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Atelosteogenesis Type II Is Caused by Mutations in the Diastrophic Dysplasia Sulfate-Transporter Gene (DTDST): Evidence for a Phenotypic Series Involving Three Chondrodysplasias

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Summary

Atelosteogenesis type II (AO II) is a neonatally lethal chondrodysplasia whose clinical and histological characteristics resemble those of another chondrodysplasia, the much less severe diastrophic dysplasia (DTD). The similarity suggests a shared pathogenesis involving lesions in the same biochemical pathway and perhaps the same gene. DTD is caused by mutations in the recently identified diastrophic dysplasia sulfate-transporter gene (DTDST). Here, we report that AOII patients also have DTDST mutations, which lead to defective uptake of inorganic sulfate and insufficient sulfation of macromolecules by patient mesenchymal cells in vitro. Together with our recent observation that a third even more severe chondrodysplasia, achondrogenesis type IB, is also caused by mutations in DTDST, these results demonstrate a phenotypic series of three chondrodysplasias of increasing severity caused by lesions in a single sulfate-transporter gene. The severity of the phenotype appears to be correlated with the predicted effect of the mutations on the residual activity of the DTDST protein.

Introduction

Atelosteogenesis type II (AO II), also called *neonatal osseous dysplasia I,* is characterized by severely shortened limbs, small chest, scoliosis, club foot of the equinovarus type (talipes equinovarus), abducted thumbs and great toes, and cleft palate. Radiographic findings include bending of the spine (cervical kyphosis, scoliosis, and lumbar hyperlordosis with horizontal sacrum), flattened vertebrae with coronal clefts, and a widened ileum with horizontal acetabulum. The distal humerus is typically bifid, and the distal femur rounded. The second and/or third metacarpals and first and second metatarsals are often larger than the other bones of the hand and the foot (fig. 1). Patients die of respiratory insufficiency shortly after birth, because of the collapse of the airways and pulmonary hypoplasia due to the small rib cage. The chondro-osseous morphology includes shortened chondrocytic columns of the growth plate and deficient interterritorial matrix with cystic lesions, fibrillar structures interconnecting the lacunae, and lacunar halos (fig. 2) (Sillence et al. 1987; Spranger and Maroteaux 1990; Nores et al. 1992; Schrander-Stumpel et al. 1994). On the basis of reports of parental consanguinity and recurrence among offspring of unaffected parents, AOII is presumed to be inherited as an autosomal recessive trait.

In many respects, AOII is similar to but more severe than the generally nonlethal autosomal recessive chondrodysplasia, diastrophic dysplasia (DTD). DTD patients have short-limbed short stature and a generalized joint dysplasia, typically with talipes equinovarus and abducted thumbs and great toes as well as kyphoscoliosis and cleft palate (Walker et al. 1972). Although there is some elevated lethality in infancy because of respiratory and neurological complications secondary to the cervical kyphosis, most DTD patients live a normal life span. Histologically and ultrastructurally, DTD is similar to but milder than AOII (fig. 2). The phenotypic similarities between DTD and AOII have caused diagnostic confusion and prompted suggestions of common etiology (Sillence et al. 1987; Stern et al. 1990; Qureshi et al. 1994, 1995; Schrander-Stumpel et al. 1994).

By positional cloning, we recently identified the DTD gene as encoding a ubiquitously expressed sulfate transporter, DTDST (Hästbacka et al. 1994). With the DTDST gene in hand, it was possible to investigate the hypothesis that DTD and AOII are genetically related.

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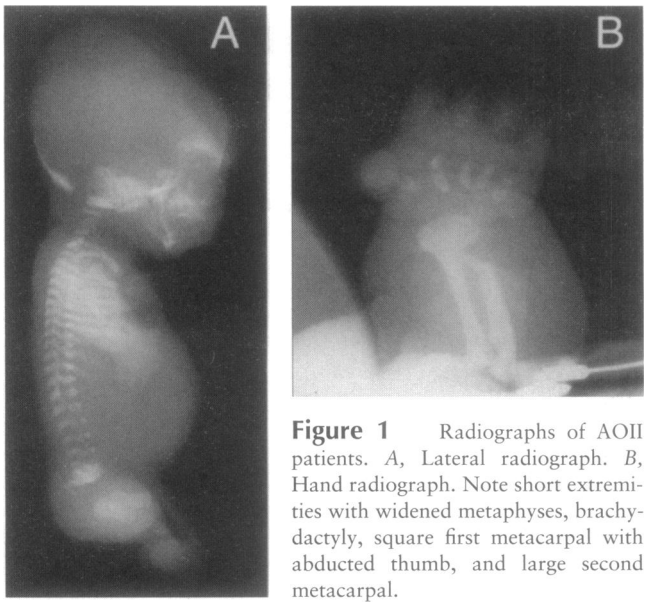

Figure 1 Radiographs of AOII patients. *A*, Lateral radiograph. *B*, Hand radiograph. Note short extremities with widened metaphyses, brachydactyly, square first metacarpal with abducted thumb, and large second metacarpal.

Here, we report biochemical and molecular studies on three AOII patients, which show defective sulfate uptake and proteoglycan sulfation in patient-derived cells and associated mutations in the DTDST gene. Together with our recent observation that achondrogenesis type IB (ACG-IB), an even more severe chondrodysplasia associated with reduced sulfation of proteoglycans is also caused by mutations in DTDST (Superti-Furga 1994; Superti-Furga et al. 1996), these results demonstrate a phenotypic series of three chondrodysplasias of increasing severity caused by lesions in a single sulfate-transporter gene.

Subjects and Methods

Study Subjects

Samples were obtained from two fetuses (patients 1 and 2) and from a term baby (patient 3) referred to the International Skeletal Dysplasia Registry. All cases were diagnosed to have typical AOII, on the basis of clinical, radiographic, and histological findings. Cases 1 and 2 were the second affected fetuses born to their parents. Patient 3 was exceptional in having survived for 2 mo with intensive care, including ventilation by a respirator. The study was approved by the institutional review board of the Cedars-Sinai Research Institute.

Sulfate-Uptake Assays

Sulfate uptake was assayed in triplicate samples with a few modifications as described elsewhere (Elgavish et al. 1985; Hästbacka et al. 1994). In brief, confluent primary skin fibroblast cultures were preincubated for an hour in Eagle's minimal essential medium (MEM) with the sulfate adjusted to be at the same concentration as that in which the uptake was to be assayed. The cells were washed three times in the appropriate uptake buffer, without the radioactive label, and were then incubated for 2 min at room temperature in the uptake buffer with varying concentrations (25–250 μM) of nonradioactive sulfate and a constant concentration of carrier-free (ICN Radiochemicals; specific activity ~43 Ci/mg) ^{35}S-Na_2SO_4 (0.13 μM). After incubation, cells were rapidly washed four times in ice-cold wash buffer and lysed. Aliquots of the lysates were used for scintillation counting and for determination of the total protein concentration.

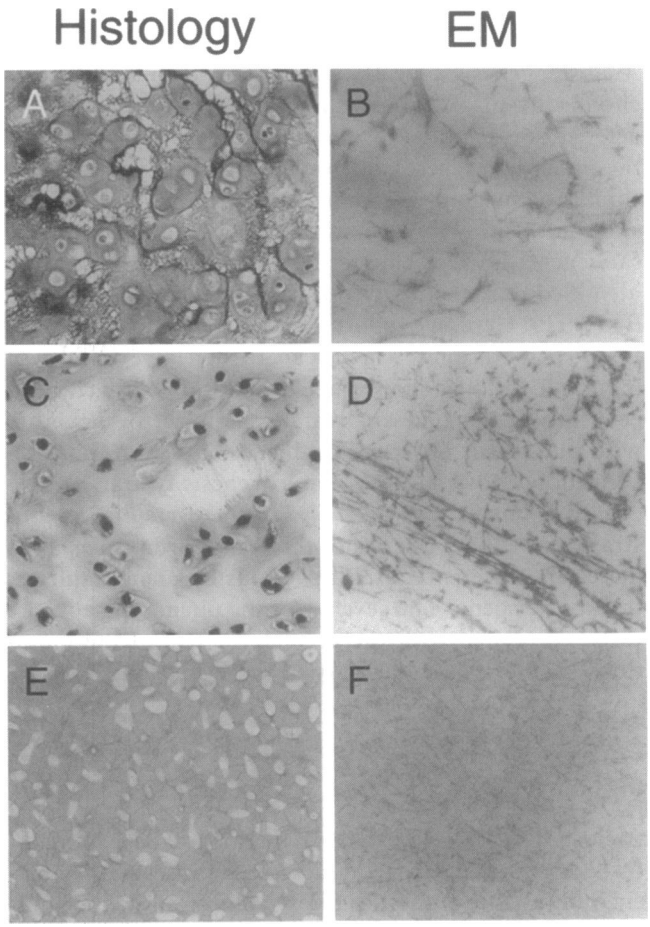

Figure 2 Histological and ultrastructural abnormalities in AOII, which are similar to but more pronounced than those seen in DTD. Chondro-osseous morphology of (*A*) AOII, (*C*) DTD, and (*E*) unaffected control (Stainsall, original magnification × 112) (Mortier et al. 1995). The AOII sample shows deficiency of the interterritorial matrix with a fibrillar, foamy appearance and thread-like radiations interconnecting the lacunae, and the DTD sample shows areas of cystic, fibrillar degeneration of the interterritorial matrix with loss of chondrocytes. Transmission electron micrographs (EM) from cartilaginous matrix of (*B*) AOII, (*D*) DTD, and (*F*) an unaffected control (magnification × 25,200). Note diminished proteoglycans and aggregated collagen fibrils. The abnormalities are more pronounced in AOII. All samples are from lower extremity of fetuses of 16–18 wk gestation.

Sulfation of Proteoglycans

Primary skin fibroblasts were grown on 8-cm^2 plates to confluency in MEM supplemented with 10% FCS and antibiotics, washed, preincubated for 15 min in sulfate-free MEM, and double labeled overnight in either standard MEM with sulfate concentration of 810 μM or sulfate-free MEM supplemented with 10% dialyzed FCS, with ^{35}S-Na_2SO_4 (200 μCi/ml) and ^{3}H-glycine (5 μCi/ml). After incubation, cells were washed twice with cold Hanks' balanced salt solution (Gibco BRL) followed by lysis in lysis buffer consisting of 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and protease inhibitors. Incorporation of radioactivity was determined by liquid scintillation counting by setting the window to 0–6 KeV for tritium and to 20–2,000 KeV for ^{35}S-sulfate. Aliquots of cell lysates, normalized for their protein content, were electrophoresed under reducing conditions in 4%–15% gradient SDS-PAGE. The gels were equilibrated with dimethyl sulfoxide, impregnated with the scintillant 2,5-diphenyloxazole, soaked in water, fixed, and dried, followed by autoradiofluorography (Laskey and Mills 1975). The experiment was carried out in duplicate samples.

SSCP

PCRs for SSCP were carried out as described elsewhere, with one of the primers being kinase labeled with γ-[^{32}P]ATP (Dietrich et al. 1992). The primer pairs used were 5′-gtagcgatgggcttctttca-3′ (nt 727–746) with 5′-tgagtgagcccacaccatta-3′ (nt 886-867) and 5′-tccaggttctgctggctc-3′ (nt 2063-2080) with 5′-gactcagaccattgggaacac-3′ (nt 2233–2213). The numbering of nucleotides refers to the previously published DTDST cDNA sequence (Hästbacka et al. 1994). PCR products were diluted 10-fold in SSCP loading buffer (95% formamide, 10 mM NaOH, 0.02% bromphenol blue, 0.02% xylene cyanol), denatured for 5 minutes at 95°C, chilled on ice, and electrophoresed at room temperature in 1 × MDE™ (mutation-detection enhancement) polyacrylamide (AT Biochem) gels containing 10% glycerol in 0.6 × Tris-borate EDTA buffer for 16 h at 10 V/ cm. The SSCPs from patients were carried out from amplification products independent from those used for direct sequencing.

Sequence Analysis

The coding region of DTDST gene was amplified as four partially overlapping fragments from the genomic DNA of patient and controls by using the following primer pairs: 5′-aggaagctgaaccatctatctcc -3′ (nt 1–23) and 5′-ctgtaaaagtgaagtcttacggct -3′ (in intron, −441 to −464 bp from the 3′ splice-acceptor site); 5′-agtgtgattctactttgatgtaaatct-3′ (in intron, −553 to −527 from the 3′ splice-acceptor site) and 5′-aagtgatacagtgatagcaaaacc-3′ (nt 1227-1204); 5′-tcactggtgcctccttcact-3′ (nt 797–816) and 5′-tggcttcataatctctgcga-3′ (nt 2056–2037); and 5′-cgctttgtagcccctctcta-3′ (nt 1771–1790) and 5′-tgtgatccagaagcctcttg-3′ (nt 2714–2695). The nucleotide numbering of primers refers to the previously published DTDST cDNA sequence (Hästbacka et al. 1994). Oligo dT–primed first-strand cDNA synthesis from skin fibroblast total RNA was carried out with Superscript II preamplification system (Gibco-BRL) according to manufacturer's recommendations. Amplification was performed using primer pairs 5′-tgctccttccttaggaatgg-3′ (nt 597–616) and 5′-tggcttcataatctctgcga-3′ (nt 2056–2037). DNA sequencing was performed by standard methods, either on ABI 373 automatic sequencer with cycle sequencing using fluorescent dye terminator chemistry or by autoradiography on sequencing gels with Sequenase polymerase (USB) and α-[^{35}S]-dATP. In the latter case, the template DNA was prepared by PCR amplification with one of the oligonucleotides being biotinylated. The biotinylated double-stranded PCR product was captured with streptavidin-coated magnetic beads (Dynal) and processed according to manufacturer's instructions to remove the nonbiotinylated strand. The resulting single-stranded PCR product was used as the sequencing template.

Results

Sulfate-Metabolism Abnormalities in Atelosteogenesis Type II

Our previous studies of DTD patients demonstrated that fibroblasts show a defect in uptake of inorganic sulfate in low-ionic-strength uptake buffer (Hästbacka et al. 1994). We performed similar sulfate-uptake assays on skin fibroblasts from the three AOII patients and found that they showed a similar defect in sulfate uptake (fig. 3).

We also studied proteoglycan synthesis in skin fibroblasts of one of the AOII patients (patient 2), using cells from DTD patients and unaffected individuals as controls. In sulfate-free MEM, the patient fibroblasts showed dramatically decreased sulfate incorporation compared to unaffected controls, whereas only slightly reduced sulfate incorporation compared to normal was observed when incubated at the high sulfate concentration (810 μM) (fig. 4).

In order to assess the ratio of synthesis of sulfated proteoglycans to the synthesis of total protein, AOII, DTD, and unaffected fibroblasts were labeled with both ^{35}S-Na_2SO_4, to measure incorporation into sulfated proteoglycans (as in fig. 4*A*), and ^{3}H-glycine, to measure incorporation into proteins. The ratio of incorporated $^{35}S/^{3}H$ was then used as a measure of the ratio of synthesis of sulfated proteoglycans to the synthesis of total protein. In sulfate-free MEM, the ratio was 0.24 for the AOII patient, 0.35–0.59 in DTD patients, and 2.94-

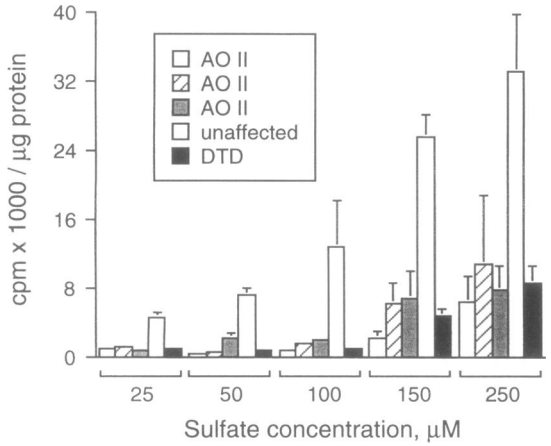

Figure 3 AOII cells, deficient in sulfate uptake. Cultured primary skin fibroblasts of three AOII patients, one DTD patients, and an unaffected control were analyzed for uptake of radioactively labeled sulfate at indicated extracellular concentrations. Incorporated radioactivity was normalized to protein concentration.

4.04 in controls. By contrast, in standard MEM (sulfate concentration 810 µM) yielded at most a slight difference between the controls and patient cells.

Mutations in the DTDST Gene in AOII Patients

In view of the clinical and biochemical similarities between the two diseases, we tested the three AOII patients for mutations in the DTDST gene by PCR-based sequencing of the complete coding region. We found evidence of five distinct mutations, accounting for all six patient chromosomes:

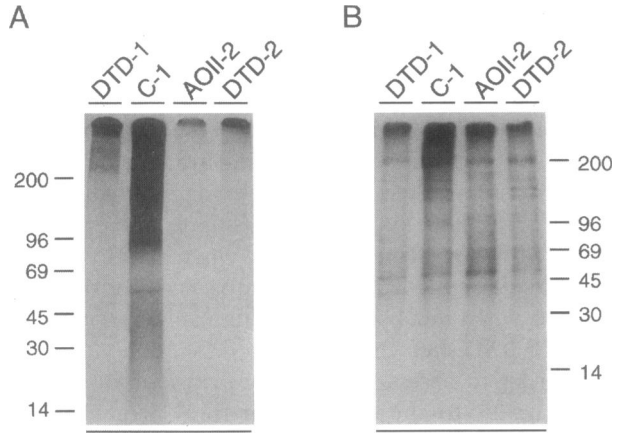

Figure 4 AOII fibroblasts, having a deficiency in incorporation of sulfate into sulfated proteoglycans. *A*, Metabolic labeling of AOII, DTD, and unaffected (C-1) skin fibroblasts with trace quantities of ^{35}S-sulfate and ^{3}H-glycine in the absence of added unlabeled sulfate. Labeled proteins were analyzed by SDS-PAGE followed by autoradiography. The migration of molecular weight markers is indicated on the side of the autoradiograph. *B*, As in *A*, except labeling was performed in the presence of 810 µM of unlabeled "cold" sulfate.

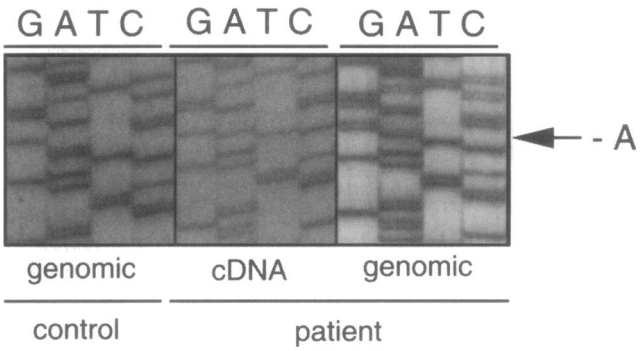

Figure 5 One allele of the DTDST gene, not expressed in an AOII patient. In a patient heterozygous for a frameshift involving deletion of an A in codon 575 (patient 2), sequencing of genomic DNA reveals both sequences (with deletion marked as "-A"), but sequencing of cDNA from fibroblasts shows only the deletion allele—indicating that expression of the other allele is not detectable.

Patient 1 was heterozygous for a single-base-pair deletion (−A, nt 1751), causing a frameshift in the codon 575 and resulting in a stop codon after nine amino acids that is predicted to truncate the last 20% of the protein. This same mutation had been seen elsewhere in three unrelated DTD patients from the Netherlands, France, and Germany (Hästbacka et al. 1994) and subsequently also in an ACG-IB patient from the Netherlands (Superti-Furga et al. 1996). This patient was also found to be heterozygous for a C-to-T transition at nt 862 resulting in an R279W substitution in the predicted third extracellular loop.

Patient 2 was also heterozygous for the same single-base-pair deletion (−A, nt 1751), seen in Patient 1. No sequence abnormalities were detected in the coding region of his other allele. However, RT-PCR experiments showed that this allele failed to be properly expressed. Specifically, we prepared first-strand cDNA prepared from the patient's fibroblasts, performed PCR amplification of a 1,460-bp region containing nt 1751, and subjected the resulting amplification product to DNA sequencing. Whereas genomic DNA showed clear heterozygosity for the deletion at nt 1751, the RT-PCR products showed exclusively the sequence of the deletion allele, indicating that the allele with the wild-type coding sequence was not detectably expressed (fig. 5).

Patient 3 was heterozygous for a G-to-A transition at nt 791, leading to a G255E substitution that introduces a negative charge in the highly conserved fifth putative transmembrane domain of the proposed protein. She was also found to be heterozygous for a C-to-T transition at nt 2171, leading to a A715V substitution close to the carboxyterminus of the protein.

To exclude the possibility that the observed sequence changes were common polymorphisms, we examined 120 control chromosomes (assaying the deletion at nt

1751 by the fact that it destroys a *Dde*I site and the other mutations by SSCP) and found that the sequence changes were not present in any of them. We did, however, find the R279W substitution in four chromosomes from DTD patients.

Discussion

The phenotype of AOII resembles DTD but has complete neonatal lethality. Our results show that the similarity extends to the biochemical and genetic level, with AOII patients showing defects in sulfate uptake and mutations in the DTDST gene. We found evidence of five distinct mutations among six AOII chromosomes, with one present twice. These included a frameshift in codon 575 that eliminates the last 20% of the protein and three missense mutations (G255E, R279W, and A715V) with the first introducing a charge into a transmembrane segment. The sequence alteration in the fifth apparent mutation was not identified, but it was shown to reduce severely the expression of DTDST mRNA. Formal proof that these changes interfere with the function of DTDST will require studies in a suitable expression system such as sulfate-transport-deficient Chinese hamster ovary cells (Esko et al. 1986). Nonetheless, it is clear that AOII patients show a high frequency of mutations in the DTDST gene. Together, the biochemical and genetic data provide a convincing case that AOII is caused by mutations in the DTDST gene.

The finding of two diseases of increasing severity caused by mutations in the same gene suggests the existence of an allelic series, and, indeed, this observation has already been extended to an even more severe chondrodysplasia, ACG-IB (Superti-Furga et al. 1996). ACG-IB is a recessive lethal chondrodysplasia characterized by extremely short limbs and characteristic radiographic appearance (Borochowitz et al. 1988; Spranger and Maroteaux 1990; Superti-Furga 1994). Affected individuals are usually born before term as stillborns or die immediately after birth. Their cartilage is abnormally soft, and the chondro-osseous morphology is similar to but more severe than in AOII, including deficient interterritorial matrix and aggregated collagen fibrils forming rings around the chondrocytes. Deficient sulfation of proteoglycans has been previously demonstrated in ACG-IB patients (Superti-Furga 1994). In a separate study, we have found that ACG-IB is also caused by mutations in the DTDST gene. Seven different mutations were identified in six ACG-IB patients, accounting for all 12 affected chromosomes. The mutations were three different frameshifts leading to a premature stop codons, with one being the same frameshift at codon 575 (nt 1751) found both in AOII and DTD patients; a nucleotide substitution creating a stop codon within the third transmembrane domain; a 3-bp deletion eliminating an amino acid residue within the seventh transmembrane segment; a missense mutation within the ninth transmembrane domain; and a missense mutation in the carboxy terminus of the protein (Superti-Furga et al., 1996). The mutations found in DTDST are presented in table 1 and figure 6.

In light of the fact that ACG-IB, AOII, and DTD all arise from mutations in the same gene, what accounts for the differences in severity? ACG-IB seems likely to represent the null phenotype of the DTDST gene, AOII seems to represent a partial loss of function, and DTD appears to be an even milder loss of function. This is supported by the following observations: (*i*) ACG-IB always shows mutations in the coding region in both alleles, with most leading to early stop codons; (*ii*) AOII tends to show mutations in the coding region in both alleles, with most involving potentially less severe changes, such as a late frameshift occurring after the last transmembrane domain or missense mutations; and (*iii*) no DTD patients have been found to possess two mutations in the coding region—all cases we have studied so far have at least one allele with an apparently intact coding region. We have previously reported that DTD patients homozygous for the haplotype common in Finland (accounting for 95% of Finnish DTD chromosomes) show undetectably low levels of mRNA on northern blots. However, by employing the very sensitive technique of RT-PCR, the presence of DTDST mRNA in these patients could be detected (J. Hästbacka, unpublished data) suggesting that these DTD patients have a low, but nonzero, level of expression of the mRNA encoding the DTDST protein. A more complete understanding of this observation must await the identification of the mutation on the common Finnish ancestral haplotype that causes this decreased expression.

Homozygotes for the apparently "leaky" Finnish DTD allele are never known to present with AOII, but there may be some overlap between DTD and AOII in compound heterozygotes carrying a mild and a more severe mutation. Although the nature of the mutations at DTDST appears to explain much of the phenotypic difference among the three diseases, other factors may also play a role. Specifically, there is significant variation in severity even among DTD patients homozygous for the ancestral mutation common in Finland or among DTD patients within a single sibship (Horton et al. 1978). Since these sibs have identical genotypes at the DTD locus, this variation points to the existence of either unlinked modifier genes or epigenetic factors. It should be noted that we have not observed marked differences in sulfate uptake or proteoglycan sulfation among DTD, AOII, and ACG-IB patients. This may, however, simply reflect the fact that the assays are rather crude and that the cells were obtained from fetuses and patients of different ages.

Table 1

Pairs of DTDST Alleles Observed in ACG-IB, AOII, and DTD

	ΔT476	C559T (R178X)	G→C at 3′ Splice Site	G791A (G255E)	C862 (R279W)	ΔGTT 1045-1047	ΔA 1221	A1300G (N425D)	ΔA1751	ΔA2010	G2060T (G678V)	C2171T (A715V)	Unknown[a]	Unknown
ΔT476	...	ACG-IB	...	...	...	...	...	...	...	...	...	...	...	...
C559T (R178X)	...	...	...	...	...	...	ACG-IB	...	...	...	ACG-IB	...	...	...
G→C at 3′ splice site	...	...	...	...	...	...	...	...	...	...	...	...	...	DTD
G791A (G255E)	...	...	...	...	...	...	...	...	...	...	...	AOII	...	...
C862T (R279W)	...	...	...	...	...	...	...	...	AOII	...	...	...	...	DTD
ΔGTT 1045-1047	...	...	...	...	...	ACG-IB	...	...	...	...	...	...	...	...
ΔA 1221	...	...	...	...	...	...	...	...	...	...	...	...	...	...
A1300G (N425D)	...	...	...	...	...	...	...	...	ACG-IB	...	...	...	...	...
ΔA1751	...	...	...	...	...	...	...	...	...	...	...	...	AOII	DTD
ΔA2010	...	...	...	...	...	...	...	...	...	...	...	...	...	DTD
G2060T (G678V)	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C2171T (A715V)	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Unknown[a]	...	...	...	...	...	...	...	...	...	...	...	...	DTD	...
Unknown	...	...	...	...	...	...	...	...	...		...	...	...	...

NOTE.—The mutations in the table are listed in 5′-to-3′ order (Hästbacka et al. 1994; Superti-Furga et al. 1996).

[a] With reduced expression demonstrated.

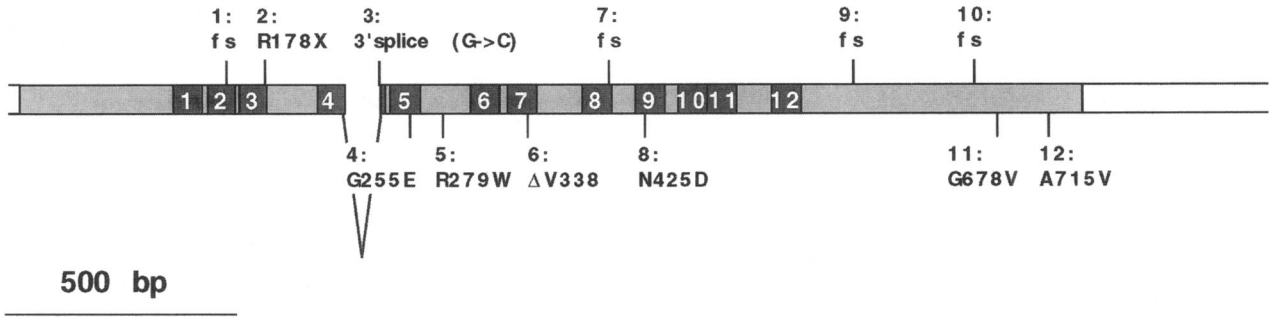

Figure 6 Structure of the DTDST gene with observed mutations. Untranslated cDNA is shown in white, the coding region in light gray, and the 12 predicted transmembrane domains within the coding region in dark gray. The fourth transmembrane domain is interrupted by the single intron within DTDST coding region. The mutations predicted to have an effect on the length of the peptide (nonsense, various frameshifts marked *fs*, splicing defect) are shown above the diagram, and the missense mutations and the small in-frame deletion are shown below it (Hästbacka et al. 1994; Superti-Furga et al. 1996).

Finally, our studies of sulfation of proteoglycans in cultured cells sheds light on how a defect in a ubiquitously expressed gene results in a tissue-specific phenotype. The physiological concentration of sulfate in human serum is ~300 μM (Cole and Scriver 1981; Pillion et al. 1984), whereas the concentration of free extracellular sulfate in normal cartilage is only ~100 μM, because of to the Donnan effect, whereby the highly negative fixed charge in cartilage extracellular matrix excludes free negative ions (Gray et al. 1988). Chondrocytes in normal cartilage are thus exposed to a lower extracellular sulfate concentration than are most other cells. Our experiments showed that, at high sulfate concentrations, the proteoglycan sulfation defect is much less pronounced than at lower concentrations. Furthermore, decreased intracellular sulfate concentrations are likely to affect proteoglycans differentially. Glycosaminoglycan sulfotransferases have different affinities for the activated sulfate donor adenosine 3′-phosphate, 5-phosphosulfate, with chondroitin sulfotransferase having a high K_M (~500 μM) and heparan N-sulfotransferase having a much lower K_M (~50 μM). Thus, limited availability of sulfate will particularly affect the sulfation of chondroitin sulfate (Humphries et al. 1988), which is the predominant glycosaminoglycan of aggrecan, the major proteoglycan of cartilage extracellular matrix. Because heparan sulfate proteoglycans are required for the dimerization and binding of fibroblast growth factors (FGFs) to their receptors, it has been suggested that the growth defect in DTD may be in part attributable to failure of FGF signaling (Erlebacher et al. 1995; Wallis 1995). It will be necessary to characterize the pattern of proteoglycan sulfation seen in DTD patients in order to assess whether this explanation is plausible. Moreover, understanding sulfate levels with DTD cells will be important to conceiving potential therapies to ameliorate the disease.

Acknowledgments

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