

Supplemental Data

Congenital Joint Dislocations Caused by Carbohydrate

Sulfotransferase 3 Deficiency in Recessive

Larsen Syndrome and Humero-Spinal Dysostosis

Pia Hermanns, Sheila Unger, Antonio Rossi, Antonio Perez-Aytes, Hector Cortina, Luisa Bonafé, Loredana Boccone, Valeria Setzu, Michel Dutoit, Luca Sangiorgi, Fabio Pecora, Kerstin Reicherter, Gen Nishimura, Jürgen Spranger, Bernhard Zabel, and Andrea Superti-Furga

Material and Methods

Genomic DNA was extracted from the subjects' peripheral leucocytes with the Gentra genomic DNA blood isolation kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's protocol. One hundred nanograms of genomic DNA was used as template in the PCR reaction. Exons 2 and 3 of the CHST3 gene are protein coding. A 391 bp product encompassing the whole of exon 2 (247 bp) and containing a 140 bp of coding sequence was generated. Exon 3 of the CHST3 gene consists of 6393 bp, of which 1.3 kb are coding. Given the size of the coding region, exon 3 was amplified in three PCR reactions with overlapping sequences of 86 bp and 93 bp, respectively. Primer information is available upon request. With this approach 66 bp of the noncoding 3'UTR and 85 bp of intron 2 were sequenced. The PCR reaction was carried out with the PCR Master Mix (Promega) in a 25 μ l reaction at an annealing temperature of 58°C for exon 2 and 60°C for exon 3 (all three parts). In addition, the PCR reactions of exon 3 were supplemented with 5% glycerol. The PCR products were purified with the PCR purification Kit from Promega (Madison, WI, USA) and directly sequenced with Dye Terminator v3.1 on a 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's recommendations. DTDST mutations had been excluded in patients 1, 4, and 5, and FLNB mutations had been excluded in patient 2 by routine molecular analysis.^{1,2}

For separation of the alleles of patient 5, the PCR products were subcloned into the pGEM T vector (Promega) and transformed into DH5 α cells. Ten single clones were picked and the plasmid DNA was isolated (Eppendorf) and sequenced with the vector-specific primers T7 and SP6, with Dye Terminator kit v3.1 (Applied Biosystems, Forster City, CA, USA).

Skin biopsies were obtained from patients 1 and 2 with informed consent. Skin fibroblasts from patients and controls were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and antibiotics at 37°C in a humidified atmosphere containing 5% CO₂.

Disaccharide analysis of glycosaminoglycan (GAG) chains synthesized in fibroblast cultures was performed as described previously.³ In brief, confluent cells in 10 cm² dishes were washed with PBS to eliminate any sulfotransferase activity that might be present in the FCS and then preincubated for 4 hr in the presence or absence of 1 mM p-nitrophenyl β -D-xylopiranoside (Sigma) in DMEM without FCS in 5% CO₂ at 37°C. Labeling with [6-³H]glucosamine (GE Healthcare) was performed in the same medium at a final concentration of 50 μ Ci/ml. After 24 hr labeling, an equal volume of 100 mM sodium acetate buffer, pH 5.8, containing 8 M urea, 4% Triton X-100, 20 mM EDTA, 20 mM NEM, and 1 mM PMSF was added to the Petri dishes, and samples were loaded on 1 ml DEAE Sephacel columns. After column washing with 50 mM sodium acetate, pH 6.0, 8 M urea, 0.15 M NaCl, 0.5% Triton X-100, and proteinase inhibitors, proteoglycans and hyaluronic acid were eluted with 1 M NaCl in the same buffer, recovered by precipitation with 9 volumes of ethanol and desalted by ultrafiltration with Centricon-10 (Millipore). PGs were then separated from hyaluronic acid by digestion with *Streptomyces* hyaluronidase (Seikagaku Corporation, Tokio, Japan) followed by ultrafiltration with Biomax Ultrafree-0.5 devices with 10kDa cut-off membrane (Millipore). PGs in the retentate were then digested with 30 mU of both chondroitinase ABC and ACII (Seikagaku Corporation) in 30 mM sodium acetate, 30 mM Tris-acetate, pH 7.35, at 37°C overnight. Undigested products were removed by precipitation with 4 volumes of ethanol;⁴ the disaccharide constituents of chondroitin sulfate (dimers of glucuronic acid and N-acetyl-D-galactosamine, either unsulfated or sulfated on the positions 4 or 6 of the GalNAc residue; Δ Di-0S, Δ Di-4S, Δ Di-6S) in the supernatants were fractionated with a Supelcosil SAX1 (Supelco, Bellefonte, USA) HPLC column (4.6 \times 250 mm) with a gradient of 5–400 mM KH₂PO₄, pH 4.2–4.5, at

room temperature, and the elution profile was measured at 232 nm.⁵ Standard unlabeled disaccharides (Δ Di-0S, Δ Di-4S, Δ Di-6S) were added as internal reference to radiolabeled samples, and the corresponding peaks at 232 nm were collected and the concentration of radioactive labeled disaccharides determined by liquid scintillation counting.

Supplemental References

1. Rossi, A. and Superti-Furga, A. (2001). Mutations in the diastrophic dysplasia sulfate transporter (DTDST) gene (SLC26A2): 22 novel mutations, mutation review, associated skeletal phenotypes, and diagnostic relevance. *Hum. Mutat.* *17*(3), 159–171.
2. Krakow, D., Robertson, S.P., King, L.M., Morgan, T., Sebald, E.T., Bertolotto, C., Wachsmann-Hogiu, S., Acuna D, Shapiro SS, Takafuta T, et al. (2004). Mutations in the gene encoding filamin B disrupt vertebral segmentation, joint formation and skeletogenesis. *Nat. Genet.* *36*(4), 405–410.
3. Rossi, A., Kaitila, I., Wilcox, W.R., Rimoin, D.L., Steinmann, B., Cetta, G., and Superti-Furga, A. (1998). Proteoglycan sulfation in cartilage and cell cultures from patients with sulfate transporter chondrodysplasias: relationship to clinical severity and indications on the role of intracellular sulfate production. *Matrix. Biol.* *17*, 361–369.
4. Harper, G.S., Hascall, V.C., Yanagishita, M., and WA Gahl, W.A. (1987). Proteoglycan synthesis in normal and Lowe syndrome fibroblasts. *J. Biol. Chem.* *262*, 5637–5643.
5. Forlino, A., Piazza, R., Tiveron, C., Della Torre, S., Tatangelo, L., Bonafè, L., Gualeni, B., Romano, A., Pecora, F., Superti-Furga, A., et al. (2005). A diastrophic dysplasia sulfate transporter (SLC26A2) mutant mouse: morphological and biochemical characterization of the resulting chondrodysplasia phenotype. *Hum. Mol. Genet.* *14*(6), 859–871.

10 20 30 40 50 60 70 80 90 100

Human MEKGLTLPQDCRDFVHSLKMRSKYALFLVFFVVFVFIIEKENKIIISR
Rat MEKGLALPQDCRDLVHNLKIRGRVYVFLFAFVVVVFVFIIEKENKIIISR
Chicken MERRSALPQDFREVLHCLKMRSKYAVLLVFFVVG-LVIIIEKENNFIISR
Mouse MEKGLALPQDFRDLVHSLKIRGRVYVFLFAFVVVVFVFIIEKENKIIISR
Bos taurus MEKGLSLPQDCRDFLHSLRMRSKYALFLFAFVVVVFVFIIEKENKIIISR
Drosophila MSTGIGSSSSSTEEQQHLELSADLEACSLLGPDPSNVDTGRTRAKQRLSLNKGRRSRMSRRANLIGICGVSSLCILLIATTOHRLPTTFNQSAGR
Canis MEKGLAFPQDCRDFLHSLRMRSKYALFLFAFVVVVFVFIIEKENKIIISR
Danio
Felis MEKGLAFPQDCRDLHSLRMRSKYALFLFAFVVVVFVFIIEKENKIIISR
Oryza -LRMKSKEYALFICVVA-LVIIIEKESIIISR
Macaca -MAPPFPMEKGLTLLQDCRDFLHSLKMRSKYALFLVFFVVFVFIIEKENKIIISR
Takifugu -RMRIKYTISVFFVA-LVIIIEKENNIIISR
Tetraodon -RMRIKYTIFIVFFLA-LVIIIEKENNIIISR
Sorex MEKGLVLPPERRDLLHGLMRGKYAFFLFAFVVVVFVFIIEKENKIIISR
Pan MEKGLTLPQDCRDFVHSLKMRSKYALFLVFFVVFVFIIEKENKIIISR
Otolemur MEKGFSLPQDCRDFMHSRMRGKYALFLFAFVVVVFVFIIEKENKIIISR
Myotis MEKGLALPQDCRDFLHSLRMRSKYALFLFAFVVVVFVFIIEKENKIIISR
Monodelphis MEKGFHFWPQDTRDLLHRLRMRSKYALFLFAFVVVVFVFIIEKENKIIISR
Tupaia MEKGLVLPQDCDFVHNLRMRSKYALFLFAFVVVVFVFIIEKENKIIISR
Gasterosteus RRRRPSAEQQHSRVMRCKKGMRIKYTIPIVFFVA-LVIIIEKENSIVSR
Erinaceus MEKGLNLPQDCDFVHSLRMRSKYALFLFAFVVVVFVFIIEKENKIIISR
Ciona -MRGALLSACDVMNPTDIWASDMRKNR
Aedes
Anopheles

110 120 130 140 150 160 170 180 190 200

Human -VSDKLLKQIPQALADAN-STDPALILAEENASLLSLELDSAFSLOLQSLRNLNLSLQGLVEPAMEAAGEEEEEQRKEE-----PPRPAVA
Rat -VSDKLLKQIPQFVADAN-STDPAALLSEENASLLSLELDSFSLHSLRSLHNLNLSLQGLIAPAMEAQQGEAFAEK-----PSQQA
Chicken -VSDKLLKQSPQVLEPEAN-ETEASPVQAENGSLASLRQLDTAFSOLRTRLRNVTLQLAGELGIAAP-----PPRPAVA
Mouse -VSDKLLKQIPHVADAN-STDPAALLSEENASLLSLELDSFSLHSLRSLHNLNLSLQGLVEPAMESQ--EAGAEK-----PSQQA
Bos taurus -VSDKLLKQIPQSLVDAN-STDPAVLVAENASLLSLELDSAFSLOLQSLRNLNLSLQGLMEPAEAEEEEQTLAEAEWEQLE--PRPPAEA
Drosophila QGASVDGGPSNSLARSIFYDRFQQQLQQQQSSQTAVNLTATIVDVLVSAQRSRILAEEMENFEYPRGGAERLTDMP-----ETN
Canis -VSDKLLKQIPQPLVDAN-STDPAALILADNASLLSLELDSAFSLOLQSLRNLNLSLQGLV-----PPRPAVA
Danio
Felis -VSDRLKQIPQPLADAN-STDPAALILADNASLLSLELDSAFSLOLQSLRNLNLSLQGLVEPAMEAAGEEAGAKAKE-----PPRPREA
Oryza -VSDKLSQMHNPHQTPETLQDYSNTTAR-SSFTVLKVLKSLPSTKGNFSNFSEEQPEVDNVGN-----S
Macaca -VSDKLLKQIPQALADAN-STDPAVLVAENASLLSLELDSAFSLOLQSLRNLNLSLQGLVEPAMEAAGEEEEEQKEE-----PPRPAVA
Takifugu -VSDKLSLQIPEASLHPSGISTATLLKLNVSSTPFNRANSAPTILIKRRLNYSKHQDVARS-----PPRPAVA
Tetraodon -MSDKLSLQIPEASLHPSGISTATLLKLNVSSTPFNRANSAPTILIKRRLNYSKHQDVARS-----PPRPAVA
Sorex -VSGKLMQIPQPAVDAN-STDPAVLVAENASLLSLELSSAFSLOLQSLRNLNLSLQGLGAEAGGAPGEEQAEHGEQE--PGVAGA
Pan -VSDKLLKQIPQALADAN-STDPAALILAEENASLLSLELDSAFSLOLQSLRNLNLSLQGLVEPAMEAAGEEEEEQRKEE-----PPRPAVA
Otolemur -VSDKLLKQIPQTLADAN-STDPAVLVAENASLLSLELDSAFSLOLQSLRNLNLSLQGLVEPAMEAAGEE--EKE-----AEASP
Myotis -VSDKLLKQIPQSLADAN-STDPAVLVAENASLLSLELDSAFSLOLQSLRNLNLSLQGLQPAEAEEEEETKREEEEAEEEEQGEQPPDPAEA
Monodelphis -VSDKLLKQIPQSLLDAN-STDPAALILAEENASLLSLELDSAFSLOLQSLRNLNLSLQGLVEPAMEAAGEE--EKE-----AEASP
Tupaia -VSDKLLKQIPQSLADAN-STDPAVLVAENASLLSLELDSAFSLOLQSLRNLNLSLQGLVEPAMEAAGEE--EKE-----AEASP
Gasterosteus -VSDKLLKQIPQSLADAN-STDPAVLVAENASLLSLELDSAFSLOLQSLRNLNLSLQGLVEPAMEAAGEE--EKE-----AEASP
Erinaceus -VSGKLSLQIPEASLHPSGISTATLLKLNVSSTPFNRANSAPTILIKRRLNYSKHQDVARS-----PPRPAVA
Ciona -KSRIGLPLVHKNKRMMTYRVFTVQLVLVVVVVFVGVTVTSNPFKRKRDHAILNMHKTDPSS-----TK
Aedes
Anopheles -PPISTAHNSTISIEDVLNYQALISAECTDSSY--GERKLPDGLTP-----ETG

210 220 230 240 250 260 270 280

Human GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFITPLPEDHLTQFMFRGSSRSL
Rat GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Chicken EPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAVGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Mouse GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Bos taurus PPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Drosophila GTPVRSVVVTSWRSGSTFLGDILNSIP-----GNFYHYEPLLDGFIKQIRDQDELAVQNLKLLNCDYADMIDVNLFG-----IHTHYL
Canis -VLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Danio -RKHILLMATTRTGSSEFVGEFFNQGDNMFYLFEPPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Felis RPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Oryza LDHKHILLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Macaca GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Takifugu -RRKHILLMATTRTGSSEFVGEFFNQGG-NMIFYLFEPPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Tetraodon -GRKHILLMATTRTGSSEFVGEFFNQGDNMFYLFEPPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Sorex GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Pan GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Otolemur APRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Myotis RRRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Monodelphis GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Tupaia GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Gasterosteus -GRRHILLMATTRTGSSEFVGEFFNQGDNMFYLFEPPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Erinaceus GPRRHVLLMATTRTGSSEFVGEFFNQGG-NIFYLFEPLWHIERIVTSFEPGGANAAGSALVYRDVLKQLFLCDLYLVEHFISPPEDHLTQFMFRGSSRSL
Ciona AKYKGVIVTNRHSGSTFTGELFNQHE-DVFIYFIEPLIQAR-----SGCGDEEDKMKILMRMLQCDLGSPEGERITQDAGVQRQRCISNFCFRE
Aedes
Anopheles GKPTRSLIITTWRSSTFLGDILNALP-----GNYYHYEPLLDIVQIRGPPNDSVALHNLRLHLLHCDYSEMENYLEFG-----R-THNYL

L259P

L307P

310 320 340 350 360 370 390 400

Human CEDPVC...
Rat CEDPVC...
Chicken CEEPVCT...
Mouse CEDPVC...
Bos taurus CEDPVC...
Drosophila FEHNTRL...
Canis CEEPVCT...
Danio CEEQVCS...
Felis CEDPVC...
Oryza CEEPVCT...
Macaca CEDPVC...
Takifugu CEESVCS...
Tetraodon CEDSVCS...
Sorex CEEPVCT...
Pan CEDPVC...
Otolemur CEDPVC...
Myotis CEDPVC...
Monodelphis CEEPVCT...
Tupaia CEDPVC...
Gasterosteus CEASVCS...
Erinaceus CEDPVC...
Ciona KSKVLC...
Aedes -----
Anopheles FSHNTRL...

410 420 R357Q E372K 50 460 470 480 490 500

Human GODDGL...
Rat GODDLS...
Chicken GEAPLQ...
Mouse GODDLS...
Bos taurus GODDLS...
Drosophila -----
Canis GQARLG...
Danio GEVPIE...
Felis GQARLG...
Oryza GQVPED...
Macaca GODDGL...
Takifugu GVTPLD...
Tetraodon GDVPLD...
Sorex GODDRL...
Pan GODDGL...
Otolemur GEDRLEE...
Myotis GODDRL...
Monodelphis GEASLRE...
Tupaia GODDRL...
Gasterosteus GDVPID...
Erinaceus GODDRL...
Ciona -----
Aedes -----
Anopheles -----

510 520 530 540 550 560 570 580 590 600

Human AHDGSG...
Rat ARDSSD...
Chicken PDSNGI...
Mouse TRDSSD...
Bos taurus AHDG--...
Drosophila KVINIG...
Canis AQDASG...
Danio ASEANG...
Felis AQDGGI...
Oryza VQEASG...
Macaca AHDGSG...
Takifugu SKKASG...
Tetraodon SKKTSG...
Sorex AQAGTG...
Pan AHDGSG...
Otolemur -----
Myotis PRDGGI...
Monodelphis SRDGGI...
Tupaia VRDGGI...
Gasterosteus SKGTSV...
Erinaceus AQDSSG...
Ciona RPKTKL...
Aedes KLDIGG...
Anopheles KQDVG...

```
.....|.....|.
Human      ERGTFWVT---
Rat        ERGTFWVT---
Chicken    EGPPTRIT---
Mouse      ERGTFWVT---
Bos taurus ERGTFWVT---
Drosophila MPPFFT-----
Canis      ERGTFWVT---
Danio      EK-----
Felis      -----
Oryza      EKQF-----
Macaca     ERGTFWVT---
Takifugu   DK-----
Tetraodon -----
Sorex      ERGTFWVT---
Pan        ERGTFWVT---
Otolemur   -----
Myotis     ERGTFWVT---
Monodelphis DRGTFWVT---
Tupaia     ERGTFWVT---
Gasterosteus D-----
Erinaceus  EGGTFWVT---
Ciona      PLCNDVTIGDC
Aedes      PLLPYSVS---
Anopheles  PLLPYSVS---
```