Loss of Dermatan-4-Sulfotransferase 1 Function Results in Adducted Thumb-Clubfoot Syndrome

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Adducted thumb-clubfoot syndrome is an autosomal-recessive disorder characterized by typical facial appearance, wasted build, thin and translucent skin, congenital contractures of thumbs and feet, joint instability, facial clefting, and coagulopathy, as well as heart, kidney, or intestinal defects. We elucidated the molecular basis of the disease by using a SNP array-based genome-wide linkage approach that identified distinct homozygous nonsense and missense mutations in *CHST14* in each of four consanguineous families with this disease. The CHST14 gene encodes N-acetylgalactosamine 4-O-sulfotransferase 1 (D4ST1), which catalyzes 4-O sulfation of N-acetylgalactosamine in the repeating iduronic acid-α1,3-N-acetylgalactosamine disaccharide sequence to form dermatan sulfate. Mass spectrometry of glycosaminoglycans from a patient's fibroblasts revealed absence of dermatan sulfate and excess of chondroitin sulfate, showing that 4-O sulfation by CHST14 is essential for dermatan sulfate formation in vivo. Our results indicate that adducted thumb-clubfoot syndrome is a disorder resulting from a defect specific to dermatan sulfate biosynthesis and emphasize roles for dermatan sulfate in human development and extracellular-matrix maintenance.

Introduction

Glycosaminoglycans such as dermatan sulfate (DS), chondroitin sulfate (CS), and heparan sulfate (HS) are long chains of repeating disaccharide subunits that are covalently bound to serine residues of a variety of core proteins via an O-linked tetrasaccharide linkage sequence O-xylose-galactose-galactose-glucuronic acid to form proteoglycans as shown schematically in Figure 1.[1] Addition of a β-N-acetylgalactosamine (GalNAc) to the linkage sequence by GalNAc transferase I (MIM 602273) distinguishes CS and DS from HS. The CS C-5 hydroxyl of glucuronic acid (GlcUA) is epimerized to iduronic acid (IdoUA) by glucuronyl C5-epimerases.[2] The addition of sulfate to the C-4 hydroxyl of GalNAc prevents further epimerization of the adjacent GlcUA or IdoUA. Four GalNAc-4-sulfotransferases have been described that differentiate between CS and DS chondroitin 4-sulfotransferases 1–3 (CHST11 [MIM 610128], CHST12 [MIM 610129], CHST13 [MIM 610124]) and dermatan-4-O-sulfotransferase 1 (CHST14 (D4ST1) [MIM 608429]).[3,4] CHST12 has been reported to transfer sulfate to both chondroitin and dermatan;[5] however, it does not demonstrate a marked preference for chondroitin or dermatan and requires high concentrations of acceptor to obtain significant levels of sulfate transfer. Dermatan sulfate can be further modified by the addition of sulfate to the C-2 hydroxyl of the IdoA and, to a lesser extent, of the GlcA, as well as to the C-4 or C-6 hydroxyls of terminal GalNAc.[6] In each case 3′-phosphoadenosine 5′-phosphosulfate acts as the sulfate donor, and transfer occurs in the *trans* network of the Golgi. The proportion of IdoUA and GlcUA and the degree of sulfation in DS vary considerably in tissues and, in the case of cultured cells, are influenced by the level of free sulfate in the culture medium.[7]

Mature proteoglycans have been implicated in a wide range of biological processes, including cell migration, proliferation, and survival, as well as modulation of growth-factor signaling. Although the roles of HS and CS in development and growth-factor signaling have been extensively studied,[8–11] the biological function of DS is less well understood.[12,13] DS proteoglycans have widespread distribution in mammalian tissues, such as blood vessel walls, skin, tendon, sclera, cartilage, and in undifferentiated mesenchymal tissue,[14] where they participate in extracellular matrix (ECM) organization, neurite outgrowth, wound repair, anticoagulant processes, and cell adhesion, migration, and proliferation.[15,16] DS proteoglycans include decorin, biglycan, versican, and endocan.[15] Interactions between DS proteoglycans and particular target molecules have been described for their core proteins as well as their DS chains. The biological activities of DS have been attributed to its content of C-4 sulfated IdoUA-GalNAc disaccharide units.[12,17]

In this study, we identified four distinct homozygous mutations in *CHST14* in patients with the adducted thumb-clubfoot syndrome (ATCS [MIM 601776]) from

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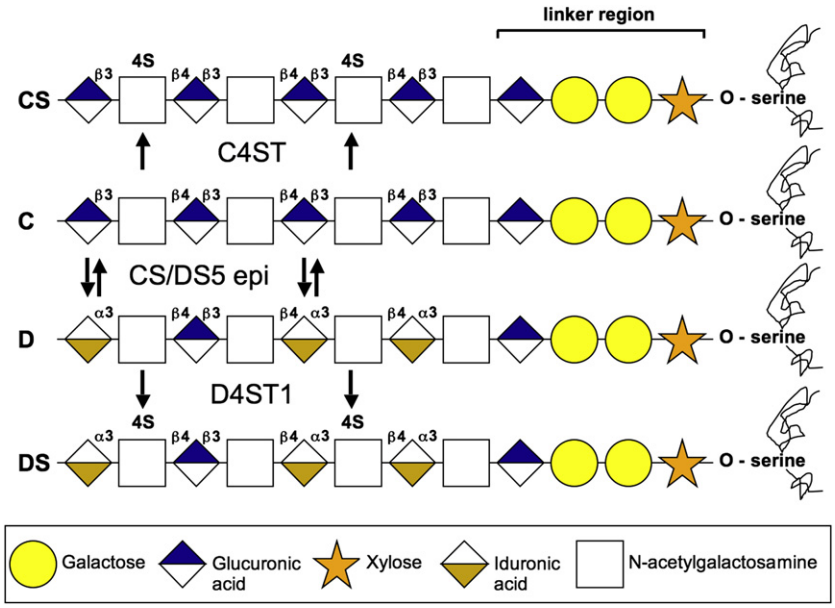

Figure 1. Biosynthesis of Dermatan Sulfate and Chondroitin Sulfate
Chondroitin sulfate (CS), dermatan sulfate (DS), and also heparin sulfate share the synthesis of a tetrasaccharide linker region that attaches the glycosaminoglycan chains to a serine within the conserved attachment site of core proteins. The activity of a unique N-acetylgalactosaminyltransferase (GalNAcT-I) that transfers the first residue onto the tetrasaccharide linker starts a growing glycosaminoglycan chain to CS. This step is followed by the activities of specific enzymes that polymerize the glycosaminoglycan chain by the alternating addition of N-acetylgalactosamine (GalNAc) and glucoronic acid (GlcUA) moieties in CS. CS chains can be modified during elongation by a Golgi resident epimerase and a number of sulfotransferases. Epimerization of GlcUA to iduronic acid (IdoUA) by 5-hydroxyl epimerase (CS/DS5 epi) followed by sulfate addition to the C4 hydroxyl of the adjacent GalNAc residue by D4ST1 generates DS from CS and prevents back-epimerization of IdoUA to GlcUA.

four families. We recently delineated ATCS as an autosomal-recessive, generalized connective tissue disorder with congenital, distal contractures, a variable degree of congenital malformations, and normal cognitive development.[18–20] The identified *CHST14* mutations abolish or decrease D4ST1 activity by early protein truncation and altered intracellular protein processing, resulting in a decrease of DS and increase of CS in patient fibroblasts and in the culture medium. To the best of our knowledge, ATCS appears to be the first disorder caused by a defect specific to DS biosynthesis, producing a generalized involvement of organs and revealing roles for DS in human development and ECM maintenance.

Material and Methods

Subjects

Eleven patients with ATCS from four consanguineous families of Austrian (family 1), Turkish (families 2 and 3), and Japanese (family 4) descent were identified in the course of this study. Initial clinical descriptions of seven patients from these four families have been published.[18–21] The patients from family 4 were originally reported as representing "a new type of distal arthrogryposis."[21] ATCS represents a generalized connective tissue disorder with normal cognitive development. ATCS can be recognized clinically by a pattern of symptoms comprising a severely wasted build and dry and translucent skin, brachycephaly, and facial characteristics such as a broad and flat forehead, hypertelorism, downward-slanting palpebral fissures, malar flatness, retrognathia, and prominent ears (Figure 2). The anterior fontanel is large at birth, and closure is delayed until after 2 years of age. Congenital contractures of the thumbs improve spontaneously within weeks, whereas congenital clubfeet require surgical treatment. Marked arachnodactyly and tapering of the fingers, as well as hypermobility of the small joints of the hands, feet, and the shoulders, are present. Delayed wound healing, ecchymoses, and hematoma formation were recorded. Detailed analyses of blood coagulation in an 8-year-old patient from family 1 revealed a prolonged bleeding time (9 min, reference range: < 7 min), indicating the presence of a coagulopathy in addition to a form of generalized connective tissue weakness. Mild osteopenia is apparent in childhood (Figure 3); however, bone densitometry of the lumbar spine was normal in the 8-year-old proband of the Austrian family but revealed osteopenia (Z score −1.6) and osteoporosis (Z score −4.6) in the two Turkish siblings at age 15 and 6 years, respectively, all three of whose photographs are shown in Figure 2. Minor degrees of cranial ventricular enlargement were present in all five patients examined. Bluish sclerae and intermittent exotropia were observed in four patients. An atrial septal heart defect was noted in two patients and mild coarctation of the aorta in one patient. One patient was found to have a horseshoe kidney, and two patients had bilateral hydronephrosis. All examined male patients were found to have undescended testes at birth. A cleft palate and cleft lip and palate were present in sibs from a Japanese family. Absence of the gastrocolic omentum was observed in association with a spontaneous volvulus of the small intestine in one Austrian ATCS patient, and a common mesentery was present in his deceased brother. This patient was born at 31 weeks of gestation with heart and kidney defects and died at birth from respiratory failure. Three affected children from two Turkish families died before four months of age and one child at age six years, and the causes of death were not recorded.

Linkage and Mutation Analysis

Written informed consent for molecular studies was obtained from all participants, and the study was conducted in accordance with the principles of the declaration of Helsinki. We carried out a genome-wide linkage scan with the Affymetrix XbaI 50K SNP arrays in four affected and 11 unaffected individuals from families 1–3 with ATCS (Figure 4). Parametric multipoint LOD score calculations and haplotypes were obtained with the ALLEGRO program[22] and an autosomal-recessive, fully penetrant model. We selected two genes from the candidate region (*THBS1* [MIM 188060], *CHST14*) on the basis of known functions and expression patterns for mutation analysis. Primer sequences were based on the National Center for Biotechnology Information (NCBI) reference entries for mRNA and genomic DNA of each gene (*CHST14*,

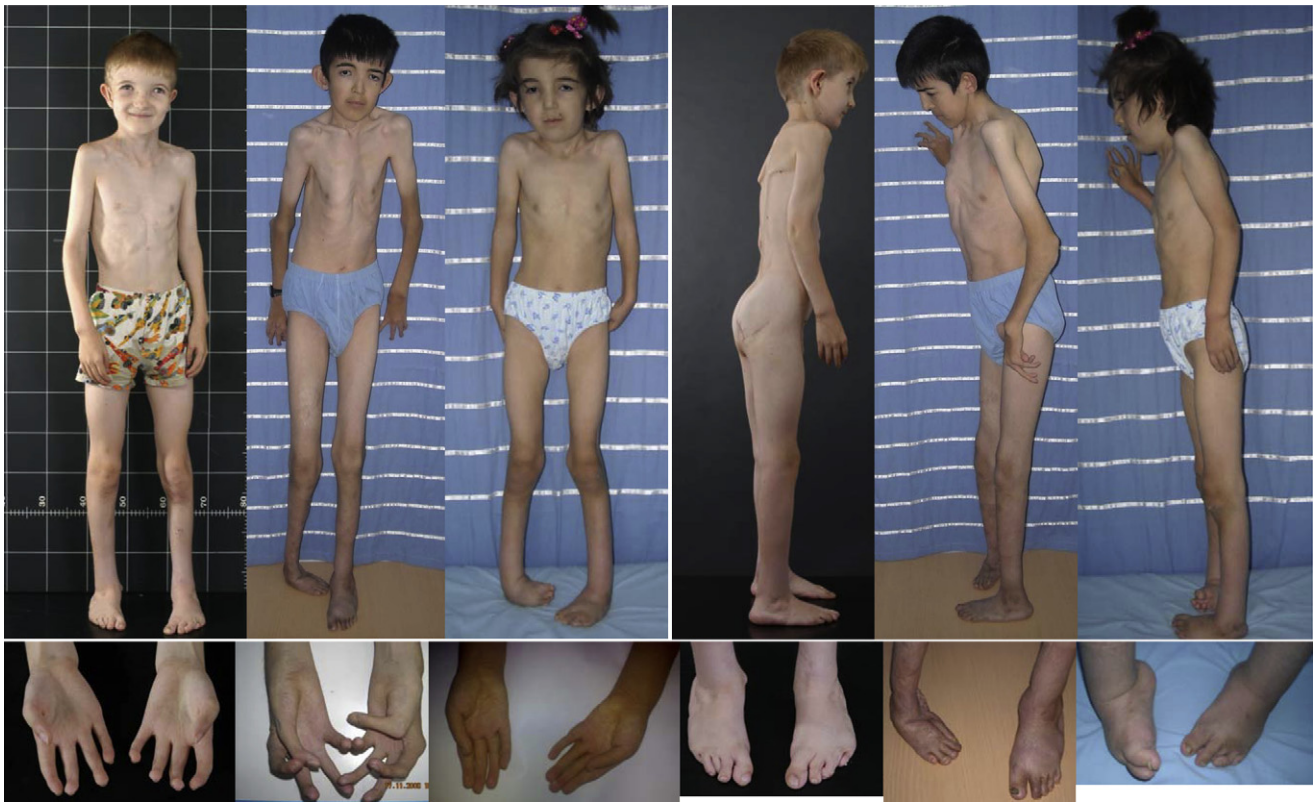

Figure 2. Clinical Features of ATCS
Left column: Austrian patient from family 1, homozygous for CHST14 mutation p.R213P at age 8 years; clinical findings were previously reported as case 2 by Janecke et al.[20]
Middle column: Turkish patient from family 3, homozygous for p.V49X at age 15 years; clinical findings were previously reported as case 2 by Dundar et al.[18]
Right column: 6-year-old sister of previous patient. Note the wasted build with weights below the 3rd centile and heights between the 25th to 50th centiles in all patients. The skin is translucent with readily visible venous pattern over the chest, abdomen, and extremities. Note long and tapering fingers, excessively wrinkled palms, and clubfeet (repaired in the Austrian patient).

NM_130468.2 and AC013356; *THBS1*, NM_003246.2 and AC037198) and are available from the authors. The coding regions and splice sites were PCR amplified and directly sequenced in one patient from each family. The sequencing reactions were analyzed on an ABI 3100 DNA sequencer, with BigDye terminator mix (Applied Biosystems, Vienna, Austria). Relatives were tested for mutations detected in a proband. A panel of 300 Austrian, 200 Turkish, and 50 Japanese DNA samples from anonymous controls was analyzed for the presence of each sequence variation.

Electron Microscopy

Skin specimens (2 × 2 mm) were fixed in 0.1 M cacodylate buffer pH 7.0 containing 3% of freshly prepared glutaraldehyde on ice for 4 hr. Then the specimens were thoroughly washed with the same buffer but without the glutaraldehyde and further processed for routine electron microscopy (EM).

Analysis of Epitope-Tagged D4ST1

Wild-type *D4ST1*, p.R213P, p.[R135G; L137Q], and p.Y293C were ligated into pcDNA3.1/V5 (Invitrogen) in frame with the carboxy-terminal epitope tag V5, and p.V49X was ligated into pcDNA3.1/V5 in frame with the aminoterminal tag V5, and they were expressed in HEK293/T cells. Cells were washed and extracted with T-PER tissue protein extraction reagent (Pierce) containing Complete Protease Inhibitor that was EDTA free (CPI-EDTA free) (Roche Molecular Biochemicals). Digestions with Endoglycosidase H (Boehringer Mannheim) and N-Glycanase (NEB) were performed as described.[23] Equivalent amounts of cell extracts and media were analyzed by SDS-PAGE with 10% NuPage Bis-Tris MOPS polyacrylamide gels (Invitrogen). After electrophoretic transfer to PVDF membranes (Millipore), epitope-tagged proteins were detected with HRP-antiV5 (Invitrogen).

Analysis of Glycosaminoglycans from Patient and Normal Fibroblasts

Metabolic incorporation of ^{34}S-sulfate in the available fibroblasts from a patient homozygous for p.R213P and in the fibroblasts from a normal control was conducted in 50% Dulbecco's modified Eagle's medium (DMEM) and 50% Ham's F-12 medium containing 10% dialyzed fetal bovine serum and 1 mM ^{34}S-sulfate. After 5 days in culture, the media were collected and replaced with fresh media containing 1 mM ^{34}S-sulfate. At day 9, media and cells were collected separately. After the homogenizing of cells with a Polytron, proteins in the media and homogenized cells were digested with Streptomyces Griseus protease (Sigma P5147), and the released glycosaminoglycans were isolated by chromatography on DEAE-Sephacel as described. Glycosaminoglycans were freed of core peptide by β-elimination followed by DEAE-Sephacel purification. Disaccharides for analysis were produced by digestion of glycosaminoglycans with chondroitinase ACII (EC 4.2.2.5),

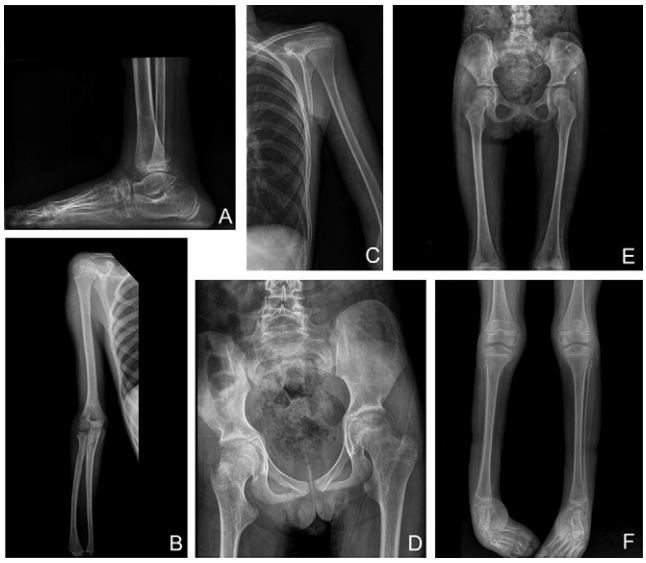

Figure 3. Radiographs Showing Generalized, Mild to Moderate Osteopenia in All Examined ATCS Patients
(A) Flat foot with plantar flexion of the talus. Rarefication of bone with reduced and coarse trabeculation, as well as thinning of the cortex, is shown.
(B–F) Slender shafts of tubular bones with thin cortex and poorly trabeculated spongiosa.
(A) and (B) show Austrian patient (family 1) homozygous for p.R213P at age 8 years; (C) and (D) show Turkish patient (family 3) homozygous for p.V49X at age 15 years; (E) and (F) show 6-year-old sister of previous patient).

chondroitinase B, or chondroitinase ABC (EC 4.2.2.4) purchased from Seikagaku. All three chondroitinases produce unsaturated uronic acid (ΔUA)-containing disaccharides. Chondroitinase ACII degrades CS, chondroitinase B degrades DS, and chondroitinase ABC degrades both CS and DS.

The reducing ends of the chondroitinase digestion products were labeled with H-aniline (normal) or D^5-aniline (patient) by reductive amination as described.[24] Separations were performed on a 0.3 × 250 mm C18 column (Zorbax 300SB, 5 μm, Agilent) with an Agilent 1100 series capillary HPLC workstation (Agilent) with Chemstation software for data acquisition, analysis, and management. The capillary high-performance liquid chromatography (HPLC) was directly coupled to the mass spectrometer. Mass spectra were acquired on a Mariner BioSpectrometry Workstation ESI time-of-flight mass spectrometer (PerSeptive Biosystems) in the negative-ion mode. Total ion current chromatograms and mass spectra were processed with Data Explorer software version 3.0. The disaccharides from patient and NORMAL fibroblasts were separately labeled with the glycan reductive isotope labels[25] (GRIL) normal aniline (H-analine) and deuterated analine (D-analine), allowing simultaneous comparison of GRIL-tagged glycosaminoglycans from two sources and eliminating variation associated with capillary HPLC-coupled MS analysis.

Results

Identification of N-Acetylgalactosamine 4-O-Sulfotransferase Gene Mutations

A genome-wide linkage scan in three families with ATCS identified a single candidate region of 3.76 Mb on chromosome 15q15 (multipoint LOD score of $Z = 5.91$ [$\theta = 0.0$] at several SNPs within this interval, Figure 4A). The boundaries were set between SNPs rs10520118 and rs10518779 by two recombinants. We identified 73 genes in this critical region in the annotated draft sequence of the human genome (NCBI map, Build 36.2), including *THBS1* encoding for thrombospondin and *CHST14* encoding for D4ST1, which we considered as plausible candidates. Thrombospondin is known to be involved in TGFB signaling,[26] and D4ST1 is known to play a role in the modification of ECM proteins.[3] Direct sequencing revealed no mutations in *THBS1*. However, we identified a 1 bp deletion (c.145_146 delG resulting in p.V49X), a missense mutation (c.638G>C; p.R213P), and a complex allele c.[404C>G; 410T>A] (p.[R135G; L137Q]) in *CHST14* in the three original ATCS families (Figure 4B, families 1–3) from Austria and from Turkey.[18–20] We also identified a homozygous point mutation (c.878A>G; p.Y293C) in a consanguineous family from Japan subsequently identified with the clinical features of ATCS (family 4, Figure 4).[21] We showed that six informative microsatellite markers spanning a 15 cM interval around *CHST14* are homozygous in both Japanese patients from this family, providing evidence for the pathogenicity of this mutation. In each case, the mutation segregated with disease status in the family and was not present in control samples.

The single *CHST14* exon encodes a type II membrane protein of 376 amino acids belonging to the family of HNK-1 sulfotransferases with a 43 amino acid cytoplasmic domain and a 316 amino acid luminal domain. A single 2.4 kb transcript is ubiquitously but most highly expressed in connective tissues, heart, liver, pancreas, and placenta.[3] Each of the identified mutations has the potential to alter D4ST1 activity and cause disease. The p.V49X mutation leads to early protein truncation. Arg-213, which is known to mediate hydrogen bonding to 3′-phosphoadenosine 5′-phosphosulfate, and Tyr-293 are highly conserved among members from the HNK1 family as well as a number of other carbohydrate sulfotransferase families. Residues Arg-135 and Leu-137 are present in a 4 amino acid sequence that is specific to D4ST1 among the HNK-1 sulfotransferases and is conserved among D4ST1 orthologs (Figure 5A). An analysis using the in silico tools PolyPhen and PANTHER predicted damaging effects of the identified missense mutations on protein function (Table 1).

Normal Structure and Ultrastructure of a Patient's Skin Biopsy

Light and electron microscopy revealed no alterations in a biopsy from a patient (family 1) homozygous for the D4ST1 mutation p.R213P (Figure 6).

Altered Intracellular Processing of D4ST1 Missense Mutations

Immunoblotting of recombinant D4ST1 revealed wild-type migrating as a major species with a Mr of 55 kD and less prominent species with a Mr of 44 kD that arises by

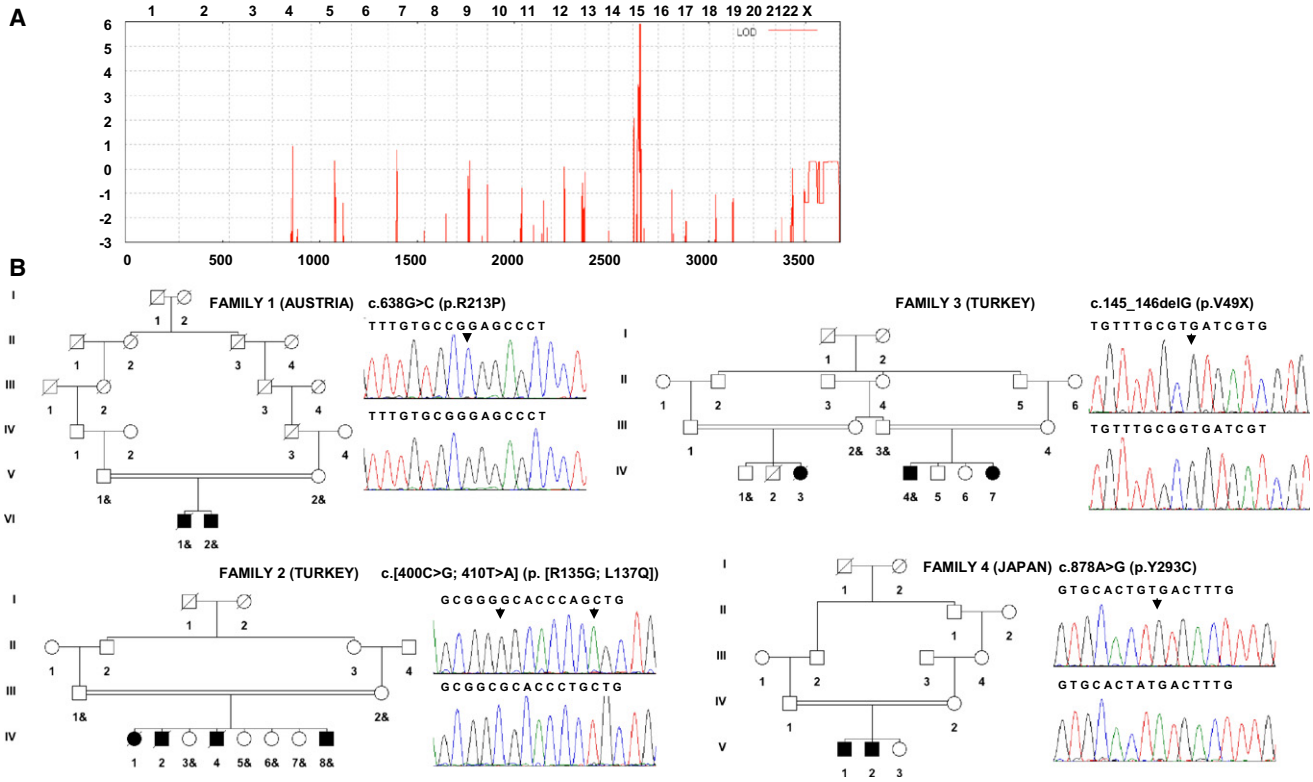

Figure 4. Pedigrees and Linkage and Mutation Analyses of ATCS Families
(A) The graph represents a parametric LOD score on the y axis in relation to genetic position on the x axis. Human chromosomes are concatenated from p-ter (left) to q-ter (right) on the x axis, and the genetic distance is given in cM. This scan identified a single region of extended homozygosity shared by all four affected individuals included in the genome scan, who are identified in (B), defining a critical interval of 3.76 Mb on chromosome 15q15.
(B) The pedigrees of the four ATCS families included in this study are shown and the corresponding *CHST14* mutations are shown on the right-hand side. A total of 15 subjects from families 1–3, identified by the & symbol, were included in the genome scan to map the disease. Family 1 originates from Austria and clinical findings were initially reported in 2001.[20] The Turkish families 2 and 3 were first reported in 2001[19] and 1997,[18] and detailed clinical findings of family 4 from Japan were reported in 2000.[21]

proteolytic cleavage in the region between the transmembrane domain and the glycosylated asparagine located at position 110 (Figure 5B). Only trace amounts of the 55 kD species are seen in cells expressing either p.R213P or p.[R135G; L137Q]; instead, the 44 kD form predominates. No product is detected for p.V49X, whereas the 55 kD species predominates in p.Y293-expressing cells. Wild-type D4ST1, p.R213P, p.Y293C, and p.[R135G; L137Q] are sensitive to digestion to both endoglycosidase H (not shown) and N-glycanase, indicating that oligomannose-type oligosaccharides are present. The intracellular processing of p.R213P and p.[R135G; L137Q] is distinct from that of wild-type D4ST1 with only trace amounts of the membrane-anchored form remaining. Wild-type D4ST1 is found in the medium of transfected 293T cells, whereas D4ST1 is not found in significant quantities in the medium of cells transfected with D4ST1 containing any of the missense mutations (Figure 5B, lower panel). The absence of the missense mutants in the medium suggests that the intracellular processing of each of these forms differs from that of wild-type and that they may not reach the Golgi but instead be degraded.

Decreased DS Biosynthesis and Increased CS Content in Patient-Derived Fibroblasts

We addressed the impact of the p.R213P mutation by comparing DS biosynthesis by cultured fibroblasts from a patient homozygous for p.R213P with normal controls. Stable isotope-labeled glycosaminoglycans obtained from the cell pellet and the medium were exhaustively digested with chondroitinase AC, B, or ABC to generate ΔUA-containing disaccharides from CS, DS, and both CS and DS, respectively. DS-derived IdoUA-GalNAc4^{32}S (m/z535) is present in cell- and media-associated glycosaminoglycans from normal fibroblasts and can be biosynthetically labeled with ^{34}S-sulfate to form IdoUA-GalNAc4^{34}S (m/z 537). No DS-derived IdoUA-GalNAc4S disaccharide is detected in cell extracts of patient fibroblasts (m/z 540) (Figure 7A). The small amount of IdoUA-GalNAc4S seen in digests of the medium from patient fibroblasts probably reflects DS present in the fetal calf serum because there is no evidence of biosynthetic incorporation of ^{34}S-sulfate into IdoUA-GalNAc4S disaccharide (m/z 542) from the medium of patient fibroblasts. In contrast, GlcUA-GalNAc4S is increased in glycosaminoglycans obtained from

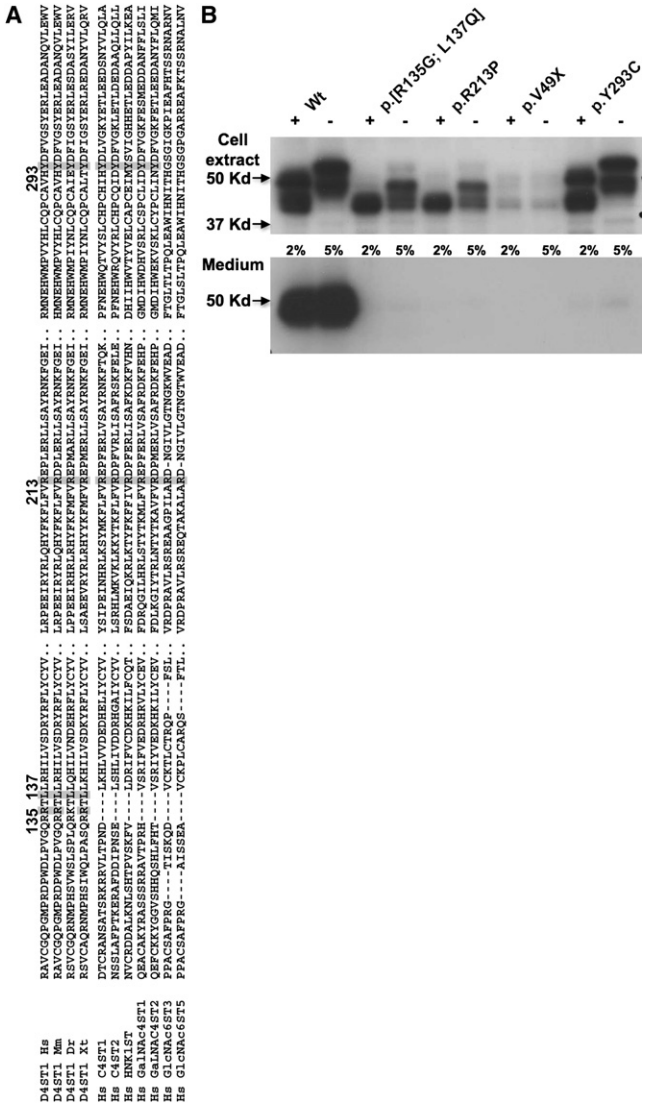

Figure 5. Consequences of D4ST1 Mutations

(A) Comparative analysis of D4ST1 orthologs and HNK-1 family members by ClustalW alignment shows a high degree of conservation of residues mutated in ATCS.

(B) Analysis of epitope-tagged D4ST1 by SDS-PAGE. HEK293/T cells were transfected with pcDNA3.2/V5 (Invitrogen) expressing D4ST1 wild-type, p.[R135G; L137Q], p.R213P, pV49X, or p.Y293C. Cells were washed and dissolved in TPER. Samples that were or were not treated with N-glycanase prior to separation by SDS-PAGE are indicated by + and −, respectively. One percent of the cell extract (upper panel) and 2% and 5% of the concentrated medium (lower panel) was analyzed for each construct. The apparent molecular weight standards are indicated by arrows. Wild-type D4ST1 migrates as a major species with a Mr of 55 kD and less prominent species with a Mr of 44 kD that arises by proteolytic cleavage in the region between the transmembrane domain and the glycosylated asparagine located at position 110.

cell extracts and media of patient fibroblasts (m/z 540) and can be biosynthetically labeled with ^{34}S-sulfate (m/z 542). Although there is evidence of biosynthetic incorporation of ^{34}S-sulfate (m/z 537 peak) into CS associated with the cell pellet, there is no evidence of biosynthetic incorporation of ^{34}S-sulfate (m/z 537 peak) into CS present in the medium of normal fibroblasts (Figure 7A). This suggests that DS rather than CS is the predominant proteoglycan produced by normal fibroblasts. Thus, the overall synthesis of cell-associated DS and CS glycosaminoglycans is reduced in patient fibroblasts, whereas the synthesis of DS and CS glycosaminoglycans that are secreted into the medium does not appear to be reduced in patient cells, suggesting that diversion of DS into CS is more extensive for the secreted glycosaminoglycans of patient fibroblasts. The amount of cell- and of medium-associated nonsulfated GlcUA-GalNAc and IdoUA-GalNAc disaccharides was increased for patient fibroblasts as compared to normal fibroblasts (Figure 7B). Thus, the levels of both GlcUA-GalNAc and IdoUA-GalNAc are increased in the absence of IdoUA-GalNAc sulfation by D4ST1.

Table 1. In Silico Analysis of CHST14 Mutations

Mutation	POLYPHEN Prediction	POLYPHEN Score1 − Score2	PANTHER SubPSEC	PANTHER Pdeleterious
p.R213P	Probably damaging	2.688	−7.74542	0.99138
p.R135G	Probably damaging	2.164	−3.19552	0.54872
p.L137Q	Possibly damaging	1.981	−2.22168	0.31468
p.Y293C	Probably damaging	2.995	−7.76921	0.99158

PolyPhen scores of $>$ 2.0 and PANTHER SubPSEC scores $<$ −3.0 indicate mutations as probably damaging protein function.

Discussion

ATCS represents a recognizable, generalized connective tissue disorder with normal cognitive development[18–21] and resembles both the progeroid and vascular types of Ehlers-Danlos syndrome (EDS, [MIM 130070 and 130050]) and the Loeys-Dietz syndrome (LDS [MIM 609192]) because of wasted build and translucent skin. The dynamic nature of distal contractures in the presence of striking hypermobility of distal joints is reminiscent of collagen VI-related muscle disorders (Bethlem myopathy [MIM 158810]) during childhood.[27] Characteristic facial features are shared in part with LDS patients[28] and autosomal-recessive cutis laxa type II, a type II N-glycosylation defect (ARCL2 [MIM 219200]).[29,30] Congenital malformations of a wide spectrum occur variably in ATCS and include the unusual coincidence of a common mesentery and absence of the gastrocolic omentum in a sib pair.

Our data suggest that ATCS is genetically homogeneous and is caused by loss-of-function mutations in *CHST14* leading to a deficiency of sulfated dermatan in affected tissues. Conversion of GlcUA to IdoUA by glucuronate C5-epimerase is a freely reversible reaction that favors the

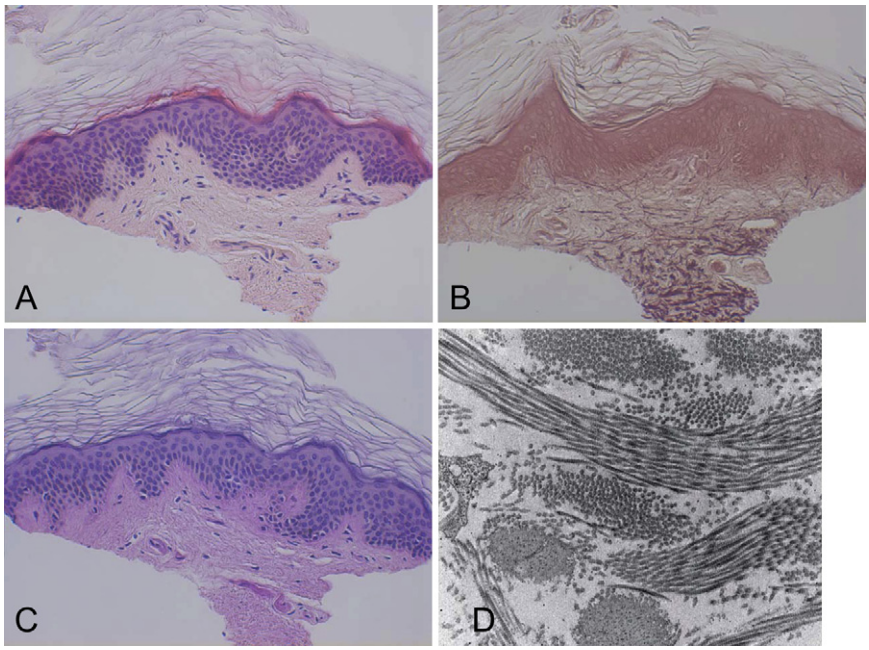

Figure 6. Light and Electron Microscopy of a Skin Biopsy from the Austrian ATCS Patient Homozygous for *CHST14* p.R213P
Normal light microscopy (LM) appearance of the skin using (A) H&E, (B) Elastica, and (C) PAS staining. (D) EM shows collagen fibrils, which are normally packed, round, and have a normal diameter and contour, as well as normal cross-sections of elastin fibers and collagen fibrils. Elastin contains normal microfibrillar material.

GlcUA form,[6] and our results support the hypothesis that the addition of sulfate to the C4 hydroxyl of GalNAc by D4ST1 prevents further epimerization of IdoUA to GlcUA. ATCS patient fibroblasts epimerize GlcUA-GalNAc to IdoUA-GalNAc but are not able to add sulfate to the IdoUA-GalNAc. Our data show that in the absence of sulfate addition to IdoUA-GalNAc by D4ST1, the levels of IdoUA-GalNAc, GlcUA-GalNAc, and GlcUA-GalNAc4S increase, and that a significant fraction of DS is, as a result, replaced by CS. Supposedly, CS cannot generally substitute for functions specially associated with DS because D4ST1 deficiency causes ATCS.

It remains a possibility that D4ST1 loss of function is incomplete in the patient in whom fibroblasts were studied. We cannot exclude the possibility that some or all mutants retain residual enzyme activity or that D4ST1 activity is compensated for in part by C4ST activity at least in some tissues. Together with the identification of an early-truncating p.V49X mutation in one family with ATCS, however, our evidence is sufficient to argue for D4ST1 loss of function as the immediate mechanism of action to cause the disease. ATCS might result both from abnormal and from loss of DS proteoglycan function. For example, osteopenia is present in ATCS patients and in null mice for biglycan, one of the most intensely studied DS proteoglycans, suggesting that biglycan's function as a positive regulator of bone formation is compromised by D4ST1 deficiency.[31,32] The shared features of ATCS and the progeroid form of EDS might result from a reduction in the amount of DS proteoglycans. A decrease of glycosaminoglycan chains on decorin and biglycan was observed in fibroblasts from patients with the progeroid form of EDS. This disease results from reduced activity of Beta4-GalT7, which catalyzes the transfer of galactose to the xylose residue forming the linkage region of proteoglycans.[33] In contrast to the progeroid form of EDS and to decorin null mice, however, which have abnormally fragile skin and irregular and loosely packed collagen skin fibers,[34] the patient's skin appeared thin and more translucent than normal, but no ultrastructural skin abnormalities were apparent in one ATCS patient, suggesting that an excess of CS might compensate for DS in the skin, at least postnatally. The same might hold true for other tissues in which D4ST1 is expressed.

DS proteoglycans are involved in the generation of morphogen gradients in epithelia.[12,13,35] Because decorin neutralizes the activity of TGFB1,[31] deficiency or substitution of DS chains by CS on decorin implicates altered TGFB1 signaling in the pathogenesis of ATCS, as was shown in LDS.[28] Altered TGFB1 signaling might lead to features shared by LDS and ATCS.[36] Intriguingly, a homozygous disruption of the C4st1 locus in mice led to a severe chondrodysplasia and affected the balance of TGFB family signaling in the cartilage growth plate.[37]

The single DS chain of decorin binds to tenascin-X (TNX [MIM 600985]), an ECM protein that binds to tropoelastin and collagen types I, III, V, XII, and XIV[38] and contributes to the integrity of the extracellular network.[39] Loss of tenascin-X results in a form of EDS with skin and joint hypermobility and poor wound healing (MIM 606408).[40] Decreased TNX binding might be responsible for the occurrence of features resembling TNX deficiency in ATCS patients.

DS activates heparin cofactor II (HCF2 [MIM 142360]) in the arterial wall after endothelial injury and thereby exerts an antithrombotic effect, and 4-O-sulfation of DS is required for this effect.[41] DS also shows a localized profibrinolytic activity.[42] D4ST1 deficiency would thus be predicted to cause both thrombophilia and delayed fibrinolysis resulting in the observed tendency to ecchymoses and hematoma formation of patients.

A large number of proteoglycan and of glycosaminoglycan biosynthetic enzymes have been identified with profound effects on growth and morphogenesis by the study of human disorders and animal models.[10,11,43] To date, these include two sulfotransferases, chondroitin

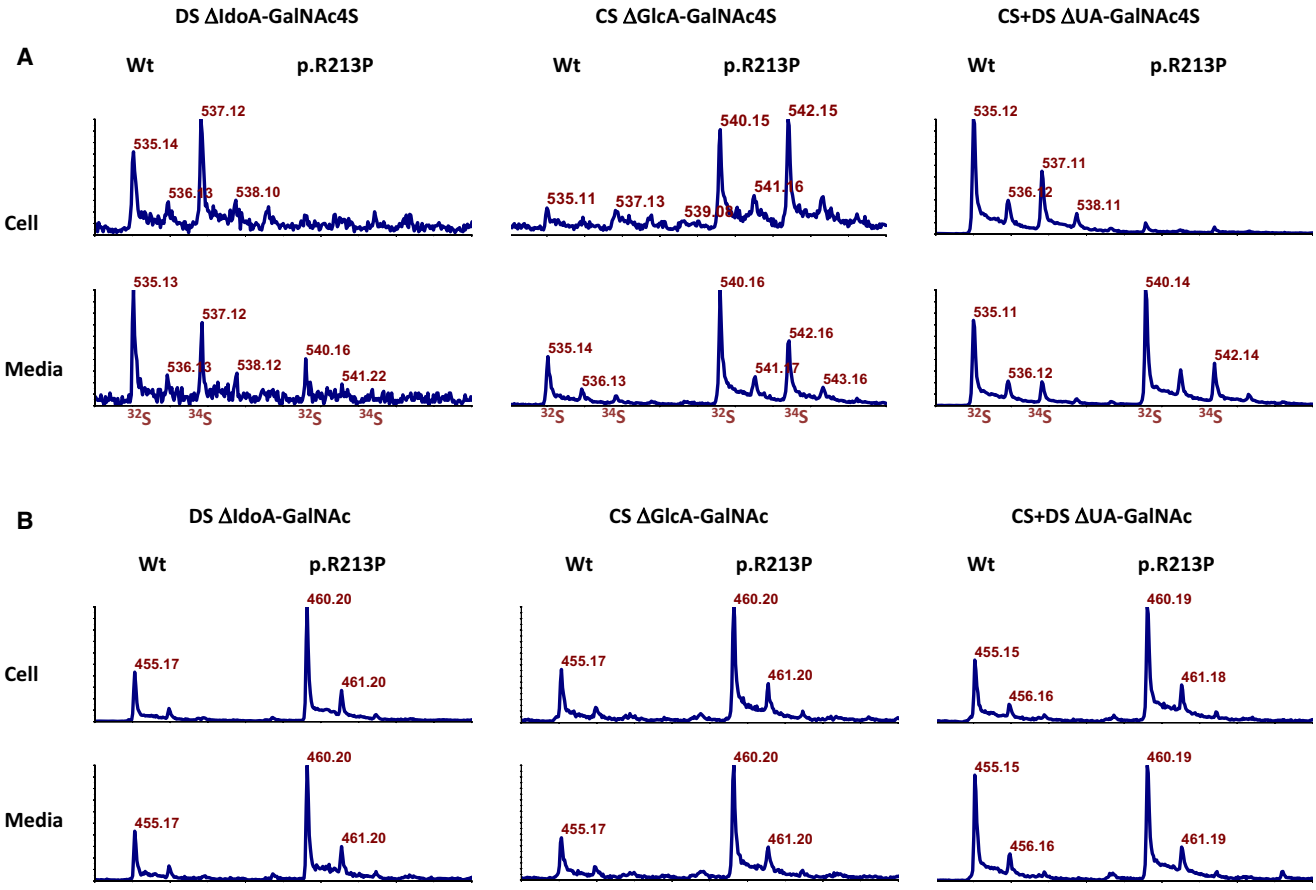

Figure 7. Defective Dermatan Sulfation and Increased CS Biosynthesis
Cultured fibroblasts from a patient homozygous for D4ST1 mutation p.R213P detected by ^{34}S-sulfate metabolic labeling and capillary HPLC/MS analysis of CS-specific, DS-specific, and CS and DS-specific monosulfated (A) and nonsulfated (B) disaccharides.
(A) Monosulfated disaccharides from cell extracts and media of patient (m/z 540) and normal fibroblasts (m/z 535) coeluted at 19 min. No DS-derived IdoUA-GalNAc4S disaccharide was detected by GRIL capillary HPLC-coupled MS disaccharide analysis of cell extracts from patient fibroblasts. In addition, GlcUA-GalNAc4S was greatly increased in glycosaminoglycans obtained from cell extracts and media of patient as compared with control fibroblasts.
(B) Nonsulfated disaccharides from patient (m/z 460) and normal fibroblasts (m/z 455) eluted at 16 min after chondroitinase A, B, and ABC digestion of their glycosaminoglycans. The amount of GlcUA-GalNAc and IdoUA-GalNAc is increased in the cell extract and the medium from patient fibroblasts as compared to normal fibroblasts.

6-sulfotransferase (CHST3 [MIM 603799]) and N-acetylglucosamine 6-O-sulfotransferase (CHST6 [MIM 605294]), whose deficiencies lead to characteristic human disorders with symptoms confined to the skeleton and cornea, respectively, Omani type of spondyloepiphyseal dysplasia (MIM 608637), recessive Larsen syndrome (MIM 245600),[44,45] and macular corneal dystrophies types I and II (MIM 217800).[46] Distinctive diseases can also be caused by decreased concentrations of intracellular sulfate and 3′-phosphoadenosine 5′-phosphosulfate, probably resulting in broad nonspecific undersulfation of glycosaminoglycan chains as demonstrated in the Pakistani type of spondyloepimetaphyseal dysplasia (MIM 612847) and diastrophic dysplasia (MIM 222600), caused by PAPSS2 (MIM 603005) and SLC26A2 (MIM 606718) deficiencies, respectively. To the best of our knowledge, ATCS represents the first disorder caused by a defect specific to DS biosynthesis, and the loss of D4ST1 activity affects a number of tissues. Furthermore, the wide and variable spectrum of congenital malformations in ATCS patients implicates roles for DS not only in connective tissue maintenance but also in embryonic development, supported by the recent observation that abrogation of endogenous DS affects the development of the primary axis, mesoderm induction, and neuronal differentiation related to FGF signaling in the *Xenopus* embryo.[35]

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Web Resources

The URLs for data presented herein are as follows:

European Bioinformatics Institute, http://www.ebi.ac.uk/index.html
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
PANTHER, http://www.pantherdb.org/tools/csnpScoreForm.jsp
PolyPhen, http://genetics.bwh.harvard.edu/pph/

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