1 monoclonal antibody clone 26 (EMD Millipore, #MAB2502, Billerica, MA, USA) was used at a 1:300 dilution. Automated Leica BOND-MAX and its detection kit (Leica Biosystems, Bannockburn, IL, USA) were used for antifibrillin-1 immunohistochemical staining. Primary antibody was incubated for 30 minutes, polymer and postpolymer for 15 minutes, 3,3′-diaminobenzidine chromogen for 10 minutes, and hematoxylin for 5 minutes. Negative controls were done likewise but used mouse serum without primary antibody. Slides were viewed at 200x magnification.

Co-Immunoprecipitation

Coimmunoprecipitation experiments were conducted according to Alcaraz, et al (16) Briefly, whole cell lysate (equivalent volume of 10 μg protein according to a BCA assay) from dermal fibroblasts was lysed as above and subjected to immunoprecipitation using the Dynabeads Protein G Immunoprecipitation Kit (Invitrogen) according to the manufacturer’s instructions. A purified rat anti-Mouse, Human, Pig TGF-β1 antibody (BD Biosciences, San Jose, CA, USA) was used to pull-down and Normal Rat IgG (R&D Systems, Minneapolis, MN, USA) antibody was used as a control for nonspecific binding. The entire eluates from the Dynabeads kit were loaded onto the gel and subjected to SDS-PAGE and western blotting with an anti-tenascin-X antibody as described above.

Statistical Analysis

Comparisons were made using the unpaired Student’s t test (Microsoft Excel). P-values are two-tailed and considered significant when ≤ 0.05. Data are represented as mean ± standard error of the mean (SEM).

Results

**Novel TNXB Variant c.12174C>G Associated with CAH-X**

Ten phenotypic CAH-X patients from 7 families were heterozygous for the novel TNXB missense variant in exon 40 defined as c.12174C>G (Figure 1A), which is predicted to result in the amino acid substitution p.C4058W within the TNX C-terminal fibrinogen-like domain. The prediction programs PolyPhen-2 and SIFT predicted that the p.C4058W change to be “probably damaging” and “deleterious”, respectively. This variant was not found in 200 healthy subjects. In our cohort, 14 CAH probands carry previously described TNXA/TNXB chimeras and 7 unrelated patients carry the novel TNXB variant resulting in a CAH-X prevalence of 8.5%.

All 10 CAH patients with the c.12174C>G variant had EDS clinical features (Table 1). Generalized joint hypermobility, a major diagnostic criterion for classical EDS, was found in all patients. Hypermobility of small (Figure 1B-1) and large joints (Figure 1B-2) and hernias (Figure 1B-3) were found in 4 (40%), 7 (70%), and 3 (30%) patients, respectively. Most adults (90%) had chronic arthralgia in 4 or more joints. Other characteristic CAH-X clinical findings included structural cardiac abnormalities, elongated uvula with midline crease, and piezogenic pedal papules. Unlike the previously-reported CAH-X CH-1 patients, a subset of patients with the novel c.12174C>G variant had skin laxity (40%). The clinical
phenotype of relatives with the c.12174C\>/G variant, but not CAH, was less severe, although the majority had hypermobile joints. To rule out possible pathogenic variants in other known EDS associated genes, we sequenced the COL3A1, COL5A1, and COL5A2 genes in the 10 novel CAH-X patients and found no pathogenic or likely pathogenic variants. Two missense variants of uncertain significance were found in the COL3A1 gene in patient I-1 from Family 1 and patient II-1 from Family 3. However, these two COL3A1 variants did not segregate with phenotypes between the parents and the probands, indicating that they are unlikely to be the primary cause of the phenotypes.

c.12174C\>/G as a Marker of a Novel TNXA/TNXB Chimeric Gene

At the locus of CYP21A2 and TNXB, the homology sequence between the pseudo and active TNX genes spans the CYP21A2 5′ upstream region to intron 31 of TNXB; thus, the junction site of a chimeric gene resulting from an unequal crossover event can vary (Figure 2A-F). The reference sequence of the pseudogene TNXA (NR 001284.2) at the corresponding c.12174 position is G, suggesting pseudogenic origin of this variation. To test this hypothesis, we sequenced TNXB exons 35 through 44 in 11 patients who were previously identified as heterozygous for TNXA/TNXB CH-1 (120 bp deletion) because the fragment spanning the exon 35/intron 35 boundary through exon 44 in the TNXA/TNXB CH-1 chimera represents the TNXA--specific sequence. All 11 patients were heterozygous for c.12174C\>/G in exon 40; analysis of 7 available parents showed that the 120 bp deletion and the c.12174C\>/G reside on the same allele (in cis). In addition to c.12174C\>/G in exon 40, a cluster of three heterozygous variants (c.12218G\>/A, exon 41; c.12514G\>/A and c.12524G\>/A, exon 43) was found in three individuals from two families, Family 3: II-1, Family 4: I-1, and Family 4: II-2 (Figure 1A, 2G). Absence of c.12174C\>/G and these three variants in 100 healthy controls (a subgroup of the 200 controls mentioned above) strongly supports that they are TNXA--specific, though only C.12174C\>/G is likely present in every copy of TNXA. Thus, c.12174C\>/G represents a novel distinguishable site between TNXA and TNXB.

Figure 1. CAH-X Pedigrees and Clinical Phenotypes. (A) The pedigrees of seven novel CAH-X families. Black represents the TNXB C4058W variant. CYP21A2 variants are represented as gray (CYP21A2 deletion due to CYP21A2/CYP21A1P chimera) or striped (other CYP21A2 pathogenic variants). Most CAH patients are compound heterozygotes. Five CAH carrier parents and 1 CAH carrier sibling also have the C4058W mutation. DNA was not available from siblings in Family 1 and 5. (B) Clinical findings in patients with CAH-X syndrome due to a C4058W mutation in TNXB. Hypermobile small (1) and large joints (2), and umbilical hernia (3).
The Ginzu prediction provided one domain for this region of the TNX primary amino acid sequence alignment. Since a full-length TNX tertiary structure was not available, the TNX C-terminal fibrinogen-like domain (residues 4023–4233) was submitted to the Robetta server for ab initio and comparative modeling. The primary protein sequence was first parsed into putative domains (Ginzu), and comparative modeling. The primary protein sequence was first parsed into putative domains (Ginzu), and the remaining larger portion of the domain is likely pathogenic and suspected to be the primary cause of the phenotypes in these patients.

**C4058W Likely Leads to Partial TNX Protein Unfolding**

A TNX primary amino acid sequence alignment showed that C4058 is highly conserved within the C-terminal fibrinogen-like domain across mammalian species (Figure 3A).

Since a full-length TNX tertiary structure was not available, the TNX C-terminal fibrinogen-like domain was submitted to the Robetta server for ab initio and comparative modeling. The primary protein sequence was first parsed into putative domains (Ginzu), and structural homologs for each domain were identified from protein structural databases and used to build structural models from homology modeling or ab initio calculations to predict protein structure (Rosetta) (22, 23).

The Ginzu prediction provided one domain for this region. Rosseta then modeled the domain with high confidence using homology and the known crystal structure for human ficolin-2, a carbohydrate-binding protein with a C-terminal fibrinogen-like domain (PDB: 2J61) (26). The ficolin-2 C-terminal fibrinogen-like domain crystal structure was overlaid with the TNX model (Figure 3B, left).

The two structures are nicely aligned, supporting the fidelity of this model. A neighboring cysteine in the tertiary structure (position 4028) was predicted to be within disulfide bonding distance of the highly conserved cysteine at position 4058 by three independent university-based online disulfide bonding state predictors (http://bioserv.rpbs-jus-sieu.fr/cgi-bin/CysState) (24, 25). This predicted disulfide bond is located at the interface between a small segment containing two β-sheets and an alpha helix at the top of the structure and the remaining larger portion of the domain at the bottom, possibly being released and unfolding upon mutation to a tryptophan, resulting in a partially unfolded TNX fibrinogen-like domain (Figure 3B, right).

**C4058W Disrupts TNX Function but not Expression**

In contrast to prior reports of CAH-X CH-1, (8) CAH-X CH-2 dermal fibroblasts did not have altered TNX expression in whole cell lysate compared to CAH controls by western blot (Figure 3C). However, CAH-X CH-2 patients had reduced Elastin-Van Gieson histology staining for elastic fibers in dermal biopsies compared to normal controls. Elastin staining in CAH-X CH-1 patients more closely resembled the controls, consistent with prior reports showing a spectrum of elastic fiber abnormalities in TNX haploinsufficient samples (15). Immunohistochemical staining using an antifibrillin-1 antibody showed disrupted fibrillin-1 organization in CAH-X CH-1 and CAH-X CH-2 dermal biopsies compared to normal controls (Figure 3D).

**CAH-X CH-2 Disrupts the Fibrinogen Domain’s Ability to Bind TGF-β1**

We hypothesized that CAH-X CH-2 patients may not fully bind TGF-β1 as a consequence of having a mutated fibrinogen-like domain. We immunoprecipitated a TNX fragment at the molecular weight of the fibrinogen domain and the remaining larger portion of the domain. Elastin staining in CAH-X CH-1 patients more closely resembled the controls, consistent with prior reports showing a spectrum of elastic fiber abnormalities in TNX haploinsufficient samples (15). Immunohistochemical staining using an antifibrillin-1 antibody showed disrupted fibrillin-1 organization in CAH-X CH-1 and CAH-X CH-2 dermal biopsies compared to normal controls (Figure 3D).

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**Table 1. Clinical Findings in Patients with CAH-X Due to the Novel TNXB Variant C4058W**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex and Age (yr)</th>
<th>Genotype</th>
<th>Other Joint Findings</th>
<th>Skin Findings</th>
<th>Cardiovascular Findings</th>
<th>Additional Clinical Features</th>
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<tbody>
<tr>
<td>Family 7: I-1</td>
<td>F/13 SW 4</td>
<td>S</td>
<td>Scoliosis Normal</td>
<td>Patent foramen ovale until age 4</td>
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<td>None</td>
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<tr>
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<td>Umbilical hernia</td>
<td>N/A</td>
</tr>
<tr>
<td>Family 6: II-2</td>
<td>F/40 carrier 2</td>
<td>S</td>
<td>Skin laxity</td>
<td>Normal</td>
<td>None</td>
<td>N/A</td>
</tr>
<tr>
<td>Family 6: II-3</td>
<td>F/13 SW 4</td>
<td>S</td>
<td>Scoliosis Normal</td>
<td>Patent foramen ovale until age 4</td>
<td>None</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Abbreviations: SW, classic salt-wasting; SV, classic simple-virilizing; NC, non-classic; LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium. n/a, not available. aHypermobility score was assessed by the Beighton scale. (19) bArthralgia is at least 3 months duration.
Figure 2. Schematic diagram of CYP21A1P/CYP21A2 and TNXA/TNXB chimera genes. (A) Schematic showing exons (rectangles) of the CYP21A2 and TNXB genes. CYP21A2 encodes the active 21-hydroxylase enzyme (blue). TNXB encodes active tenascin-X (red). The sizes of the exons/introns are scaled, with the exception of introns with a slash (e.g., CYP21A2 exon 9 is ~100 bp). The homology sequence spans the CYP21A2 5′ upstream region to intron 31 of TNXB. The solid triangle at the boundary of TNXB exon 35 and intron 35 denotes the 120 bp fragment absent in TNXA.

(B) Unequal crossover during meiosis can occur at the CYP21A2 (blue cross) or TNXB (red cross) locus, resulting in a CYP21A1P/CYP21A2 or TNXA/TNXB chimera. Pseudogenes CYP21A1P and TNXA are in gray and are framed with the color of the corresponding functional gene. RP1 encodes a serine/threonine nuclear kinase (gray), and RP2 is the corresponding truncated pseudogene (gray). C4 encodes the fourth component of serum complement (gray). The open triangle at the boundary of exon 35 and intron 35 denotes the 120 bp deletion in TNXA.

(C) The CYP21A1P/CYP21A2 and TNXA/TNXB chimeric genes result from unequal crossover. (D) Schematic showing exons (rectangles) of a representative CYP21A1P/CYP21A2 chimeric gene (with a junction site in exon 8) and the intact TNXB gene. CYP21A1P/CYP21A2 chimeric genes have been classified into nine types (CH-1 to CH-9) based on the junction site location. (E) Schematic showing exons (rectangles) of the
domain using a TGF-β1 antibody in CAH-X CH-2 patient whole cell lysate compared to CAH controls (16). Serum TNX exists as the full-length form, as well as multiple functional fragments, which is also likely reflected at the tissue level (6, 16, 27). Less TNX fragment was pulled down by the TGF-β1 antibody in the CAH-X CH-2 patients compared to CAH controls by western blot, suggesting a disrupted interaction with TGF-β1 (Figure 3E).

Discussion

In this study, we describe increased CAH-X syndrome prevalence relative to our previous report (8) in a large cohort of patients with CAH due to 21-hydroxylase deficiency. Through detailed genetic studies, we identify a pseudogene-derived variant c.12174C>G (p.C4058W) representing a novel TNXA/TNXB chimera that does not...
involve a 120 bp deletion in exon 35. Patients with this novel variant have the cardinal EDS features, such as joint hypermobility and chronic joint pain, as well as midline defects and skin hyperextensibility in a patient subset (8). All patients with CAH-X syndrome described to date have a similar phenotype, disrupted extracellular matrix, and altered TGF-β pathway, although we describe here mutation-specific effects on TNX protein expression (8).

We previously identified CAH-X in 7% of CAH patients; however, the genetic cause underlying the CAH-X phenotype in a patient subgroup was unknown. The lack of unequivocally distinguishable sites between intron 8 of CYP21 and intron 35 of TNX made identification of chimeric gene junction sites challenging. Due to the homologous sequence in this region, an in-depth understanding of the sequence differences between the active genes and pseudogenes is required. Unlike CYP21A2 and its pseudogene CYP21A1P, which have been extensively studied and well characterized, (28) TNXB and TNXA sequences are still largely unknown. A reference sequence for TNXA (NR 001284.2) exists; however, the variations within this pseudogene have never been investigated in detail. In our current study, we utilized the TNXA-specific sequence present in the previously identified TNXA/TNXB chimera (120 bp deletion in exon 35) to compare with allelic sequences in unaffected controls. TNXA and TNXB variation profiling and a variant comparison between them in a proper sample size in the general population is essential for future genetic studies of CAH-X and EDS. Cantürk et al showed that CYP21A1P-specific variants present with various allele frequencies within CYP21A1P (28). Similarly, our data suggest that the c.12174C>G variant is present in every copy of TNXA, while the other three identified TNXA-specific variants (c.12218G>A, c.12514G>A, and c.12524G>A) may have a lower allele frequency.

The TNX protein is known to regulate collagen deposition and crosslinking, (4, 15, 29) though the mechanism is largely unknown. Recent studies with recombinant TNX constructs and the discovery of a link between TNX and the TGF-β signaling pathway is beginning to elucidate this mechanism. The highly conserved C4058W variant found in this study is located in the fibrinogen-like domain and is likely disulfide bonded, and upon disruption would potentially lead to at least partial protein misfolding and a functional impact. Prior studies of CAH-X and EDS patients carrying the previously identified TNXA/TNXB CH-1 gene showed reduced dermal and serum TNX expression compared to controls, supporting a haploinsufficient mechanism (7, 8). However, TNX expression was unchanged in our CAH-X patients carrying the TNXA/TNXB CH-2 or TNXB variants, suggesting a dominant negative mechanism. Previous studies showed disrupted elastin fibers and reduced fibrillin-1 staining in patients with autosomal recessive complete TNX deficiency (30) and, to some extent, patients with partial deficiency due to TNX haploinsufficiency (15). Our data revealed reduced elastin staining in dermal biopsies from CAH-X CH-2 patients compared to controls, while the reduction in elastin staining in CAH-X CH-1 samples was less dramatic. Similarly, variable effects of TNX haploinsufficiency on elastin have been reported (15). In our study, fibrillin-1 organization was highly disrupted in all CAH-X patients. Though fibrillin-1 is a part of the elastic fiber and microfibrillar network, it also exists independently and is believed to interact with TNX indirectly to stabilize elastic fiber assembly (30).

TGF-β is known to regulate collagen, (31) and disrupted TGF-β signaling is implicated in a number of connective tissue disorders, (32–36) including CAH-X (17). We previously reported elevated TGF-β biomarkers in both dermal fibroblasts and tissue, as well as in circulation and secreted from cells in patients with CAH-X due to the TNXA/TNXB CH-1 chimera (17). We hypothesized that TGF-β pathway disruption could also be responsible for some of the connective tissue phenotypes in our CAH-X CH-2 patients, though likely through a different mechanism. Alcaraz and coworkers showed that the TNX C-terminal fibrinogen-like domain activated the TGF-β pathway by directly interacting with the latent TGF-β complex and inducing a conformational change, causing release of active TGF-β1 and subsequent downstream Smad signaling (16). Similarly, we found that less TNX fibrinogen domain fragment was bound by TGF-β1 in dermal fibroblast whole cell lysate from CAH-X CH-2 patients compared to controls, suggesting a disrupted interaction between TNX and TGF-β1. Further studies are needed to analyze this interaction in CAH-X CH-2 patients.

The clinical impact of carrying a TNXB defect includes the risk of chronic joint pain in adulthood, joint subluxations, hernias, and/or organ prolapse, as well as developmental cardiac defects. Unlike previously described patients with CAH-X, several patients with CAH-X due to the novel TNXA/TNXB CH-2 had skin laxity with normal healing, reflecting a phenotypic spectrum that may or may not be variant specific. Most hypermobile EDS cases are unexplained (37). Novel ways in which TNX is disrupted, including the novel TNXA/TNXB chimera described here, represent potential genetic causes of hypermobile EDS of unknown etiology and requires further study.

A strength of our study is the large cohort size with extensive phenotyping. However, limited sequence infor-
mation on the TNXA and TNXB genes remains a challenge. Also, the lack of an appropriate CAH-X mouse model is one limitation to fully dissecting the mechanism behind TNX deficiency, though the development of in vitro TNX constructs could address this issue.

Our study shows that multiple TNXA/TNXB chimeric genes can lead to CAH-X syndrome, and likely further chimeras remain to be discovered. TNX deficiency effects can be seen through a disrupted ECM and TGF-β signaling pathway in CAH-X patients, though the mechanism behind these effects is likely specific to the nature of the TNXB variant. This study reveals novel ways in which connective tissue features can manifest in CAH patients; therefore, more patients may be affected by CAH-X syndrome than previously recognized. We can now estimate that approximately 9% of CAH patients have CAH-X syndrome, expanding the phenotypic spectrum of CAH. Therefore, evaluation for a connective tissue dysplasia is warranted in CAH patients and further studies of TNXB may provide insight into the pathogenesis of EDS.

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References

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