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Tenascin-X, Congenital Adrenal Hyperplasia, and the CAH-X Syndrome

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Abstract

Mutations of the *CYP21A2* gene encoding adrenal 21-hydroxylase cause congenital adrenal hyperplasia (CAH). The *CYP21A2* gene is partially overlapped by the *TNXB* gene, which encodes an extracellular matrix protein called Tenascin-X (TNX). Mutations affecting both alleles of *TNXB* cause a severe, autosomal recessive form of Ehlers-Danlos Syndrome (EDS). Rarely, patients with severe, salt-wasting CAH have deletions of *CYP21A2* that extend into *TNXB*, resulting in a 'contiguous gene syndrome' consisting of CAH and EDS. Heterozygosity for *TNXB* mutations causing haploinsufficiency of TNX may be associated with the mild 'hypermobility form' of EDS, which principally affects small and large joints. Studies of patients with salt-wasting CAH found that up to 10% had clinical features of EDS, associated joint hypermobility, haploinsufficiency of TNX and heterozygosity for *TNXB* mutations, now called 'CAH-X'. These patients have joint hypermobility and a spectrum of other co-morbidities associated with their connective tissue disorder, including chronic arthralgia, joint subluxations, hernias and cardiac defects. Other disorders are beginning to be associated with TNX deficiency, including familial vesicoureteral reflux and neurologic disorders. Further work is needed to delineate the full spectrum of TNX-deficient disorders, with and without associated CAH.

Introduction

Congenital adrenal hyperplasia (CAH) caused by 21-hydroxylase deficiency is one of the most common disorders of the adrenal gland and is familiar to most endocrinologists. In 'classic CAH' the adrenal cannot produce cortisol adequately and overproduces androgens and androgen precursors resulting in prenatal virilization of female fetuses, whereas in 'non-classic CAH' cortisol production is essentially normal and adrenal hyperandrogenism is minimal or mild. Classic CAH is further subdivided into 'salt-wasting CAH', a severe, potentially life-threatening disorder in which the synthesis of both cortisol and aldosterone are impaired, and 'simple virilizing CAH' in which there is no salt loss, but there is

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hyperandrogenism and impaired cortisol synthesis. All CAH is caused by mutations in the single functional adrenal 21-hydroxylase gene, *CYP21A2*; the three 'forms' of CAH simply reflect the degree to which the *CYP21A2* gene is impaired, and represent convenient clinical pictures in a continuous spectrum of disease severity. CAH is a complicated disorder that is fairly well understood and requires intensive management [1]; this review focusses on a newly described variant called 'CAH-X' in which CAH is accompanied by defects in the overlapping gene for Tenascin-X (TNX).

21-hydroxylase genes

Although small amounts of 21-hydroxylase activity (especially in the synthesis of aldosterone) can be catalyzed by some hepatic enzymes [2], only deficiency of the adrenal 21-hydroxylase causes CAH. Adrenal 21-hydroxylase activity is catalyzed by an enzyme called P450c21 or CYP21. The gene encoding CYP21 lies in the middle of the human leukocyte antigen (HLA) locus on chromosome 6p21.33, hence CAH is closely linked to specific HLA types [3]. The region containing the *CYP21* genes is duplicated and contains several other very closely linked (sometimes overlapping) genes, thus this locus is among the most complex in the human genome [4,5]. The duplicated 30 kb units contain the functional *CYP21A2* gene that encodes adrenal 21-hydroxylase and the non-functional *CYP21A1P* pseudogene duplicated in tandem with the *C4A* and *C4B* genes that encode the fourth component of serum complement [6–8]. In some literature, the functional, centromeric, *CYP21A2* gene is termed 21B (because it is in the 'B unit') and the nonfunctional, telomeric *CYP21A1P* gene is termed 21A. Somewhat surprisingly, *CYP21A1P* pseudogene is transcribed, but the resultant RNAs do not encode protein [9,10]. The *CYP21A2* and *CYP21A1P* genes are about 3.4 kb long, are divided into 10 exons, and differ in only 87 or 88 bases [11–13]. This high degree of sequence similarity indicates that these two genes are evolving in tandem through intergenic exchange of DNA. This happens because the HLA locus has a very high rate of genetic recombination. Thus, 99% of mutations causing CAH derive either from gene conversion events where some or all of the *CYP21A1P* pseudogene replaces the corresponding area of the *CYP21A2* gene, of from gene deletoins where homologous recombination excises the functional gene. This high rate of genetic recombination explains why 21-hydroxylase deficiency is so much more common than deficiencies of other steroidogenic enzymes. The *CYP21* genes of most, but not all mammals are duplicated similarly, albeit with different duplication boundaries; however, while only the *CYP21A2* gene functions in human beings, only the *cyp21a1* gene corresponding to *CYP21A1P* functions in mice [14,15] and both genes function in cattle [16,17]. Sequencing of the gene duplication boundaries show that the human locus duplicated after mammalian speciation [18], consistent with data that indicate that other mammals have single copies of this locus [19].

Other genes in the 21-hydroxylase locus

Several other genes lie in the *CYP21* gene locus, and several adrenal-specific transcripts have been identified that do not have known functions (Fig. 1). The best-studied of these neighboring genes are the tandemly duplicated *C4A* and *C4B* genes that encode the two isoforms of C4, the fourth component of serum complement. The C4B protein has >99%

amino acid sequence identity with C4A but has more hemolytic activity [20]. The *C4A* and *C4B* genes are ~22 kb long, although C4B may be only 16 kb long on some alleles due to a deletion within one intron [21]. Whereas in most regions of the genome where genes are separated by long segments of intergenic DNA, the 3' ends of the *C4A* and *C4B* genes are only 2466 base pairs upstream from the transcriptional start sites of the *CYP21A1P* and *CYP21A2* genes, respectively. The *C4* and *CYP21* genes have remained in close physical association throughout evolution because the promoter sequences responsible for adrenal-specific expression of the mouse [22] and human [23] *CYP21* genes lie far upstream within intron 35 of the *C4* genes. Just upstream from *C4A* is a gene now called *STK19*; it was originally called G11 [24] or RP [25], but the name *STK19* is is now accepted because it encodes a Serine/Threonine Kinase [26]. The STK19 protein is localized to the nucleus, suggesting a role in gene transcription, but its function remains unknown. There is no homologue of *STK19* immediately upstream from the *C4B* gene because much of the coding DNA in this region was lost during the duplication of the *C4/CYP21/TNX* locus, so that only 914 bases of the 3' end of the gene lie upstream from C4B [24,25].

The *TNXA* and *TNXB* genes lie on the opposite strand of DNA from the *C4* and *CYP21* genes and hence have the opposite transcriptional orientation. These genes partially overlap the 3' ends of the *CYP21*genes: the last exon of *TNXA* and *TNXB* lies within the 3' untranslated region of exon 10 in *CYP21A1P* and *CYP21A2*, respectively [27], and contain fibronectin type III repeats [28]. The *TNXA* gene was truncated during the duplication of the ancestral *C4/CYP21/TNX* genetic unit, but nevertheless it is transcribed in the adrenal [18]. Immediately upstream of *TNXB* lies a gene called *CREB-RP*, which encodes a protein related to the CREB (cyclic AMP response element binding protein) transcription factor [29]. Transcription of *TNXB* is initiated from multiple start sites in or near *CREB-RP* [30,31]. Thus *TNXB* us unique in having both ends overlapping other genes. The *TNXB* gene is very large, spanning 68.2 kb of DNA and includes 43 exons encoding a 12kb mRNA that encodes the extracellular matrix protein, Tenascin-X (TNX) [30,32]. The *TNXB* gene also encodes a truncated 74 kDa form of Tenascin-X, called XB-S (XB-Short), which is identical to the carboxy-terminal 673 amino acids of TNX, arises from an intergenic promoter and is expressed uniquely in the adrenal [33]. Expression of XB-S is induced by hypoxia [34], and proteomics studies indicate it associates with mitotic motor kinesin Eg5, but the precise function of XB-S remains unclear [35]. In addition, RNA transcripts termed YA and YB arise from the *CYP21A1P* and *CYP21A2* promoters, respectively, but do not encode protein [9]. Transcripts having an open reading frame, termed ZA and ZB, arise from a promoter element within intron 35 of the C4 genes, but it is not clear whether or not they encode protein [36].

Tenascin-X

The tenascins are a widely-expressed family of extracellular matrix proteins. Their functions typically oppose those of fibronectin and are largely associated with anti-adhesive effects but extend beyond cellular architecture [37]. There are four mammalian tenascins: according to the nomenclature first proposed by Bristow et al [32], these are now called tenascin-C (TNC, formerly called cytotactin) [38,39], tenascin-R (TNR, formerly called 'restrictin' or 'janusin') [40,41], tenascin-X (TNX), and tenascin-W (TNW, also tenascin-N) [42,43]. The

tenascin proteins are characterized structurally from N-terminus to C-terminus by: i) an N-terminus with 7-amino acid repeats flanked by cysteine residues (TNX has 4 such repeats); ii) a series of repeats that resemble epidermal growth factor (TNX has 18.5); iii) a stretch of fibronectin type III repeats that vary in number as a result of alternative RNA splicing (TNX has 33); iv) a large C-terminal domain structurally related to fibrinogen [32,44]. The N-terminal heptad repeats mediate oligomerization; TNX [45] and TNR [46] form homo-trimers; TNC [44] and TNW form homo-hexamers. Each TNX monomer is 4267 amino acids long with a mass of about 450 kDa; TNX is variably glycosylated and forms trimers whose masses approach 1.4 million daltons; TNX is expressed in most tissues, especially connective tissues [47].

Identification of a CAH patient with a 'contiguous gene syndrome' comprising a deletion of both the *CYP21A2* and *TNXB* genes, demonstrated that Tenascin X deficiency results in Ehlers-Danlos syndrome (EDS) [48]. The causative role of TNX deficiency in connective tissue disorders is confirmed in *tnx*-knockout mice [49]; it is noteworthy that mouse knockouts of other tenascins do not yield identifiable phenotypes [50–52]. EDS is typically caused by autosomal dominant mutations in collagen, but recessive forms can be caused by mutations in genes for collagen-modifying factors, such as TNX, which is associated with and stabilizes collagen fibrils [45,53]. Tenascin-X deficiency causes a clinically distinct, more severe form of EDS, either with or without associated CAH [54–56]. Heterozygosity for severe *TNXB* mutations causing haploinsufficiency for TNX may cause ‘hypermobility type EDS’, characterized by joint hypermobility, recurring joint dislocations and joint pain. Among 20 obligate heterozygotes for a severely defective *TNXB* allele, 9 of 14 females but no males had hypermobility EDS [57]. The diagnosis of TNX-deficient EDS or TNX haploinsufficiency is facilitated by the existence of a 140 kDa proteolytic fragment of TNX in normal serum, but not in the sera of TNX-deficient patients; this 140 kDa fragment of TNX can be measured easily [54, 58].

Beyond classical EDS, TNX is important in development as it promotes epithelial-mysenchymal transitions via TGF-β [59], and may be associated with tumor invasion [60,61]. TNX deficiency has been associated with primary myopathy [62,63], recurrent gastrointestinal perforation [64], and missense mutations in TNX have been found in vesicoureteral reflux [65,66]. TNX, which is expressed in the leptomeninges and choroid plexus [67,68], may play a role in brain and behavior. Single nucleotide polymorphisms in *TNXB* are associated with schizophrenia [69,70], and *tnxb* knockout mice have increased anxiety, improved memory and higher sensorimotor coordination than control animals [71].

CAH-X

The initial report of TNX deficiency was in a single patient with CAH and EDS [48]. To evaluate whether isolated TNX deficiency was associated with EDS, Schalkwijk *et al* [54] evaluated 151 patients with EDS of unknown etiology and found that 5 patients had TNX deficiency, one with CAH. These initial studies of TNX deficiency were consistent with an autosomal recessive pattern of inheritance and the two patients with concomitant CAH had 30 kb deletions involving both the *CYP21A2* and *TNXB* genes. Relatives who were

heterozygous for *TNXB* mutations were either asymptomatic or had mild hypermobility-type EDS of unknown clinical importance [57].

The first evaluation of the potential clinical implications of *TNXB* heterozygosity in CAH patients was performed in an ongoing observational study of CAH at the National Institutes of Health Clinical Center. In a prospective study, 193 consecutive unrelated patients with CAH were evaluated clinically for manifestations of EDS and genetically for *TNXB* mutations. Heterozygosity for a *TNXB* deletion was present in 7% of CAH patients; these CAH patients were more likely than age-and sex-matched CAH patients with normal *TNXB* to have joint hypermobility, chronic joint pain, multiple joint dislocations and a structural cardiac valve abnormality by echocardiography [72]. Six of 13 probands had a cardiac abnormality including the rare finding of a quadricuspid aortic valve [73], a left ventricular diverticulum and an elongated anterior mitral valve leaflet [72]. Six parents were also evaluated representing relatives who did not have CAH but were heterozygous for a *TNXB* deletion. Similar to the earlier findings of relatives of the autosomal recessive TNX deficient EDS patients, parents displayed variable symptomatology ranging from no EDS manifestations to hypermobility-type EDS, two with cardiac findings (dilated aortic root and redundant anterior mitral leaflet) [72]. In general, carrying a contiguous deletion of the *CYP21A2* and *TNXB* genes resulted in a more severe EDS phenotype in CAH patients than in their CAH unaffected relatives. As a result of this study, the term CAH-X was coined to describe the subset of CAH patients who display an EDS phenotype due to the monoallelic presence of a *CYP21A2* deletion extending into the *TNXB* gene.

The study of CAH-X has provided insight into the recombination events that occur in the class III region of the MHC locus. This region of the genome is predisposed to genetic recombination due to the presence of duplicated genes; both the *CYP21A2* and *TNXB* genes have corresponding homologous pseudogenes (*CYP21A1P* and *TNXA*) giving rise to misalignment during meosis. Approximately 20% of deleterious *CYP21A2* mutations are 30 kb gene deletions that result in *CYP21A1P/CYP21A2* chimeric genes [74–76]. Nine types of *CYP21A1P/CYP21A2* chimera have been identified and named chronologically CH-1 to CH-9 following determination of the junction sites [77]. Similarly, chimeric recombination between *TNXB* and *TNXA* occurs (Fig. 2A). Such a recombination event deletes *CYP21A2*, and therefore also represents a CAH disease-causing allele. The contiguous gene deletion described in the first studies of *TNXB* in EDS [48, 54] and CAH populations [72] was a *TNXA/TNXB* chimeric gene [48] which was later termed *TNXA/TNXB* CH-1 or CAH-X CH-1, in keeping with *CYP21A1P/CYP21A2* terminology [78]. CAH-X CH-1 retains a *TNXA* pseudogene derived 120-bp deletion and renders the gene nonfunctional, resulting in reduced dermal and serum TNX expression supporting a haploinsufficent mechanism [54, 72].

After the initial description of CAH-X in a large cohort of patients with CAH, novel *TNXA/TNXB* chimeras, named CAH-X CH-2 and CAH-X CH-3 were identified [78, 79]. CAH-X CH-2 is characterized by the variant c.12174C>G (p.C4058W) derived from the *TNXA* pseudogene [78] and CAH-X CH-3 is characterized by a cluster of 3 closely linked mutations (p.R4073H, p.D4172N, and p.S4175N), also derived from the *TNXA* pseudogene [79] (Fig. 2B). Unlike CAH-X CH-1, both CAH-X CH-2 and CAH-X CH-3 cause structural

changes in the TNX protein. CH-2 (p.C4058W) deletes a cysteine that forms a disulfide bond, and computational studies show that it alters protein structure [78]. CH-3 changes three residues (p.R4073H, p.D4172N, and p.S4175N). The p.R4073H change is predicted to reduce protein folding energy by interfering with a cation-pi interaction between p.R4073 and p.F4080 [79]. The changes p.D4172N, and p.S4175N are not predicted to significantly affect the folding energies in the models, but computational analysis is imperfect and future experimental verification is warranted. Because CAH-X CH-2 and CH-3 produce altered proteins, rather than reducing TNX expression, we no longer use the term 'haploinsufficiency' in describing the monoallelic presence of a *TNXA/TNXB* chimera. Similarly, the term 'autosomal recessive' is not used to describe patients with CAH-X who have *TNXB* disease causing mutations on both alleles because 'autosomal recessive' implies that having one affected allele does not result in a clinical phenotype. This is not the case with CAH-X.

To date, 24 patients (19 families) with monoallelic CAH-X [72, 78] and 5 patients (5 families) with biallelic CAH-X [48, 54, 79] have been described. Approximately 10 percent of patients with CAH due to 21-hydroxylase deficiency are now estimated to be affected by CAH-X [78]. Overall, CAH-X patients have generalized joint hypermobility, subluxations, chronic arthralgia and about 25% have cardiac abnormalities (Table 1, Figure 3). CAH-X CH-2 causes a more severe phenotype than CAH-X CH-1, characterized by greater skin and joint involvement [78] (Table 1). Patients heterozygous for CAH-X CH-1 have normal skin, while 40% of CAH-X CH-2 patients have skin laxity. Gastrointestinal disorders, such as chronic gastroesophageal reflux and irritable bowel syndrome, and hernia or rectal prolapse are more commonly found in patients heterozygous for CAH-X CH-2 than CAH-X CH-1 (Table 1). Other clinical findings in monoallelic CAH-X include bifid uvula (n=4), scoliosis (n=3), pectus excavatum (n=1) and early onset osteoarthritis (n=1). CAH-X CH-3 has only been described in biallelic CAH-X, so the CAH phenotype associated with heterozygosity for CAH-X CH-3 is unknown. Once again, relatives of CAH-X patients who carry a CAH-X CH-2 or CAH-X CH-3 allele but do not have CAH had a milder phenotype than CAH-X patients, although the majority of relatives had hypermobile joints and two had cardiac findings (atrial septum aneurysm with patent foramen ovale, and chamber enlargement) [78, 79].

All patients with biallelic CAH-X show severe skin hyperextensibility (Fig 3G,H), and significant joint hypermobility, and two (19 yo male, 26 yo male) had delayed wound healing [48, 79]. Other EDS manifestations in CAH-X biallelic patients include multiple joint dislocations, chronic arthralgia, chronic tendonitis and/or bursitis, rectal prolapse, severe gastroesphagelal reflux and cardiac abnormalities (ventricular enlargement in two). Biallelic CAH-X patients appear to have a more severe phenotype than biallelic TNX deficient type EDS patients without CAH who have normal wound healing [54, 56, 80]. *TNXA/TNXB* chimeras may lead to a more severe phenotype than *TNXB* missense or nonsense mutations, but the hormonal factors characteristic of CAH including chronic glucocorticoid treatment may also influence phenotype.

Most previously reported *TNXB* mutations causing EDS are located in the region encoding the EGF-like repeats or the fibronectin type III domain of the tenascin protein, while *TNXA/*

TNXB chimeras either interfere with TNX production (CAH-X CH-1) or affect the fibrinogen-like domain (CAH-X CH-2, CAH-X CH-3). The junction site of a *TNXA/TNXB* chimera could be anywhere between exons 32 and 44, the homologous region between *TNXA* and *TNXB*. Unlike *CYP21A2* and *CYP21A1P*, variations in the sequences of *TNXB* and *TNXA* are not well characterized and novel chimeras may yet exist. Chimeric junction sites can be clinically meaningful. Two rare *CYP21A1P/CYP21A2* chimera (CH-4 and CH-9) result in a milder CAH phenotype than other *CYP21A1P/CYP21A2* chimeras [77, 81]. Mutation specific effects on TNX protein expression have been described between CAH-X chimeras and might occur across the spectrum of *TNXB* mutations. Further studies are needed as variations within the *TNXA* and *TNXB* genes have not been investigated in detail.

Extensive studies of the *CYP21A2* gene led to the discovery of *TNXB* and have expanded our understanding of the spectrum of TNX-related disorders. The study of CAH-X led to the discovery of different *TNXA/TNXB* chimera; 3 have been identified to date. The TNX matrix protein interacts with TGF-β [59] but the precise molecular mechanisms have yet to be determined. The identification of CAH-X syndrome and additional *TNXB*-related diseases promise to expand our understanding of this widely expressed extracellular matrix protein in various disease processes.

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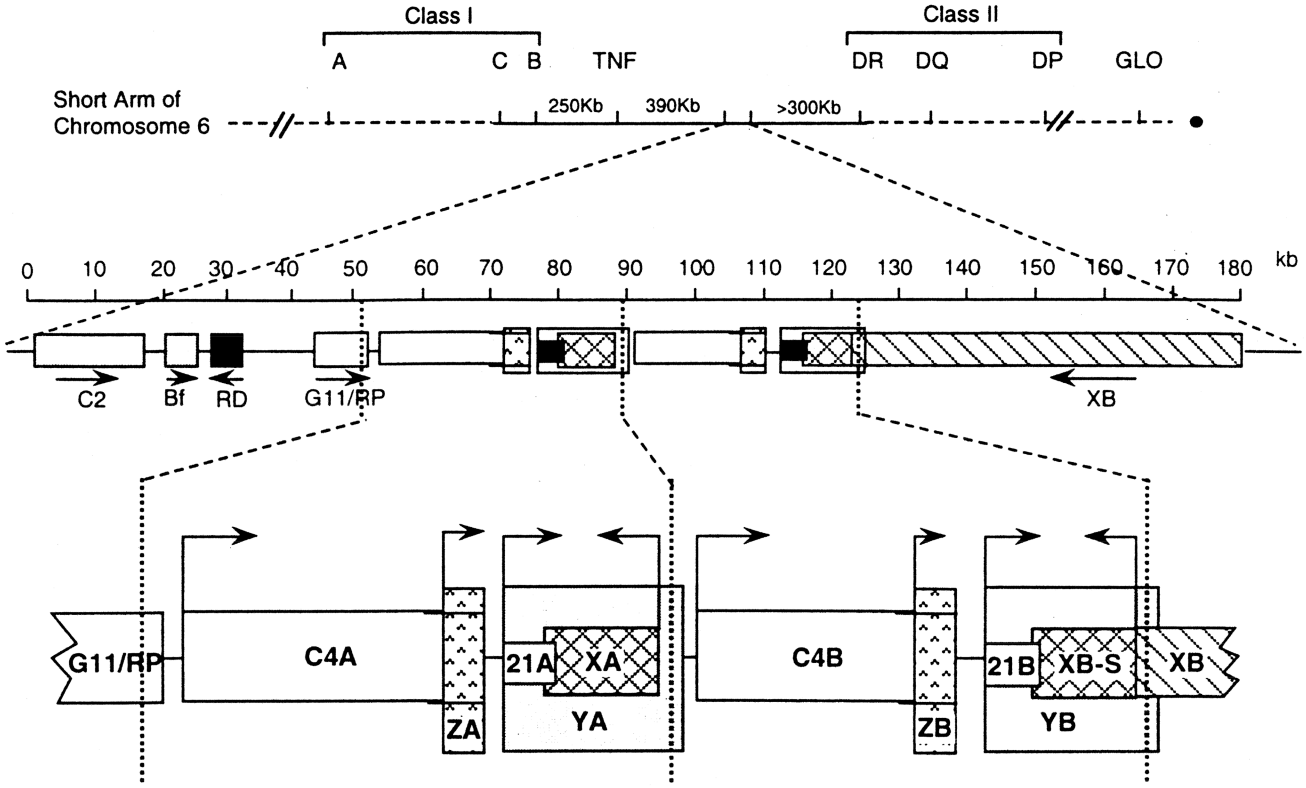

Fig. 1.

The 21-hydroxylase gene locus. Top: diagram of the short arm of chromosome 6, showing the HLA Class I and Class II regions; the telomere is to the left and the centromere to the right. Middle: enlarged view of ~180 kb showing the Class III region on chromosome 6p21.33. C2, complement factor C2; Bf, properedin factor Bf; RD (now known as *NEFLE*), negative elongation factor subunit E; G11/RP (now known as *STK19*), serine/threonine kinase 19. The arrows indicate transcriptional orientation. Bottom: the duplicated 30 kb 'A' and 'B' units: C4A and C4B, genes for complement component 4; 21A, the inactive *CYP21A1P* pseudogene; 21B the active *CYP21A2* gene; XA, YA, and YB, adrenal transcripts that lack open reading frames; XB, the *TNXB* gene for TNX; XB-S a short, adrenal-specific form of TNX of unknown function; ZA and ZB, adrenal-specific transcripts with open reading frames arising from promoters within the C4 genes; the ZA and ZB promoters are essential components of the *CYP21A1P* and *CYP21A2* promoters. The vertical dotted lines designate the boundaries of the genetic duplication event that resulted in the A and B regions. © WL Miller

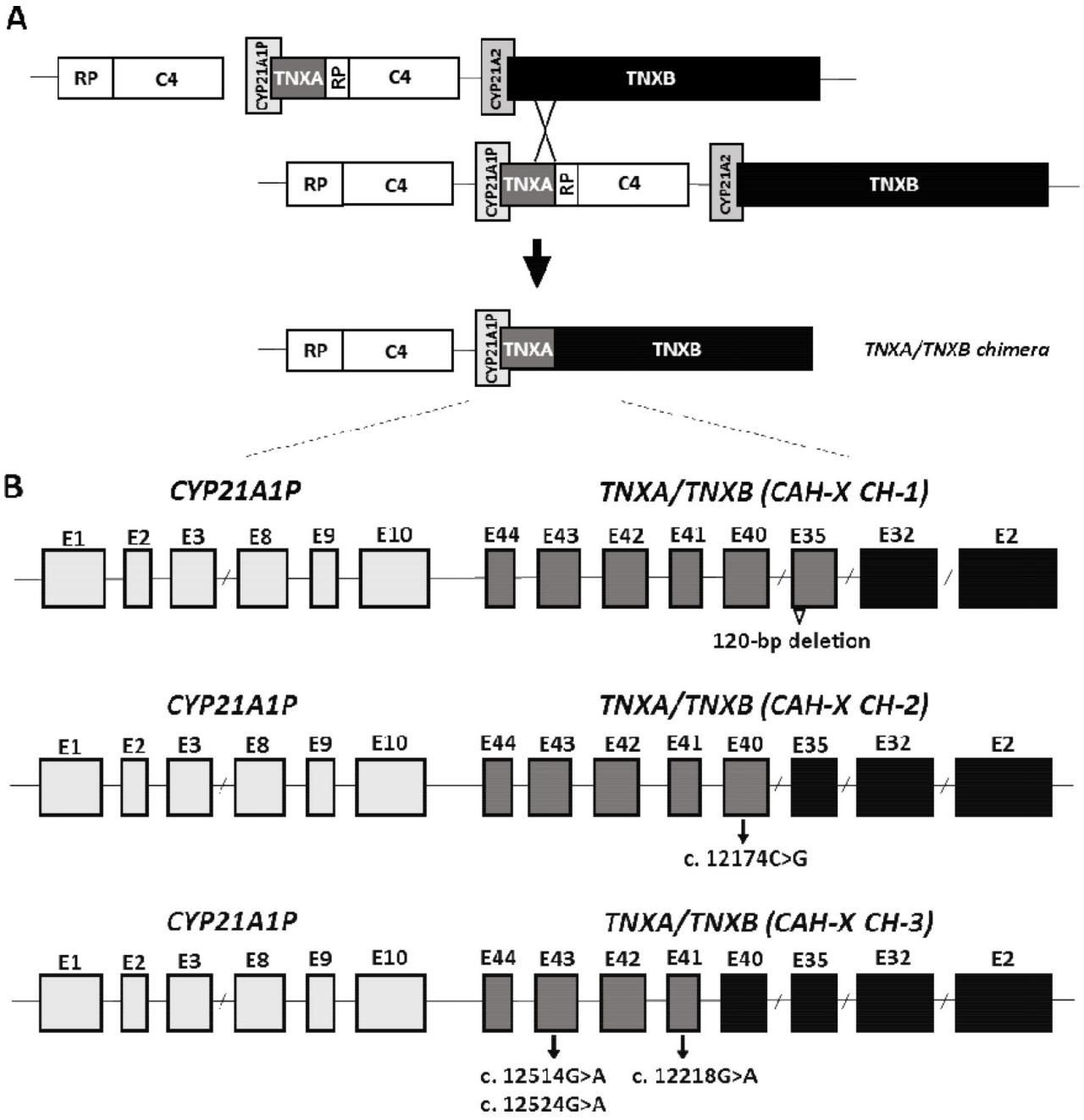

Fig. 2.

Schematic diagram of the *TNXA/TNXB* chimeric genes. Most common is a bimodular *RP-C4-CYP21-TNX* region. *TNXB* encodes the active tenascin-X gene (black) and *TNXA* encodes the corresponding pseudogene (dark grey). *CYP21A2* encodes the active 21-hydroxylase gene (medium grey) and *CYP21A1P* encodes the corresponding pseudogene (light grey). Panel A shows formation of a *TNXA/TNXB* chimeric gene due to misalignment during meiosis resulting in deletion of the *CYP21A2* gene. Panel B is a schematic of exons (rectangles) of representative *TNXA/TNXB* chimera. *TNXA/TNXB* chimeric genes have been classifed into 3 types (CH-1 to CH-3) based on the junction site

location. The 120-bp deletion at the boundary of exon 35 and intron 35 of *TNXB* is shown by an open triangle and present in CAH-X CH-1. The c.12174C>G pseudogene variant in Exon 40 identifies CAHX CH-2, and CAH-X CH-3 is characterized by a cluster of 3 pseudogene variants: c.12218G>A in exon 41, and c.12514 G>A, and c.12524 G>A in exon 43. Adapted from reference [78]; with permission.

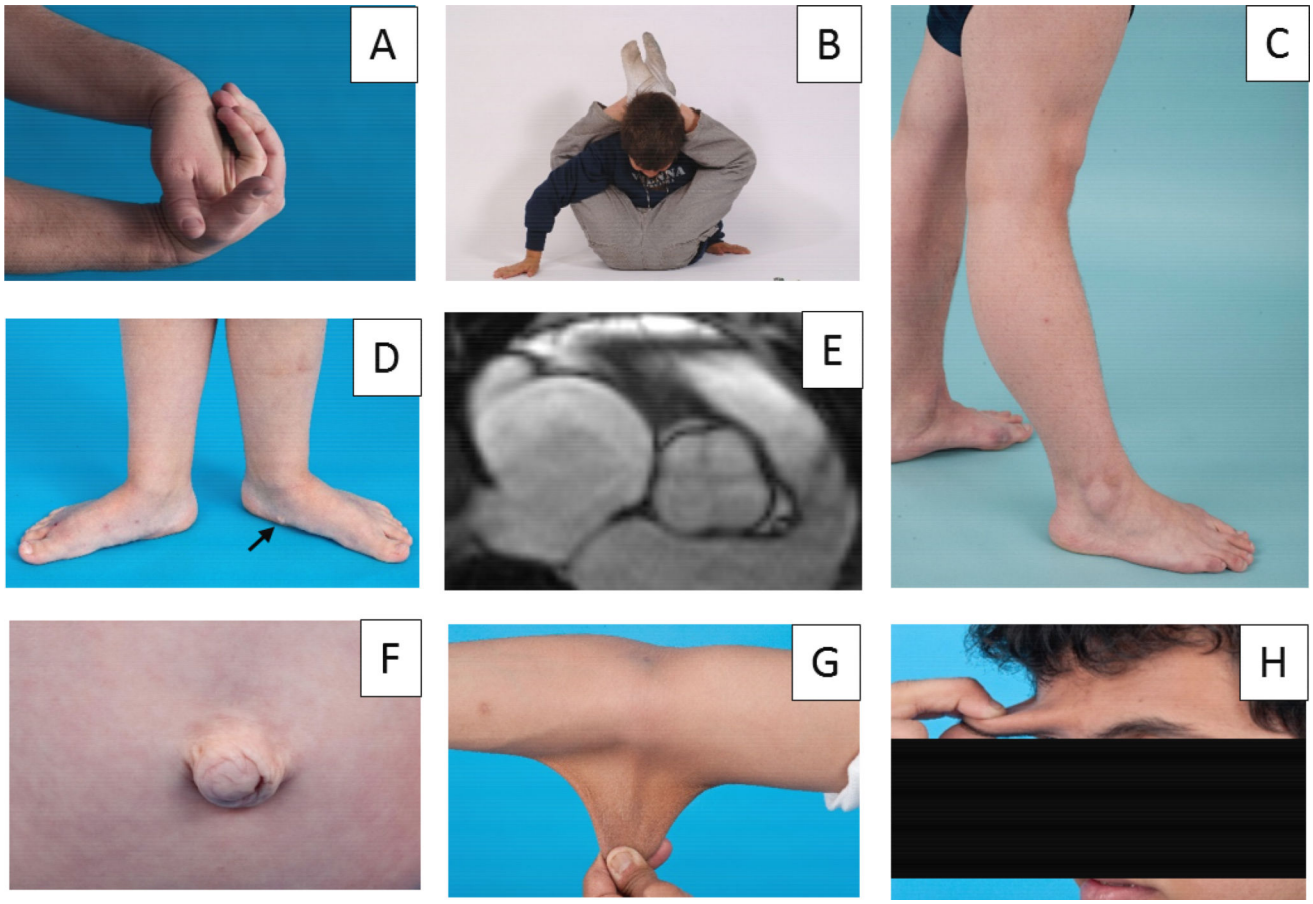

Fig. 3.

Clinical manifestations of CAH-X. Patients with CAH-X due to heterozygosity of a *TNXA/TNXB* chimera commonly have hypermobility of small joints, shown in Panel A, and large joints, shown in Panels B and C. Pes planus and piezogenic papules (arrow), shown in Panel D, are frequently observed. Approximately 25 percent of patients have a congenital cardiac defect. Panel E shows a quadricuspic aortic valve in a patient heterozygous for CAH-X CH-1 [73]. Hernias are most often observed in patients heterozygous for CAH-X CH-2, shown in Panel F, or biallelic for CAH-X. Hyperextensible skin is observed with CAH-X CH-2, and most severe with biallelic CAH-X, shown in Panels G and H. Panels C and F from reference [78]; with permission.

Table 1

Clinical Characteristics of Patients with CAH-X

	Monoallelic CAH-X CH-1[a] (n=14)	**Monoallelic CAH-X CH-2[b] (n=10)**	**Biallelic CAH-X[c] (n=5)**
Age (years, mean ± s.d., range)	18.1 ± 8.3 (8–32)	20.8 ± 16.4 (2–45)	24.0 ± 7.4 (14–32)
Females [no, (%)]	8 (57.1)	6 (60.0)	1 (20.0)
Musculoskelatal (no, (%)			
Generalized hypermobility[d]	7 (50.0)	10 (100.0)	5 (100.0)
Subluxations	5 (35.7)	4 (40.0)	2 (40.0)
Chronic arthralgia	5 (35.7)	4 (40.0)	2 (40.0)
Chronic tendonitis, bursitis or fasciitis	2 (11.8)	3 (30.0)	2 (40.0)
Dermatologic			
Hyperextensible skin'	0	4 (40.0)	5 (100.0)
Wide scars	3 (21.4)	2 (20.0)	2 (40.0)
Easy bruising	5 (35.7)	4 (40.0)	5 (100.0)
Poor wound healing	0	0	2 (40.0)
Gastrointestinal			
Chronic disorder[e]	1 (7.1)	4 (40.0)	1 (20.0)
Hernia or rectal prolapse	0	3 (30.0)	2 (40.0)
Cardiac			
Congenital defect[f]	3 (21.4)	3 (30.0)	0
Chamber enlargement	2 (11.8)	0	2 40.0)
Enlarged aortic root	0	2(20.0)	0

[a] In Merke *et al* 2013 [72].

[b] In Morissette *et al* 2015 [78].

[c] In Burch *et al* 1997 [48], Schalkwijk *et al* [54], Chen *et al* 2016 [79].

[d] Generalized hypermobility defined as a Beighton score 5 of 9 or greater for children and 4 of 9 or greater for postpubertal adolescents and adults.

[e] Chronic disorder includes gastroesphageal reflux or irritable bowel syndrome.

[f] Congenital heart defect includes structural valve abnormality, left ventricular diverticulum and patent foramen ovale.