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The Phenotype of the Musculocontractural Type of Ehlers-Danlos Syndrome due to *CHST14* Mutations

Andreas R. Janecke,[1,2]* Ben Li,[3] Manfred Boehm,[4] Birgit Krabichler,[2] Marianne Rohrbach,[5] Thomas Müller,[1] Irene Fuchs,[1] Gretchen Golas,[6] Yasuhiro Katagiri,[7] Shira G. Ziegler,[6] William A. Gahl,[6] Yael Wilnai,[8] Nicoletta Zoppi,[9] Herbert M. Geller,[7] Cecilia Giunta,[5] Anne Slavotinek,[3] and Beat Steinmann[5]

[1]Department of Pediatrics I, Innsbruck Medical University, Innsbruck, Austria

[2]Division of Human Genetics, Innsbruck Medical University, Innsbruck, Austria

[3]Department of Pediatrics, Division of Genetics, University of California, San Francisco, California

[4]Translational Medicine Branch NHLBI-NIH, Bethesda, Maryland

[5]Division of Metabolism, Connective Tissue Unit and Children's Research Center, University Children's Hospital, Zurich, Switzerland

[6]NIH Undiagnosed Diseases Program, Common Fund, Office of the Director, and Office of the Clinical Director, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland

[7]Developmental Neurobiology Section, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

[8]Division of Medical Genetics, Department of Pediatrics, Stanford University Medical Center, Stanford, California

[9]Division of Biology and Genetics, Department of Molecular and Translational Medicine, Medical Faculty, University of Brescia, Brescia, Italy

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The musculocontractural type of Ehlers-Danlos syndrome (MC-EDS) has been recently recognized as a clinical entity. MC-EDS represents a differential diagnosis within the congenital neuromuscular and connective tissue disorders spectrum. Thirty-one and three patients have been reported with MC-EDS so far with bi-allelic mutations identified in *CHST14* and *DSE*, respectively, encoding two enzymes necessary for dermatan sulfate (DS) biosynthesis. We report seven additional patients with MC-EDS from four unrelated families, including the follow-up of a sib-pair originally reported with the kyphoscoliotic type of EDS in 1975. Brachycephaly, a characteristic facial appearance, an asthenic build, hyperextensible and bruisable skin, tapering fingers, instability of large joints, and recurrent formation of large subcutaneous hematomas are always present. Three of seven patients had mildly elevated serum creatine kinase. The oldest patient was blind due to retinal detachment at 45 years and died at 59 years from intracranial bleeding; her affected brother died at 28 years from fulminant endocarditis. All patients in this series harbored homozygous, predicted loss-of-function *CHST14* mutations. Indeed, DS was not detectable in fibroblasts from two unrelated patients with homozygous mutations. Patient fibroblasts produced higher amounts of chondroitin sulfate, showed intracellular retention of collagen types I and III, and lacked decorin and thrombospondin fibrils compared with control. A great proportion of collagen fibrils were not integrated into fibers, and fiber bundles were dispersed into the ground substance in one patient, all of which is likely to contribute to the clinical phenotype. This report should increase awareness for MC-EDS. © 2015 Wiley Periodicals, Inc.

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*Correspondence to:
Andreas R. Janecke, M.D., Department of Pediatrics I & Division of Human Genetics, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria.
E-mail: Andreas.Janecke@i-med.ac.at
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INTRODUCTION

The musculocontractural type of Ehlers-Danlos syndrome (MC-EDS; OMIM 601776), which is also referred to as Adducted Thumb-Clubfoot Syndrome (ATCS; OMIM 601776), represents a recently described autosomal recessively inherited condition [Dündar et al., 2009; Kosho et al., 2010; Malfait et al., 2010; Miyake et al., 2010; Syx et al., 2015]. Neonates with MC-EDS present with distal arthrogryposis and muscular hypotonia. MC-EDS thus represents a differential diagnosis within the congenital neuromuscular and connective tissue disorders. Characteristic features with a spectrum of clinical symptoms have been reported in 34 patients with MC-EDS, and bi-allelic mutations have been identified in *CHST14* (carbohydrate [N-acetylgalactosamine 4–0] sulfotransferase 14, OMIM 608429) in all but three patients [Dündar et al., 2009; Malfait et al., 2010; Miyake et al., 2010; Voermans et al., 2012; Winters et al., 2012; Mendoza-Londono et al., 2012; Syx et al., 2015], who displayed *DSE* (dermatan sulfate epimerase, OMIM 605942) mutations [Muller et al., 2013; Syx et al., 2015]. *CHST14* and *DSE* encode two enzymes that are necessary for dermatan sulfate (DS) biosynthesis. DS proteoglycans are components of diverse connective tissues and their deficiency and/or replacement by chondroitin sulfate (CS) in proteoglycans such as decorin results in abnormal regulation of collagen fibril assembly [Shimizu et al., 2011]. Moreover, DS interacts with heparin cofactor II and inhibits thrombin in vessel walls following disruption of the endothelium, and thus modulates thrombus formation [He et al., 2008].

In this study, we report seven additional patients from four families with MC-EDS, including the oldest patient with this condition described so far, and identify bi-allelic *CHST14* mutations in all probands. MC-EDS emerges further as a recognizable condition with both consistent and variable clinical features, known disease causing genes (*CHST14* and *DSE*), and thus available molecular testing.

MATERIALS AND METHODS

Subjects

We evaluated seven individuals from four different families presenting with a history of arthrogryposis, muscular hypotonia, hypermobility of finger, elbow, and knee joints, and sub-luxations of large joints, skin hyperelasticity and fragility, craniofacial dysmorphism, and large subcutaneous bleedings.

Molecular Studies

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 in family one using the HumanCytoSNP-12v2 BeadChip (Illumina, CA) according to the manufacturer's instructions. Raw single nucleotide polymorphism (SNP) call data were processed with the Genotyping Analysis Module in GenomeStudio 1.6.3 (Illumina). Homozygosity mapping was performed with the Merlin program [Abecasis et al., 2002]. Detection of large homozygous chromosomal regions in the index patient from family 2 was performed using the same SNP array.

Direct genomic sequencing of the coding exon of *CHST14* was performed. Briefly, genomic DNA was isolated and a polymerase chain reaction (PCR) amplified the coding regions and splice sites. The PCR products were sequenced in both directions using sense or antisense primers and the Big Dye terminator kit (Applied Biosystems, Vienna, Austria). The same primers (available upon request) were used for PCR and sequencing reactions. Linear amplification products were then separated on an automated capillary sequencer (ABI 3100 Genetic Analyzer, Applied Biosystems). Electropherogram-derived sequences were compared to reference for *CHST14* (NCBI ref.seqs.: NM_130468.2 and NG_017074.1) using Sequencher software 4.8. To confirm genetic segregation, relatives were tested for mutations detected in probands.

Cell Cultures

Primary dermal fibroblast cultures from individuals with MC-EDS from families 1 and 2 were established from skin biopsies by routine procedures. Control samples were obtained from normal skin of age- and sex-matched patients undergoing dermatologic procedures. Fibroblasts were routinely maintained in Dulbecco's modified Eagle medium (DMEM; PAA, Somerset) supplemented with 10% fetal calf serum (PAA, Somerset) at 37°C in 5% CO2. Fibroblasts were expanded until full confluency was achieved, and then harvested by trypsin treatment at the same passage number.

Indirect Immunofluorescence (IF) Microscopy

Polyclonal antibodies (Abs) against collagen types I, III, and V and mouse monoclonal Abs (mAbs) against collagen type VI (clone 3C4), α2β1 (clone BHA.2) and α5β1 (clone JBS5) integrins were obtained from Millipore Chemicon International (Billerica, MA). Ab against the human fibronectin (FN) and mAbs against all of the human isoforms of tenascins (TNs), including tenascin C (TNC, clone BC-24) and the CS glycosaminoglycans (GAGs; clone CS-56), ascorbic acid, and tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-goat antibody were from Sigma–Aldrich (Taufkirchen, Germany). The anti-decorin (DCN) mAb (clone 115402), recognizing the core protein, was from R&D Systems, Inc. (Minneapolis, MN) and the anti-heparan sulfate (HS) GAGs mAb was from USBiological Life Sciences (Swampscott, MA). The anti-thrombospondin (TSP) mAb was from NeoMarkers, Lab Vision (Fremont, CA). The fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and TRITC-conjugated goat anti-mouse secondary Abs were from Calbiochem-Novabiochem Int. (San Diego, CA).

Fibroblasts used for IF microscopy were grown for 48 hr on glass coverslips, as described above. To analyze the organization of the collagen types I, III, and V, FN, and TN, cells were fixed in cold

methanol and incubated with the specific Abs, as previously reported [Zoppi et al., 2004]. To analyze the collagen type VI organization, fibroblasts were cultured for 7 days in the presence of 0.25 mM ascorbic acid, refed every 48 hr, fixed in methanol and immunoreacted with the anti-collagen type VI mAb. The α5β1 and α2β1 integrin organization was analyzed on cells fixed in 3% paraformaldehyde (PFA)/60 mM sucrose and permeabilized in 0.5% Triton X-100, and reacting for 1 hr at room temperature with 1 μg/ml anti-α5β1 and anti-α2β1 integrin mAbs. To analyze the DCN, HS and CS GAGs, and TSP organization, the cells were fixed for 10 min in 3% PFA, and reacted with 25 μg/ml anti-DCN, 5 μg/ml anti-HS GAG and anti-CS GAG and 1 μg/ml anti-TSP mAbs, respectively. Cells were subsequently incubated for 45 min with 10 μg/ml FITC- or TRITC-conjugated anti-rabbit, anti-goat, or anti-mouse IgG and IgM. IF signals were acquired by a charge-coupled device black and white TV camera (SensiCam-PCO Computer Optics GmbH, Germany) mounted on a Zeiss fluorescence-Axiovert microscope and digitalized by Image-Pro Plus program (Media Cybernetics, Silver Spring, MD).

CHST14 Immunoblot and Quantitative RT-PCR for *CHST14* and DCN

Quantitative RT-PCR was performed as described previously [Wang et al., 2008]. Briefly, total RNA was isolated from cultured fibroblasts with the Absolutely RNA purification kit (Agilent, Santa Clara, CA). Genomic DNA was removed by DNaseI treatment following the manufacturer's protocol. RNA was reverse transcribed using SuperScript III (Life Technologies, Grand Island, NY) and real-time PCR was performed on a Chromo4 (Bio-Rad) with iQ SYBR Green (Bio-Rad, Hercules, CA). All samples were run in triplicate and results were normalized to the level of GAPDH. The primer sequences are as follows: CHST14: 5′-TCCAAGGAAA GGAAAACGGA AG-3′ and 5′-CAGCCACAGT CACAACCTCA AAG-3′; DCN: 5′-CATCTCTGCT GTTGACAATG GCTC-3′ and 5′-TGCTGAAAAG ACTCACACCC G-3′; and *GAPDH*: 5′-ACTCCTCCAC CTTTGACGCT G-3′ and 5′-GGGTCTCTCT CTTCCTCTTG TGCTC-3′. cDNAs for *CHST14*, *DCN*, and *GAPDH* were used as a template for PCR to obtain standard curves. Immunoblot analysis for CHST14 was performed as described previously [Wang et al., 2008] with anti-CHST14 antibody (#ARP48994_P050, Aviva Systems Biology, San Diego, CA) using Immuno-enhancer reagent (Wako, Richmond, VA). Monoclonal anti-actin antibody (Sigma, St. Louis, MO) was used for loading control of the samples.

Glycosaminoglycan Analyses

Cultured fibroblasts in 10 cm dishes were dehydrated and delipidated by extraction with acetone, air-dried, and used for extraction of GAGs essentially as reported previously [Li et al., 2008] with some modifications. Briefly, the dried acetone powder was digested with Proteinase K (Ambion, Grand Island, NY) in 200 μl of 10 mM Tris HCl (pH 8.0) at 50°C for 48 hr. Following incubation, samples were treated with 5% trichloroacetic acid, and the resultant precipitate was removed by centrifugation. The supernatant was extracted with ether to remove trichloroacetic acid. After neutralization with 1 M sodium carbonate, the aqueous phase was adjusted to contain 80% ethanol and 1% sodium acetate and kept at 4°C overnight. The precipitated crude GAGs were recovered by centrifugation. One half of the crude GAGs was digested with chondroitinase B (Sigma, St. Louis, MO) in 10 mM Tris HCl buffer (pH 7.5) for 16 hr. The other half was digested with chondroitinase ABC (Seikagaku, Tokyo, Japan) in 20 mM Tris acetate buffer (pH 8.0) for 16 hr. The digests treated with 80% ethanol were centrifuged and the supernatants containing the disaccharides were recovered and ethanol was evaporated. Cleaved saccharides were separated in 150 mM ammonium bicarbonate with size-exclusion chromatography (Superdex Peptide, 1.0 × 30 cm, GE Healthcare Life Sciences, Piscataway, NJ) attached to a Shimadzu UFLC system at a flow rate of 0.3 ml/min. Unsaturated disaccharides were monitored by absorbance at 232 nm. CS-B (DS from Sigma) was used as a positive control for chondroitinase digestions and size fractionations. Fractions containing disaccharides were collected and repeatedly evaporated; the resulting disaccharides were labeled with the fluorescent tag 2-AB (Sigma) and disaccharide composition was analyzed with HPLC [Wang et al., 2008].

RESULTS

Clinical Findings

The main clinical findings of the four index patients and the three affected sibs together with those MC-EDS patients published previously are listed in Table I. There were common characteristic craniofacial features (Fig. 1), as well as common features of the hands and feet (Fig. 2). The parents of all patients were healthy.

The clinical history of the Pakistanian siblings P1/I and P2/I (Figs. 1 and 2) from birth until 20 and 18 years of age, respectively, has been reported [Steinmann et al., 1975; Steinmann et al., 2002]. Their parents were first-degree cousins, and the two younger brothers were healthy. Female P1/I was born at term weighing 2,700 g. There were clubfeet, marked muscular weakness, weak crying, inability to suck and bottle-feeding possible only by use of teat with especially large opening. The skull was reported to be very soft (like a "balloon filled with water" until age 8 months) and the skin was paper-thin. She sat at one year and walked at three and a half years of age. Her hips dislocated without significant trauma at age 19 years.

Intellectual development was normal, and she was a university student at 20 years of age. She measured 153 cm, weighed 57 kg, head circumference 53.5 cm, with narrow shoulders, heavy thighs and legs and flat thorax, and arm span of 141 cm. She had small mammae and inverted nipples. On clinical examination, her muscle mass appeared greatly decreased.

Laxity of joints, frequent bruising, delayed wound healing, skin fragility with prolonged bleeding, and dermal hyperelasticity were present. Grasping her hand was like taking a cherry stone pillow. There was hyperalgesia to pressure, so that even blood pressure measurements were painful. Electromyogram (EMG) and creatine kinase (CK) were normal. She had a moderate scoliosis, and radiographs showed that the vertebral bodies were narrow, high and osteophytic. Diaphyses of phalanges and metacarpals were narrow with thickening of the corticalis and markedly reduced marrow space, and the metaphyses were osteoporotic. She frac-

TABLE I. Features Associated With *CHST14* Mutations (Including Indels) and with Two *DSE* Mutations in MC-EDS Patients Reported in This Study and in the Literature

Always present
Habitus/skeletal/muscular
Congenital multiple contractions
Generalized joint laxity
Scoliosis, kyphoscoliosis (increasing with age)
Myopathy, muscular weakness, decreased muscle mass
Tapering/slender and cylindrical fingers
Fine palmer creases
Craniofacial
Hypertelorism
Protruding, large, low-set, posteriorly-rotated or dysplastic ears
Short nose, long philtrum
Bleeding disorder
Large subcutaneous hematomas
Cryptorchidism in males
Mostly present (>90% of individuals)
Habitus/skeletal/muscular
Marfanoid or asthenic habitus
Pectus deformities
Recurrent/chronic joint dislocations
Gross motor delay
Craniofacial
Large fontanelle
Down-slanting palpebral fissures
Blue sclerae
Small mouth (early childhood)
Thin upper lip
High palate
Prominent nasolabial folds in childhood
Skin
Hyperextensible to redundant skin with bruisability, atrophic scarring
Hyperalgesia to pressure or tension (such as measuring blood pressure)
Common (>50% of individuals)
Craniofacial
Microretrognathia
Strabismus
Myopia, astigmatism
Elevated intraocular pressure, glaucoma
Retinal detachment (following glaucoma surgery and age-related)
Skin
Recurrent subcutaneous infections
Cardial
Heart valve abnormalities (possibly increasing with age)
Gastrointestinal
Constipation
Occasional anomalies
Cleft lip/cleft palate
Craniosynostosis
Hearing impairment
Cerebral ventricular enlargement
Pneumothorax
Congenital heart defects
Anomalous insertions of flexor muscles
Absence of gastrocolic omentum, common mesentery, volvolus of small intestine, intestinal malrotation, diverticular perforation
Inguinal hernia
Horseshoe kidney
Nephrolithiasis, hydronephrosis
Bladder dysfunction

Data from 34 persons with MC-EDS from 22 unrelated families were included in this compilation; no clinical details were available from 3 deceased subjects.

tured her tibial tuberosity following increased tension exerted by her quadriceps tendon, and hyperelasticity of her cruciate ligaments was judged extraordinary on surgery. She acquired large cutaneous and subcutaneous hematomas following minor trauma, and had a prolonged bleeding time with normal clotting factors. She had down-slanting palpebral fissures, bluish sclerae, microcorneae (diameter 9–10 mm), myopia (-2 diopt.), a few circumscript retinal degenerations at the periphery but no signs of retinal detachment. She experienced retinal detachment on both eyes at 45 years of age, causing right-sided blindness. The patient died at age 59 years after she fell to the ground and was found to have a large intracerebral hemorrhage as the cause of death.

P2/I, the younger brother of P1/I, was born at term weighing 2,720 g. His development and clinical course such as muscular hypotonia, feeding difficulties, gross motor delay, and growth along the 3rd centile were similar to that of his older sister. His Achilles tendons were lengthened at age 5 years, and abnormal flaccidity and fragility of all soft tissues were noted. Frequent bruising, vulnerability of skin and prolonged bleeding were reported throughout childhood. Large subcutaneous haematomas of legs frequently required surgical removal. At 18 years of age, he measured 175 cm and weighed 46.5 kg, with a head circumference of 55 cm, an arm span of 170 cm, and a slender build (as opposed to his sister) with slim arms and legs, and arachnodactyly (positive wrist sign, negative thumb sign). He had down-slanting eyes, drooping lower eyelids, bluish sclerae, myopia (-2 diopt.), microcorneae (8–9 mm) but no signs of retinal detachment, and posteriorly-rotated very soft and floppy ears. He had mild scoliosis. He had one small and one undescended testicle. On clinical examination, his muscle mass appeared greatly decreased. He had mitral valve prolapse with moderate insufficiency and hyperalgesia to pressure such as measuring blood pressure. The bleeding time was repeatedly prolonged with normal coagulation factors. Serum CK at age three and half years was normal.

He died at 28 years of age from fulminant endocarditis despite the prescribed endocarditis prophylaxis.

P3/II (Figs. 1 and 2), a Hispanic boy, was born at term to first cousins once removed, with birth weight 2.9 kg, and length 45.7 cm. Three older sibs were healthy. Prenatal ultrasound at 17 weeks of gestation suggested hand and foot anomalies.

Skeletal survey at birth showed possible intracranial calcifications that could represent resolving hematomas, and the skull was very thin over the fronto-parietal region. He was referred to Medical Genetics at 2 weeks of age for multiple congenital anomalies, i.e., hypotonia, bilateral adducted thumbs, bilateral clubfeet, bilateral inguinal hernias, and bilateral undescended testes. Serial casting of feet from 3 to 12 weeks of age was followed by a Dennis Browne splint for 3 months, and Achilles tendon release.

He underwent surgeries for bilateral inguinal hernias and undescended testes, as well as surgery for bilateral extensor tendon transfers from the ulnar dorsal side of the hand to the thumb extensor at 1 year of age. During surgery he was found to have a single flexor tendon to the thumbs and a single tendon to the dorsum of the index fingers. Significant joint laxity and weakness were noted.

At 19 months of age, paper-thin, pale distensible skin with multiple bruises, and muscular hypotonia were noted. He was unable to support himself in a sitting or standing position. He had

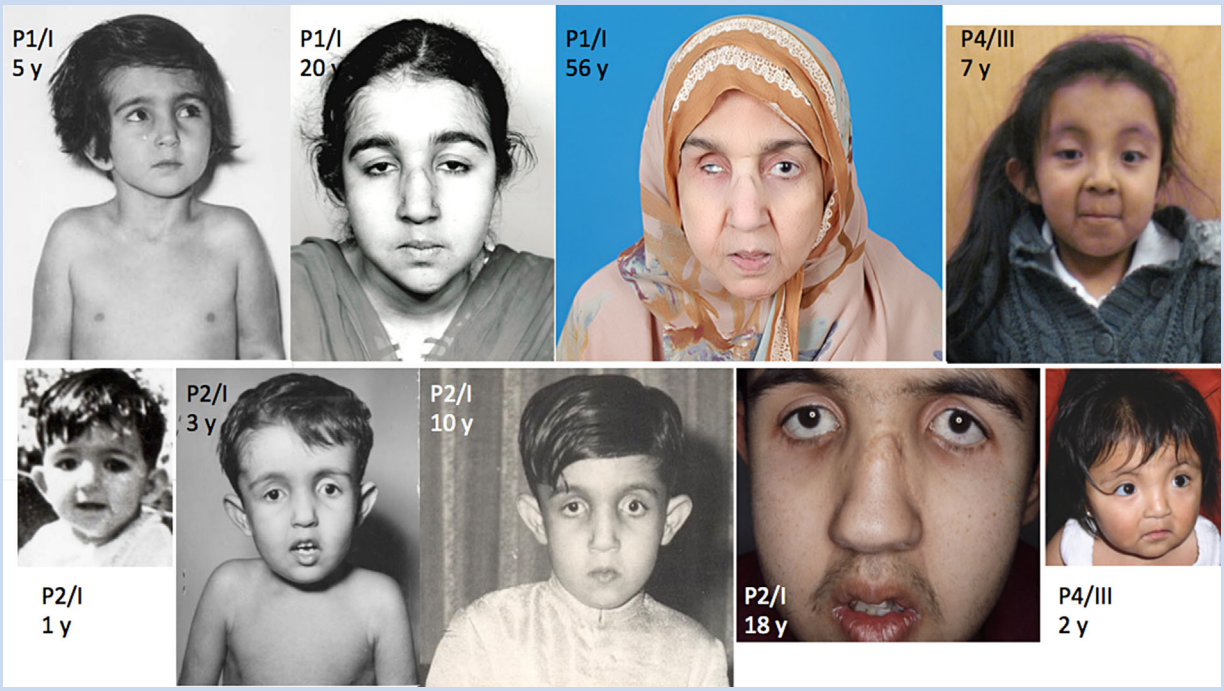

FIG. 1. Characteristic craniofacial aspects of MC-EDS. Note hypertelorism, down-slanting palpebral fissures, microcorneae, prominent nasolabial folds, a thin upper lip vermillion, prominent and often low-set and posteriorly-rotated ears. The face appears myopathic. These aspects are present at different age. y, years; m, months.

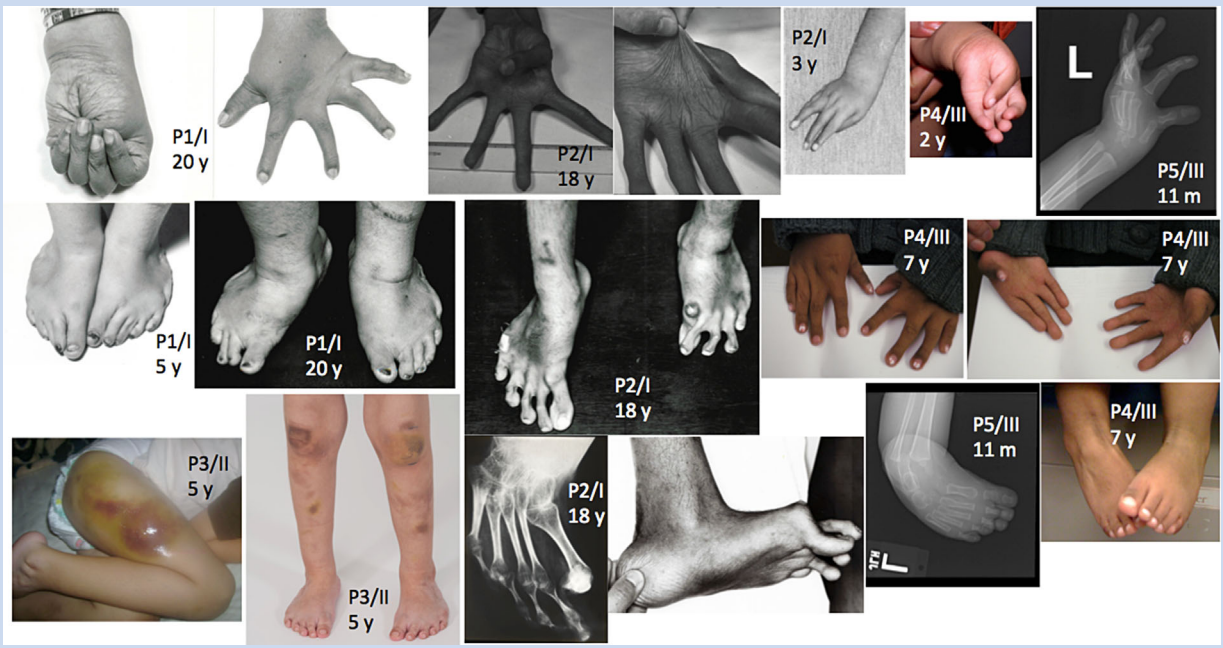

FIG. 2. Characteristic findings of hands and feet of MC-EDS patients, and an example of hematoma-formation. Note arachnodactyly, partial syndactyly, fine palmar creases, and tapering fingers and toes. Radiographs show congenital talipes valgus and planus, with slight diaphysial narrowing of the phalanges and metatarsals in infancy, and marked narrowing of diaphyses of metatarsals and phalanges, and osteoporotic metaphyses in P2/I at age 18 years. (some figures of P1/I and P2/I are taken from [Steinmann et al., 1975] with permission of the Publisher and from [Steinmann et al., 2002], respectively).

mild elbow contractures, increased joint laxity of wrists and ankles, and adducted thumbs. Language skills and social development were normal. CK was 3,000 U/L (<305) and Ullrich congenital muscular dystrophy was suspected.

At age 2 years, he developed epidural bleeding associated with obstructive hydrocephalus after a minor fall and required surgical evacuation. He still had a widely patent anterior fontanelle. Every 2–4 months after 2 years of age, he had extensive soft tissue and joint hemorrhages following minor trauma, primarily affecting the knees, buttocks, and thighs. The frequency of soft tissue bleeds decreased markedly after age 5 years.

When seen at age 6 years, he had soft, doughy elastic skin, thin scarring, and lax joints. Dysmorphic features included flat facies with malar hypoplasia, hypertelorism with down-slanting palpebral fissures, depressed nasal bridge, high arched palate, low set, slightly posteriorly-rotated ears, short neck, and a shield chest with widely spaced nipples. Microcornea was apparent but the cornea diameter was not measured. Remarkably, he was hypersensitive to tactile stimuli such as applying or removing an electrocardiogram (ECG) electrode. His cognitive development was normal. At age 8 years, he was able to communicate by email. He does not participate in normal sport activities for fear of injury. Fresh frozen plasma and desmopressin (DDAVP, 1-Desamino-8-D-Arginine-Vasopressin) administration were considered beneficial in the case of hemorrhage.

Cranial magnetic resonance imaging (MRI) scans revealed normal findings at 5 weeks, and at 26 months of age. Magnetic resonance angiography did not detect any vascular malformations. He had a prolonged bleeding time and a decreased ristocetin response on platelet aggregometry. There were normal results for complete blood counts (CBC), platelets, prothombin time (PT), partial prothrombin time (PTT), fibrinogen, factors 8, 9, 11, and 13, platelet function testing (PFA-100), alpha-2 antiplasmin, von Willebrand disease, plasminogen activator inhibitor-1, tissue plasminogen activator, collagen-specific platelet glycoproteinIb/IIb/IIIa surface receptors, overall fibrinolysis as measured by the euglobulin lysis time and shear wave elastography quantification of blood elasticity during clotting. Serum CK levels were mildly elevated at serial measurements (1.5 years: CK 2,944 U/L; normal range 60–305), 2 years: 878 U/L (<228), 3.5 years: 336 U/L (<149), 5.5 years: 405 U/L (<149)). An echocardiogram at 5 years of age was normal.

P4/III (Figs. 1 and 2), the index patient, was a girl born by emergency Cesarean section at 42 weeks gestation to healthy Hispanic parents, who denied consanguinity. The infant emerged with poor respiratory effort and Apgar scores of 1 at 1 min and 8 at 5 min. The birth weight was 3,180 g, length was 45.5 cm, and head circumference (OFC) was 33 cm. The child had multiple craniofacial anomalies with brachycephaly, hypertelorism, down-slanting palpebral fissures, a broad nasal bridge and a shallow palate. Her skeletal findings comprised bilateral talipes equinovarus, which were reducible and bilateral adducted thumbs which were difficult to extend. She had muscular hypotonia. She required oxygen over the first 24 hr and had feeding difficulties secondary to a poor and uncoordinated suck. However, she did not require tube feeding. She had mild sensorineural hearing loss on the right with normal hearing on the left. She had a normal cranial ultrasound, a normal cranial computerized tomography (CT) scan and MRI scan, a normal echocardiogram and a normal skull radiograph. She was discharged home at 5 days of life with mild jaundice and improved feeding.

She was followed intermittently in Genetics clinic until age 7 when, despite gross motor delays, cognition was normal, head circumference was 49 cm, and she was doing well in school. Serum CK level was elevated at one measurement (CK 698 U/L; normal range 39–189 U/L). She also had a history of recurrent joint dislocations with at least three episodes of glenohumeral dislocation resulting from minimal trauma. She developed a large temporal hemorrhage after minor trauma. Based on clinical findings, she was suspected to have MC-EDS.

P5/III (Figs. 1 and 2), the 6-year younger brother of P4/III, was born by repeat Cesarean section at term due to oligohydramnios and decreased fetal movement. Apgar scores at 1 and 5 min were 8 and 9, respectively. The birth weight was 3,225 g. Multiple craniofacial anomalies included brachycephaly, hypertelorism, down-slanting palpebral fissures, bilateral talipes equinovarus and adducted thumbs. Neurologically, he had hypotonia and weak suck, and like his sister, he stayed in hospital after birth due to feeding difficulties. At 4 months of age, head circumference was 40 cm, a cranial ultrasound showed mild ventriculomegaly, a cranial MRI was normal and a skeletal survey showed talipes as described above. Serum CK level was elevated at one measurement (CK 1838 U/L; normal range 39–189 U/L).

Both sibs had blue sclerae, apparent microcornea (cornea diameter not measured), low-set and posteriorly-rotated ears, downturned corners of the mouth, micrognathia, fine palmar creases, reduced distal muscle bulk, soft and doughy skin and long and tapering fingers. The girl had glaucoma from age 3 years that required medical and surgical management. Her EMG was myopathic, and a muscle biopsy showed lipid and glycogen accumulation with mitochondrial hypertrophy.

P6/IV, the index, is a 4-year-old girl born at term by normal spontaneous vaginal delivery to a 30-year-old mother and a 31-year-old father of Hispanic descent. Consanguinity was not reported, but the parents came from the same village in Mexico. Birth weight was 3,200 g and length was 47 cm. After birth, she was noted to have distal arthrogryposis with bilateral clubfeet and bilateral hand deformity. At age 1 month, she had casting for talipes for 5 weeks and then wore braces at night for a period of time.

At age 4-years her length was 111 cm and weight was 23.2 kg. Her distinctive craniofacial features included low-set posteriorly-rotated ears with prominent and overfolded helices, bilaterally attached lobules, hypertelorism with down-slanting palpebral fissures, bluish sclerae, and apparent microcornea (cornea diameter not measured). Dimples were present in the shoulders and elbows. She had ulnar deviation of her hands with abnormal shallow palmar creases. Fingers and toes had a spatula-like appearance. Her skin felt doughy. Psychomotor development was normal. Following negative genetic work-up for Freeman-Sheldon syndrome, she was suspected to have MC-EDS on clinical findings.

P7/IV, the younger brother of P6/IV, was born at term by spontaneous vaginal delivery; a left foot contracture and right hand contracture were diagnosed prenatally. Birth weight was

2,860 g and length was 48 cm. Apgar scores were 9 at 1 and 5 min. After birth, contractures of extremities were confirmed, and he had an undescended testis and dysmorphic features. He had casting of his left clubfoot and left tendon Achilles tendon lengthening.

At age 18-months his length was 80 cm, weight was 10 kg and head circumference 47.5 cm. Several dysmorphic craniofacial features were noted: low-set posteriorly-rotated ears with prominent, cupped helices and bilaterally attached ear lobules, hypertelorism with down-slanting palpebral fissures, bluish sclerae, and apparent microcorneae. He had ulnar deviation of the right hand with abnormal, shallow palmar creases. His fingers and toes had a spatula-like appearance. Severe eczema was noted. Psychomotor development was normal. Echocardiography was recommended but not performed on either sibling.

Molecular Genetic Investigations

P1/I and P2/I were clinically diagnosed with EDS at 5 and 3 years of age, respectively, in 1959, and with EDS VI due to an apparent deficiency of collagen lysyl hydroxylase in cultured skin fibroblasts producing hydroxylysine-deficient collagen by the Manchester group. However, the hydroxylysine content was only moderately reduced in dermis and was normal in tendon and bone as determined by the Zurich group [Steinmann et al., 1975], who alluded to this biochemical discrepancy in the title of the paper. Later studies could not confirm the deficiency of lysyl hydroxylase activity by direct [Royce et al., 1989] or indirect measurement [Royce et al., 1989; Steinmann et al., 2002] and the disease was called EDS VIB by Victor McKusick as opposed to EDS VIA with enzyme deficiency. Finally, homozygosity mapping in this family, including both affected and two unaffected sibs, identified four candidate regions on chromosomes 8, 9, 11, and 15. The chromosome 15 Locus contained *CHST14*, which had been reported to be mutated in MC-EDS/ATCS at the time of the linkage analysis [Dündar et al., 2009; Miyake et al., 2010]. The shared clinical features with published MC-EDS/ATCS patients prompted *CHST14* sequence analysis. A homozygous c.453dup (p.(Cys152Leu*fs**10)) mutation causing early protein truncation was found to segregate with the disease in the family. Array-based high-resolution chromosome analyses were normal in both patients. The parents were heterozygous for the same mutation.

P3/II underwent sequencing of *COL3A1*, *COL6A1*, and *COL6A2*, which ruled out initial diagnoses of the vascular type of EDS (OMIM 130050) and Ullrich muscular dystrophy/Bethlem myopathy (OMIM 254090 and OMIM 158810), respectively. A SNP array analysis revealed a 26 Mb-region of homozygosity on chromosome 15q15.1 in the patient but not in the two unaffected sibs. This region contained *CHST14*, which was found to harbor a homozygous c.638G>C (p.(Arg213Pro)) mutation. The same mutation had been reported previously in a patient with ATCS and affects an invariably conserved residue of CHST14 [Dündar et al., 2009]. Array-based high-resolution chromosome analysis was normal in the patient. The parents were heterozygous for the same mutation.

P4/III, the index, was suspected with MC-EDS which was proven by the homozygous c.977_980dup (p.(Trp327Cys*fs**29)) mutation of *CHST14* in her as well as in her younger brother P5/III. Her mother was heterozygous for this change. Samples from the father and her younger sibling were unavailable and thus a deletion on the paternal allele rather than homozygosity for the same mutation cannot be excluded. The identified frameshift mutation causes a truncation of the 49 C-terminal residues of the wild-type protein. Array-based high-resolution chromosome analyses were normal in both children.

P6/IV was suspected to have MC-EDS, which was proven by the homozygous c.784G>A (p.(Glu262Lys)) mutation of *CHST14* in P6/IV and her affected brother P7/IV, while the parents were heterozygous for this mutation. This novel mutation affects a highly conserved residue in CHST14. Array-based high-resolution chromosome analyses were normal in both children. Testing for Freeman-Sheldon syndrome (OMIM 193700) had been performed prior to *CHST14* testing including sequencing and deletion/duplication analysis of the *TNNI2*, *TNNT3*, *MYH3*, and *TPM2* genes and the results were normal. The parents were heterozygous for the same mutation.

Altogether, *CHST14* mutations were identified in all probands, bringing the total number of known *CHST14* mutations to 18, of which 8 are protein-truncating mutations and 10 are missense mutations (Fig. 3).

Immunostaining of Fibroblasts and Electron Microscopy

To investigate the pathophysiological consequences of CHST14 deficiency, the distributions of well-characterized ECM proteins were examined in cultures of dermal fibroblasts derived from P1/I and P2/I with the homozygous p.(Cys152Leu*fs**10) mutation. These cells were not covered by a reticular ECM of FN and TNs, as compared with control skin fibroblasts. The highly decreased levels of TNs assembled in the ECM of CHST14-deficient cells suggest a significant reduction of TNC as well. Patient fibroblasts lacked DCN and TSP fibrils in the extracellular environment, and FN and fibrillar collagen receptors α5β1 and α2β1 integrins, respectively, on the cell surface. The mutant cells showed intracellular retention of collagen types I and III, and a reduced deposition of collagen types I, III, V and VI fibrils in the ECM, as compared to control fibroblasts. These cells produced and stored higher amounts of CS GAG in the cytoplasm, which was not organized in the ECM, and normal amounts of HS GAG as compared with control fibroblasts (Fig. 4). Previous investigation of the reticular layer of dermis from P2/I by electron microscopy revealed that a great proportion of collagen fibrils was not integrated into fibers and fiber bundles. As a result, fiber bundles were poorly delineated, and non-integrated fibrils formed a rather irregular texture (Fig. 5).

Analysis of Glycosaminoglycan Chain Composition, *CHST14* and DCN Expression

A fifty percent reduction in *CHST14* mRNA was found in P3/II fibroblasts with the homozygous p.(Arg213Pro) mutation (Fig. 6A). This reduction was confirmed on the protein level by immunoblot analysis (Fig. 6B), whereas *DCN* mRNA, encoding one of the major DS proteoglycans [Moreth et al., 2012], showed no substantial changes compared to control fibroblasts.

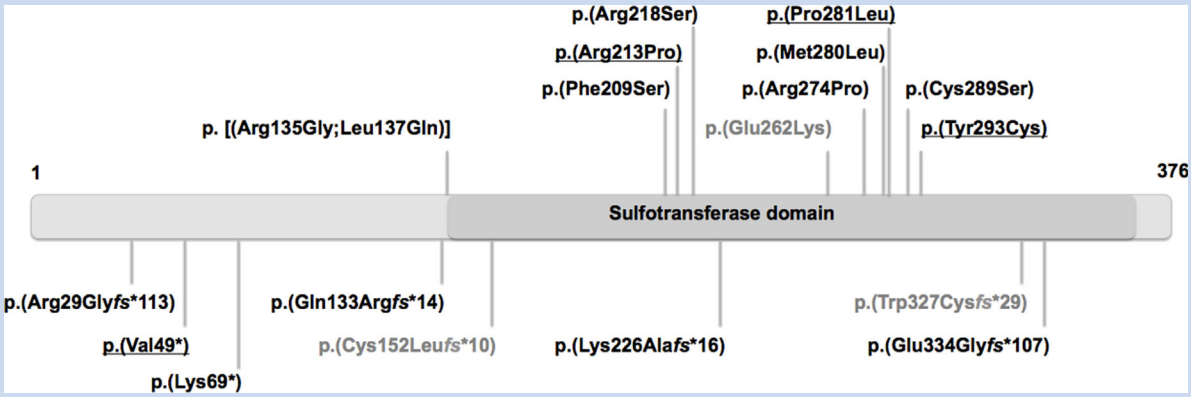

FIG. 3. Schematic compilation of known *CHST14* mutations. In gray, novel mutations; underlined, recurrent mutations found in unrelated MC-EDS patients; above missense mutations, below truncating mutations.

In order to examine the effects of the homozygous *CHST14* p.(Cys152Leu*fs**10) and p.(Arg213Pro) mutations on carbohydrate properties, GAG fractions were prepared from cultured fibroblasts of patients P1/I and P3/II and were extensively digested with either chondroitinase ABC or chondroitinase B. Whereas chondroitinase ABC generates disaccharides from CS as well as DS, chondroitinase B converts only DS to disaccharides. Very small amounts of DS were detected in fibroblasts from patient P1/I and P3/II by chondroitinase B digest, whereas similar total amounts of disaccharides from CS and DS digests by chondroitinase ABC were generated in control and patients' fibroblasts, indicating that CS production in patient fibroblasts was increased (Fig. 7). To further define the alteration in their GAGs, disaccharide fractions were collected and composition analyses were performed (Table II). While the major sulfation of chondroitin/dermatan sulfate was 4-sulfation (35.5%) in control fibroblasts and in fibroblasts with the homozygous p.(Cys152Leu*fs**10) mutation, the homozygous p.(Arg213Pro) mutation in CHST14 reduced 4-sulfation (10.7%), possibly via binding of the residual mutant enzyme to GAGs without any 4-sulfation activity but thereby blocking access of other chondroitin sulfotransferases. Both mutations resulted in increased 6-sulfation (52.6 and 58.7%).

DISCUSSION

Musculocontractural EDS (MC-EDS) represents one of several recently recognized disorders of GAG synthesis [Mizumoto et al., 2013]. Because of variability in severity of clinical presentation, the disorder has received the different designations of ATCS, EDS Kosho type, EDS type VIB and Dündar syndrome [Dündar et al., 2009; Kosho et al., 2010; Malfait et al., 2010; Miyake et al., 2010; Shimizu et al., 2011 Kosho et al., 2011; Janecke et al., 2011; Mendoza-Londono et al., 2012]. Recently, it has been classified among the Ehlers-Danlos syndromes because of shared common characteristics, such as joint hypermobility, skin hyperextensibility, and tissue fragility [De Paepe and Malfait, 2012]. MC-EDS types 1 and 2 are distinguished by the underlying mutations in *CHST14* (OMIM 601776) and *DSE* (OMIM 615539), respectively.

Our identification of seven additional patients with MC-EDS increases awareness of the condition and corroborates our knowledge regarding the clinical manifestations. Furthermore, this report should facilitate patient identification for targeted molecular analysis of MC-EDS. Indeed, the diagnosis of MC-EDS was made on clinical findings in the index patients of families III and IV at 7 and 4 years of age, respectively, just after the molecular basis of MC-EDS was established, and targeted mutation analysis was initiated. The patients of families I and II (P1/I, P2/I, and P3/II) were diagnosed with MC-EDS based on results from homozygosity mapping and subsequent *CHST14* analysis.

Our current study and previous reports on MC-EDS point out that MC-EDS can be recognized based on the presence of congenital distal arthrogryposis, i.e., adducted thumbs or clenched fists and talipes equinovarus, as well as hands with atypically shallow palmar creases, tapering fingers, neonatal muscular hypotonia, and characteristic craniofacial features (Table I). The latter consist of brachycephaly, large fontanelle, hypertelorism, down-slanting palpebral fissures, microcorneae, strabismus, a short philtrum, prominent nasolabial folds, a thin upper lip vermillion, small mouth and high palate, microretrognathia, prominent and often low-set and posteriorly-rotated ears. MC-EDS patients show muscular hypoplasia and weakness, which has been confirmed by ultrasound and electromyography [Voermans et al., 2012]. Mildly elevated CK levels have so far been reported in one case of MC-EDS, and CK levels have been normal in all other reported patients; our finding of mildly elevated CK levels in three of seven patients supports the evidence for a myopathy in MC-EDS. Distal arthrogryposis and myopathy might explain the delayed motor development in all reported MC-EDS patients. Our series of patients confirms current knowledge that intellectual development in this disorder is normal.

The pattern of clinical findings at birth should allow to distinguish between MC-EDS and other differential diagnoses of hypotonic and weak infant with arthrogryposis, i.e., congenital muscular dystrophies such as laminin a2 deficiency (OMIM 607855), congenital myopathies, congenital myasthenic syndromes, congenital metabolic myopathies, very early spinal muscular atrophy (SMA, OMIM 253300),

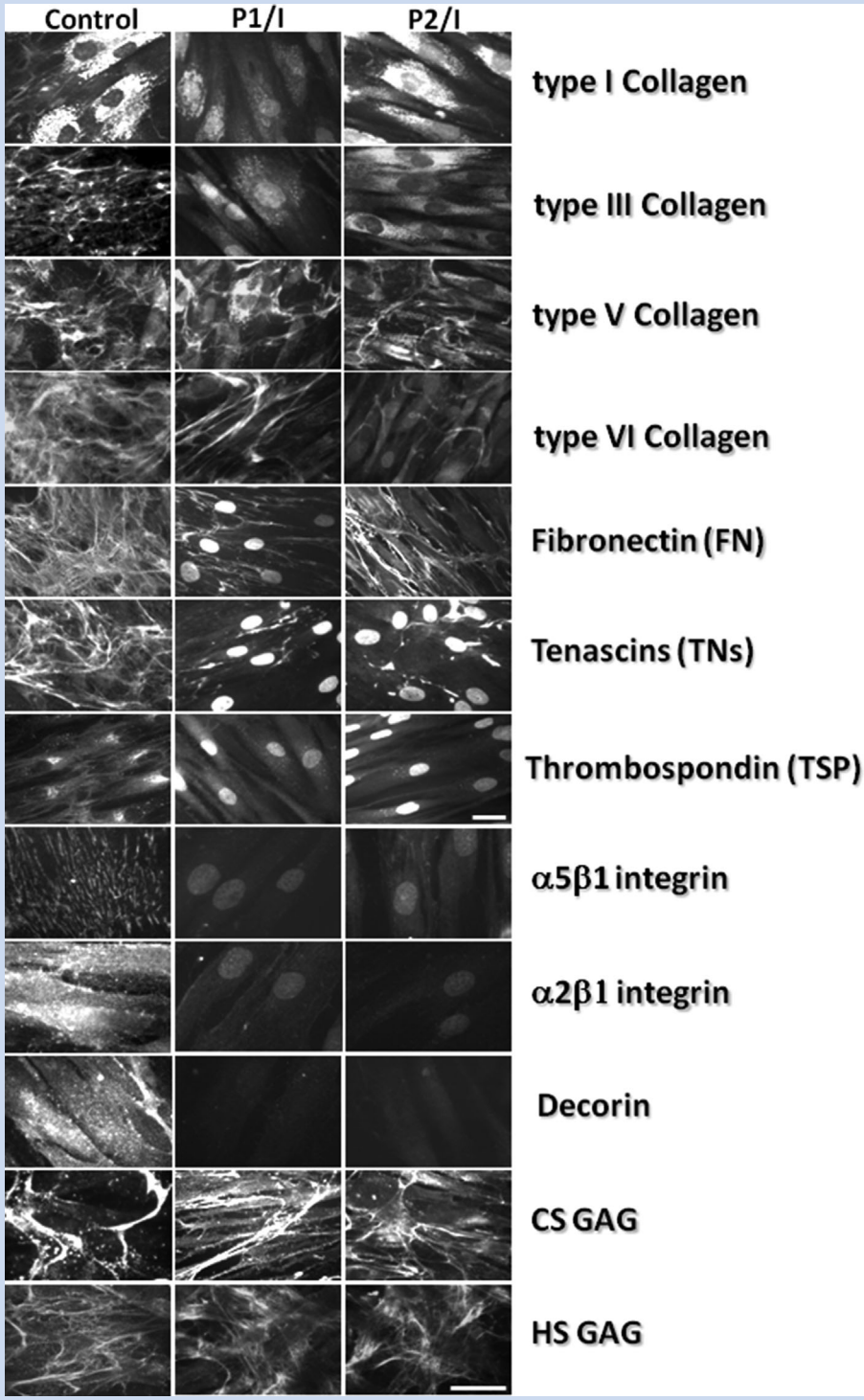

FIG. 4. Representative micrographs depicting immunodetection of ECM components in 2-day-old cultures of dermal fibroblasts derived from skin biopsies from P1/I and P2/I. Mutant fibroblasts show increased intracellular levels of CS GAGs, that are not organized in the ECM, lack of DCN and TSP fibrils, and reduced deposition of collagens I, III, V and VI, FN and TNs. The FN and fibrillar collagens receptors α5β1 and α2β1 integrins, respectively, are not recruited on the mutant cells membrane. Scale bar: 10 μm.

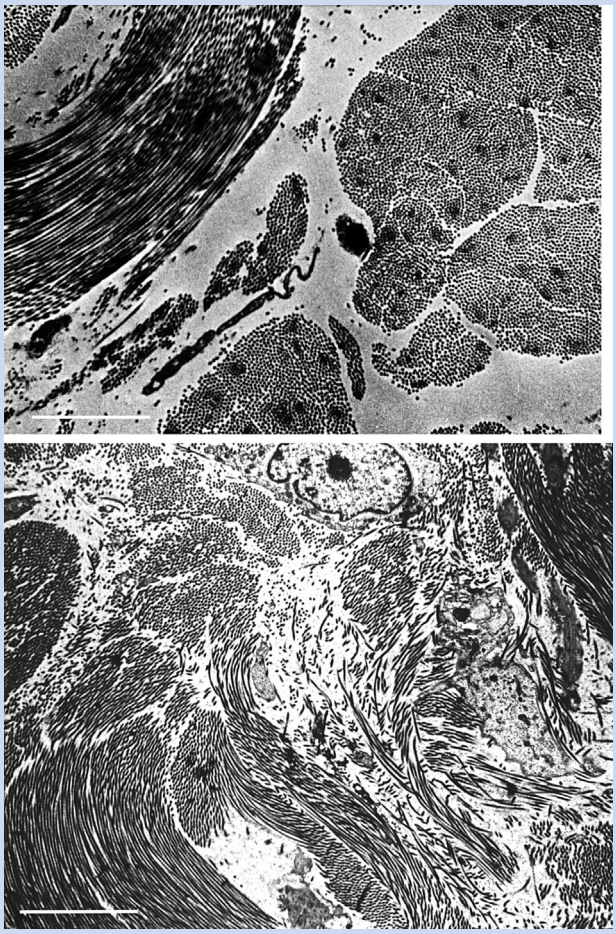

FIG 5. EM of the reticular layer of dermis from P2/I (lower panel) shows that a great proportion of collagen fibrils is not integrated into fibers and fiber bundles but dispersed into the ground substance as compared with a control (upper panel). Consequently, fiber bundles are poorly delineated, and non-integrated fibrils form a rather irregular texture; (taken from [Steinmann et al., 1975] with permission of the Publisher) Scale bar: 50 μm.

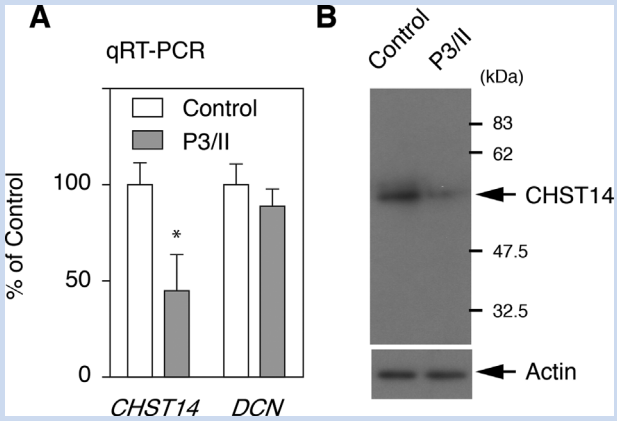

FIG. 6. *CHST14* p.(Arg213Pro) mutation causes a reduction of *CHST14* mRNA and CHST14, but not *DCN* mRNA. (A) RNA was prepared from cultured fibroblasts and quantitative RT-PCR was performed. Open bar, control fibroblasts; closed bar, P3/II fibroblasts. Data are normalized to GAPDH. (B) Cell lysates were prepared from cultured fibroblasts and subjected to immunoblot analysis with anti-Chst14 antibody. Anti-actin antibody was used for sample loading control.

the kyphoscoliotic (OMIM 225400) and FKBP14-related (OMIM 614557) types of EDS, and Prader–Willi syndrome (OMIM 176270). The collagen VI-related Ullrich muscular dystrophy and Bethlem myopathy might emerge as differential diagnoses because hypermobility of large joints can be seen with contractures of the small joints simultaneously [Bonnemann, 2011]. The kyphoscoliotic type of EDS (EDS VIA) shares some facial and dermal features with MC-EDS [Rohrbach et al., 2011; Yeowell and Steinmann, 2013; Abdalla et al., 2014]; patients P1/I and P2/I were originally diagnosed with EDS VIA, which was later questioned by the authors. The original report of these two patients represents the first description of MC-EDS [Steinmann et al., 1975].

MC-EDS patients develop a Marfanoid habitus with narrow shoulders and spinal and pectus deformities. Hyperextensibility and bruisability of the skin, a tendency to atrophic scars, hyperalgesia to pressure, and recurrent subcutaneous hemorrhages are common. Endocarditis should be considered in MC-EDS patients with sudden deterioration, as P2/I in this study died from fulminant endocarditis despite endocarditis prophylaxis, and endocarditis in the presence of aortic and mitral valve regurgitation had been treated by surgery in a 9-year-old MC-EDS patient [Kosho et al., 2010]. Aortic and mitral valve regurgitation, mitral and tricuspid prolapse, and aortic root dilation have occurred in several MC-EDS patients, and warrant attention.

Formation of large subcutaneous hematomas with only minor trauma represents a common and painful experience in MC-EDS, increases the risk of hypovolaemia, and frequently requires surgical treatment. Intranasal administration of 1-desamino-8-D-arginine vasopressin (DDAVP) after injuries has been reported to prevent further hematoma formation [Kosho et al., 2005; Kosho et al., 2010], as in our patient 3/II. A prolonged bleeding time or a decreased ristocetin response on platelet aggregometry with normal results for standard blood coagulation parameters was noticed in patients P1/I, P2/I, and P3/II and was reported in two patients previously [Dündar et al., 2009; Kosho et al., 2010]. However, three other patients had a normal bleeding time [Kosho et al., 2005; Shimizu et al., 2011], indicating that serial testing might be necessary to confirm blood coagulation status. The hematomas have been hypothesized to be caused by rupture of subcutaneous arteries or veins; the risk of rupture of arteries or veins of the brain and internal organs, as seen in the vascular type of EDS [De Paepe and Malfait, 2012] and in the FKBP14-EDS [Baumann et al., 2012], is difficult to estimate. The potential for intracranial bleeding is suggested by the sudden death at 59 years of age of P1/I in this series.

Our data also point to childhood or juvenile onset glaucoma, retinal detachment and microcorneae as common findings in MC-EDS. Cryptorchidism was present in all male patients to

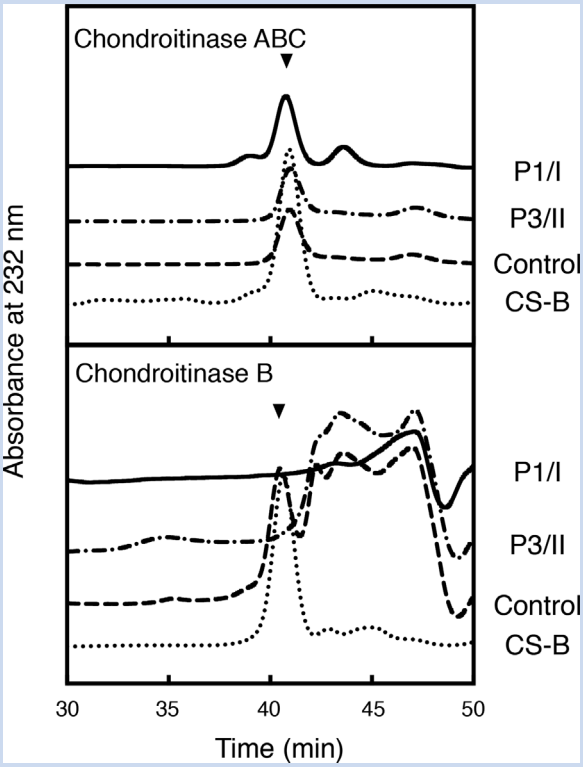

FIG. 7. *CHST14* mutations p.(Cys152Leu*fs10) and p.(Arg213-Pro) identified in patients P1/I and P3/II, respectively, cause a considerable reduction of DS, but not CS in fibroblast culture. GAGs extracted from cultured fibroblasts of patients P1/I and P3/II were digested with chondroitinase ABC (upper panel) and chondroitinase B (lower panel), followed by size exclusion chromatography. Disaccharides generated by chondroitinase digestion were eluted at 40.8 min as a peak (arrowhead). Dashed line, control fibroblasts; solid line, P1/I fibroblasts; dashed-dotted line, P3/II fibroblasts; dotted line, chondroitinase digest of porcine intestinal DS used as standard reference.**

date probably due to muscular hypotonia, and small mammae were reported in post-pubertal female patients.

Counseling of families with MC-EDS regarding long-term outcome and management, and reproductive plans is presently based on the description of 34 affected persons from 22 unrelated families [Steinmann et al., 1975; Dündar et al., 1997; Sonoda and Kouno, 2000; Dündar et al., 2001; Janecke et al., 2001; Kosho et al., 2005; Dündar et al., 2009; Kosho et al., 2010; Malfait et al., 2010; Shimizu et al., 2011; Mendoza-Londono et al., 2012; Voermans et al., 2012; Winters et al., 2012]. MC-EDS had been established as a disease entity by the identification of *CHST14* mutations at least in the index patient of each family reported to date (Fig. 3), and by a homozygous *DSE* mutation in three patients [Muller et al., 2013; Syx et al., 2015].

CHST14 is a one-exon gene, and the mutant transcripts with premature termination codons are predicted to be translated into truncated proteins that completely or partially lack the sulfotransferase domain. The two most C-terminal mutations result in loss of the 42 and 49 C-terminal residues of the 376-residue protein. Eight missense mutations are clustered within or immediately adjacent to the sulfotransferase domain, and seven of these mutations affect highly or invariably conserved residues. Abnormal intracellular enzyme processing and degradation, a decrease in sulfotransferase activity, and/or deficiency of DS-derived disaccharides in fibroblasts have been demonstrated for six missense mutations [Dündar et al., 2009; Miyake et al., 2010]. In this study, we confirmed by disaccharide analysis of intracellular GAGs following chondroitinase digests that fibroblasts derived from P3/II with a homozygous p.(Arg213Pro) mutation do not produce DS because of decreased *CHST14* mRNA and CHST14 protein expression. Immunofluorescent analysis on fibroblasts derived from P1/I and P2/I with a homozygous CHST14 p.(Cys152Leu*fs**10) mutation revealed increased intracellular amounts of CS and an atypical retention of collagen I (Fig. 5), findings consistent with the previous analysis of fibroblasts with a homozygous p.(Arg274Pro) mutation [Mendoza-Londono et al., 2012].

Biosynthesis of CS/DS is a process requiring at least 22 enzymes directly involved in the assembly and modification of the chain; CHST14 and DSE catalyze the conversion of CS to DS, the last steps of this process, and introduce iduronic acid-containing domains in

TABLE II. Composition Analysis of Chondroitin/Dermatan Disaccharides in Fibroblasts of P3/II

	Chondroitinase ABC			Chondroitinase B		
% Mol	**Control**	**P1/I**	**P3/II**	**Control**	**P1/I**	**P3/II**
ΔDi-0S[a]	16.88	4.16	17.25	40.97	N.d.	N.d.
ΔDi-4S	35.51	37.86	10.66	37.50	N.d.	N.d.
ΔDi-6S	28.89	52.58	58.67	8.18	N.d.	N.d.
ΔDi-2,6S	7.88	5.39	9.00	7.94	N.d.	N.d.
ΔDi-4,6S	10.84	0.00	4.41	5.41	N.d.	N.d.
ΔDi-2,4,6S	0.00	0.00	0.00	0.00	N.d.	N.d.

Extracted GAGs were digested with chondroitinase ABC and chondroitinase B, respectively. Disaccharide fractions were labeled with 2-AB and composition analysis was performed.
[a]ΔDi-0S = ΔHexUA-GalNAc; ΔDi-6S = ΔHexUA—GalNAc(6-*O*-sulfate); ΔDi-4S = ΔHexUA-GalNAc(4-*O*-sulfate); ΔDi-2,6S = ΔHexUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate); ΔDi-4,6S = ΔHexUA-GalNAc(4,6-*O*-disulfate); ΔDi-2,4,6S = ΔHexUA(2-*O*-sulfate)-GalNAc(4,6-*O*-sulfate); N.d., not detectable.

the GAG chain. CHST14 and DSE deficiencies cause a shortage of sulfated dermatan, while all prior enzyme deficiencies would affect CS and/or heparin sulfate synthesis [Mizumoto et al., 2013]. DS domains have a key role in mediating the functions of DS proteoglycans [Malmström et al., 2012], and CS/DS proteoglycans are found in the extracellular matrix of the connective tissues including basement membranes, at the cell surface, and intracellularly. DS proteoglycans are found in blood vessel walls, skin, tendon, sclera, cartilage, and undifferentiated mesenchymal tissues [Rosenberg et al., 1985]. *CHST14* mutations identified in MC-EDS patients were shown or predicted to be loss-of-function mutations and deficiency of CHST14 leads to a loss of DS and/or a partial replacement of DS by CS and thus the CS type GAGs on proteoglycans such as decorin results in abnormal regulation of collagen fibril assembly (Fig. 5) [Miyake et al., 2010]. Indeed, abnormal collagen fibril and fiber distribution had originally been observed in patient P2/I [Steinmann et al., 1975]. The sulfated polysaccharide DS forms proteoglycans with a number of other core proteins, such as biglycan and versican [Malmström et al., 2012] whose deficiencies might also contribute to the range of clinical problems in MC-EDS patients. Secreted biglycan interacts via its core protein or GAG chains with type I, II, III, and VI collagen and elastin [Nastase et al., 2012], and thus their tissue expression corresponds to the phenotypic expression in MC-EDS.

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