CSGALNACT1-congenital disorder of glycosylation: A mild skeletal dysplasia with advanced bone age

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Abstract
Congenital disorders of glycosylation (CDGs) comprise a large number of inherited metabolic defects that affect the biosynthesis and attachment of glycans. CDGs manifest as a broad spectrum of disease, most often including neurodevelopmental and skeletal abnormalities and skin laxity. Two patients with biallelic CSGALNACT1 variants and a mild skeletal dysplasia have been described previously. We investigated two unrelated patients presenting with short stature with advanced bone age, facial dysmorphism, and mild language delay, in whom trio-exome sequencing identified novel biallelic CSGALNACT1 variants: compound heterozygosity for c.1294G>T (p.Asp432Tyr) and the deletion of exon 4 that includes the start codon in one patient, and homozygosity for c.791A>G (p.Asn264Ser) in the other patient. CSGALNACT1 encodes CSGalNAcT-1, a key enzyme in the biosynthesis of sulfated glycosaminoglycans chondroitin and dermatan sulfate. Biochemical studies demonstrated significantly reduced CSGalNAcT-1 activity of the novel missense variants, as...
Congenital disorders of glycosylation (CDGs) are monogenic inherited conditions that result from abnormal glucan biosynthesis resulting in a broad spectrum of pathologies. These clinical entities often include skin laxity, skeletal dysplasias, neurodevelopmental disorders, and endocrine abnormalities. So far, more than 125 different CDG have been identified among several glycosylation pathways (Bui et al., 2014; Ng & Freeze, 2018). One of these pathways is the O-linked xylose (O-Xyl) glycosylation pathway, which is governed by several genes that encode enzymes responsible for the biosynthesis and structural diversity of sulfated glycosaminoglycans (GAGs; Mizumoto, Yamada, & Sugahara, 2015a; Sato et al., 2011; Watanabe et al., 2010). The biosynthesis of GAGs starts from the formation of a common tetrasaccharide linker region, which is attached covalently to the serine residues of the PG core proteins (Lindahl & Rodén, 1965). Four glycosyltransferases subsequently polymerize the common tetrasaccharide linker region in CS, DS, and HS biosynthesis. This is followed by the stepwise polymerization of either HS or CS/DS chains. The first hexosamine transfer to the tetrasaccharide linker region determines selectively whether HS or CS/DS is produced. CS N-acetylgalactosaminyltransferase-1 (CSGALNACT1, ChGn-1) encoded by CSGALNACT1 (MIM # 616615) initiates CS/DS synthesis by catalyzing the transfer of a GalNAc residue from uridine diphosphate (UDP)-GalNAc onto the tetrasaccharide linker (Izumikawa et al., 2015; Uyama, Kitagawa, Tamura Ji, & Sugahara, 2002).

Recently, we and others reported two patients with biallelic CSGALNACT1 variants and a mild skeletal dysplasia with advanced bone age (Meyer et al., 2019; Vodopiutz et al., 2017). Here, we report two additional affected individuals with biallelic CSGALNACT1 variants and we show altered GAG synthesis in fibroblasts from one patient. We hereby suggest that CSGALNACT1 loss-of-function causes a distinct disease entity, the congenital disorder of glycosylation, CSGALNACT1-CDG.

**KEYWORDS**
advanced bone age, cartilage and brain development, CSGALNACT-1, CSGALNACT1-CDG, glycosaminoglycan, joint laxity, macrocephaly, proteoglycan, short stature

1 INTRODUCTION

1.1 Patients

Two patients (P2, P3) with skeletal dysplasia with advanced carpal bone age in infancy (Figures 1a–o and 2a–c), were examined by consultants specialized in pediatrics, radiology, and clinical genetics. Sharing of patient-related data was facilitated by the GeneMatcher tool (Sobreira, Schiettecatte, Valle, & Hamosh, 2015). Fibroblasts from patient P2 and from the previously reported patient (P1) with CSGALNACT-1 deficiency (Vodopiutz et al., 2017) were studied. Written informed consent for genetic and metabolic research investigations was obtained from all participants, and the ethics committees of the Medical University of Innsbruck, Austria (P1), Medical University of Vienna (P2), Duke University Health System (P3), and of the Meijo University, Nagoya, Japan, approved the study. Genomic DNA was extracted from peripheral blood from all participants by standard procedures.

1.2 Whole-exome sequencing (WES)

WES was performed on patient P2 and his parents using SureSelect V6 exome enrichment kit (Agilent Technologies, Waldbronn, Germany) and the 150 bp (base pairs) paired-end mode on an Illumina HiSeq4000 instrument (GATC-Biotech, Konstanz, Germany). Sequencing reads were aligned to the human genome (hg19) with Burrows-Wheeler transformation (Li & Durbin, 2009), polymerase chain reaction (PCR) duplicates removed with PICARD (http://picard.sourceforge.net), and single-nucleotide variants (SNVs) and small indels were identified with the samtools mpileup software. All variants were submitted to SeattleSeq (http://snp.gs.washington.edu/SeattleSeqAnnotation/) for annotation, categorization into synonymous and nonsynonymous SNPs or indels, and for filtering using the data from dbSNP, the Exome Sequencing Project (ESP), and the Exome Aggregation consortium (ExAC), genome aggregation database (gnomAD). A spreadsheet-based filtering for rare and private variants was performed. Copy-number variants (CNVs) were detected using the panelcn.MOPS software package (Povysil et al., 2017).

WES was performed on patient P3 and her parents as described (Jordan et al., 2015) with VCRome 2.1 in-solution exome probes (Bainbridge et al., 2011) and 100 bp paired-end reads on an Illumina HiSeq 4000 instrument; data were processed with CASAVA 1.8.
software (Illumina), and mapped to the reference human genome (hg19) with BWA (Li & Durbin, 2009). Variant calls were performed using Atlas-SNP and Atlas-indel (Shen et al., 2010), and data were filtered to retain functional variants predicted to alter messenger RNA (mRNA) splicing or protein amino acid sequence with minor allele frequency ≤1% in public SNP databases (dbSNP, ESP, ExAC, and gnomAD) that segregated with disease.

2.3 Sanger sequencing and quantitative PCR

SNVs and CNVs detected by WES were confirmed by Sanger sequencing and quantitative PCR, respectively, using an ABI 3730s automated sequencer, with BigDye terminator mix and an ABI real-time PCR cycler. CSGALNACT1 PCR and sequencing primer sequences were based on the ENSEMBL reference entries for mRNA (ENST00000454498.2, corresponding to NCBI reference sequence for mRNA NM_001130518.1), and genomic DNA (ENSG00000147408). Quantitative SYBR green PCRs targeting 20 different loci from CSGALNACT1 intron 3 to intron 4, and normalized to ACTB as a control gene, were used to delineate the large intragenic deletion in P2 and to enable breakpoint sequencing. Primer sequences and PCR conditions are available from the authors upon request. Nucleotide numbering reflects complementary DNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

2.4 In silico analysis of variants

The significance of SNVs resulting in missense changes was predicted using the following four web-based programs: Mutation Taster (http://www.mutationtaster.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/index.php/), and CADD (complete annotation dependent depletion; https://cadd.gs.washington.edu/).
Variants were considered to be likely pathogenic if they had low allele frequencies (<0.005) in the normal population (ExAC database; http://exac.broadinstitute.org/), and were predicted to be damaging or disease-causing by at least three of four prediction programs, and segregated with the phenotype in the pedigree. Amino acid conservation was assessed by aligning a set of CSGALNACT1 orthologous protein reference sequences obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/gene/) using ClustalOmega.

2.5 | Chromosomal microarray analysis

Chromosomal microarray analysis was performed in patients P2 and P3 using the genome-wide Human SNP Array 6.0, genotyping console 4.0, and ChAS 1.2.2 software (Affymetrix).

2.6 | Glycosyltransferase assay of recombinant, mutant, and wild-type CSGalNAcT-1

The human CSGalNAcT-1 (wild-type [WT]) expression vector, p3xFLAG-CMV8/hCSGALNACT1 was used to generate p.Asp432Tyr and p.Asn264Ser variants in CSGALNACT1 as described previously for patient P1 with the pPro384Arg CSGALNACT1 variant (Vodopiutz et al., 2017). The variants were introduced by overlapping extension PCR (Zhao, Zhang, & Padmanabhan, 1993). The expression plasmid (6 µg) was transfected into HEK293T cells (~50% confluency) in a T-75 flask or 100-mm plate using FuGENE 6 HD (Promega) and GalNAc-transferase activity was determined as described (Uyama et al., 2002; Vodopiutz et al., 2017). Briefly, the GalNac-transferase assay mixture contained 10 µl of enzyme-bound anti-FLAG affinity resins, 50 mM 2-(N-morpholino)ethanesulfonic acid-NaOH (pH 6.5), 10 mM MnCl2, 10 mM MgCl2, 0.1 mM UDP-GalNAc (ultra-pure grade; Promega) as the sugar donor substrate, and 100 µg chondroitin (Seikagaku Corp., Tokyo, Japan) or 2 mM p-nitrophenyl-β-D-glucuronic acid (pNP-GlcUA; Sigma-Aldrich) as the sugar acceptor in a total volume of 50 µl. The reaction mixture was incubated at 37°C for 4 hr. The reaction product, the UDP moiety released from UDP-GalNAc, was mixed with UDP detection reagent, which contains an enzyme to convert UDP to adenosine triphosphate (ATP), in a UDP-Glo™ Glycosyltransferase Assay kit (Promega). The newly synthesized ATP was measured using a luciferase/luciferin reaction, and the luminescent signals were detected in a luminometer, Victor X4 or EnSpire (PerkinElmer).

2.7 | Primary fibroblast culture

Skin fibroblasts were available from patients P1 and P2. We cultured skin fibroblasts from patients and controls in Dulbecco’s modified Eagle medium with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM l-glutamine (Invitrogen) in a humidified atmosphere containing 5% CO2.
2.8 Determination of the CS/DS chain number on core proteins by cell-based enzyme-linked immunosorbent assay (ELISA)

Cell-based ELISA was carried out as described (Job et al., 2016) to determine the relative number of CS/DS chains on core proteins. Briefly, fibroblasts from the patients and a control subject were cultured on 96-well plates (5,000 cells/well) for a day, washed with PBS, and treated with chondroitinase ABC at 37°C for 30 min. The chondroitinase-treated cells were fixed with 4% paraformaldehyde, incubated with the primary antibodies, a mixture of anti-CS-stub antibodies (1B5, 2B6, and 3B3, Cosmobio; Tokyo, Japan), and subsequently incubated with the secondary antibody, an alkaline phosphatase-conjugated anti-mouse immunoglobulin G. Then, the cells were incubated with the substrate, p-nitrophenyl phosphate, and analyzed by absorbance at 405 nm using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA).

3 | RESULTS

3.1 Genetic analysis

We generated sequencing reads for patients P2, P3, and parents of P3 yielding an average coverage of >120× with >95% of target sequenced at >20× coverage; sequencing reads for the parents of patient P2 yielded an average coverage of 40× with >82% of target sequenced at >20× coverage (low-coverage exomes). WES identified novel and biallelic CSGALNACT1 variants in patients P2 and P3 with skeletal dysplasia with advanced bone age: P2 was compound-heterozygous for c.1294G>T (p.Asp432Tyr) and an intragenic deletion of 84,966 bp that removes exon 4 (NM_001130518.1:c.−297+17058_634+22070delinsT; Figures 3a,b and S1a). The CSGALNACT1 expression level in fibroblasts from P2 was comparable to controls, with exon 4 missing in approximately half of the transcripts (Figure S1bc and data not shown). Exon 4 contains the translation start codon as well as 211 out of 532 codons of CSGALNACT1. This deletion, therefore, precludes the synthesis of a functional CSGALNACT1 protein and has not been reported in ClinVar and DECIPHER databases. Patient P3 was homozygous for c.791A>G (p.Asn264Ser), due to complete paternal isodisomy for chromosome 8, which harbors CSGALNACT1. The healthy father was heterozygous for this variant and the healthy mother was WT CSGALNACT1 (Figure 3a,b and Table 1). The identified CSGALNACT1 missense variants p.Asp432Tyr and p.Asn264Ser change evolutionary highly conserved amino acids (Figure 3c), are predicted to be damaging by multiple in silico algorithms, and all variants are very rare in the ExAC and gnomAD public databases (Table 1). WES did not reveal potentially pathogenic variants in any of the other known skeletal dysplasia genes in both patients, collectively indicating CSGALNACT1 as the disease-causing gene. The CSGALNACT1 variant information was submitted to the Leiden Open Variation Database. Chromosomal microarray analysis was normal in patient P2. Chromosomal microarray analysis of P3 revealed a region of homozgyosity consistent with complete isodisomy of chromosome 8. WES data and clinical genetic testing of polymorphic DNA markers on chromosome 8 confirmed paternal isodisomy of chromosome 8 and interphase FISH (fluorescence in situ hybridization) revealed 3.5% mosaic trisomy 8 in leukocyte-derived DNA from P3 (Supplemental Fig. S2). P3 was also homozygous for a VPS13B variant NM_181661.2:c.1336_1351del, (p.Cys409Asnfs*5) due to paternal isodisomy 8. Recessive variants in VPS13B cause Cohen syndrome (MIM: 216550; Kolehmainen et al., 2003). However, we considered the VPS13B change as an unlikely cause of P3’s phenotype: her clinical features do not overlap with Cohen syndrome and the variant only impacts a short alternate isoform.

3.2 Patients’ clinical characteristics

We performed detailed clinical assessment and analysis of skeletal radiographs for patients P2 and P3 (Figure 1) compared to the previously reported patients P1 (Vodopiutz et al., 2017) and P4 (Meyer et al., 2019; Table 1). The four patients with CSGALNACT1 variants were all born at term and are now between 5 and 12-year old. Three patients displayed a fetal length below the fifth centile, and skeletal dysplasia was suggested prenatally in P1, P2, and P4 by short fetal femurs. An advanced bone age within the first year of life was observed in patients P1–3 (Figure 1a, b, m, o) and was reported at age 6 in P4. A mild spondyloepiphyseal dysplasia with short and plump long bones, flat acetabular roofs, and monkey wrench appearance of the proximal femur was documented in P1 and P2 (Figure 1a–l), for whom neonatal skeletal radiographs were available.

Relative macrocephaly, rhizomelia, hyperlordosis and joint laxity are present in P1, P2 and P4, whereas muscular hypotonia in infancy, mild neurodevelopmental delay, congenital heart defects, and dysplastic external ears are present in P2 and P3. Structural defects of the central nervous system, apnoes, and endocrine abnormalities are variable (Table 1).

3.3 Case reports

The male patient P2 is the second child of healthy, nonconsanguineous northern European parents. P2 had cardiorespiratory monitoring at home in the first years of life due to the occurrence of apparent life-threatening events. At the age of 5 years 7 months, he has nonproportionate short stature, macrocephaly, brachydactyly, hyperlordosis, mild scoliosis, progressive pectus excavatum, pes planus, 2/3-toe syndactyly, and facial dysmorphism. Mild language delay and attention deficit hyperactivity disorder (ADHD) were noted. Cranial magnetic resonance imaging (MRI) showed mild ventriculomegaly, and reduced volumes of the hippocampi and cavum vergae. Electroencephalography, nerve conduction velocity studies, electromyography, metabolic and endocrine investigations were normal and family history is unremarkable (Figures 2a,b and 3a; and Table 1).

The female patient P3 is the second child of healthy, nonconsanguineous parents of Jewish and northern European descent (Figure 3a). At the age of 10 years, she presents with height at the third percentile, facial dysmorphism, scoliosis, overlapping fingers, 2/3-toe syndactyly, camptodactyly of the fifth distal interphalangeal joints, clinodactyly of second finger, and limited extension of elbows and
**FIGURE 3** Identification and characterization of CSGALNACT1 variants. (a) Simplified pedigrees showing segregation of identified CSGALNACT1 variants. Individuals carrying biallelic CSGALNACT1 variants are indicated by solid symbols, whereas unaffected individuals are indicated as open symbols. (b) Schematic of the human CSGALNACT1 locus with exons displayed as gray (untranslated) and black (translated) boxes and the localization of reported and newly identified CSGALNACT1 variants on complementary DNA and protein level indicated. (c) The two newly identified and one previously reported CSGALNACT1 missense variants affect invariantly conserved amino acids. (d) Schematic illustration of GAG synthesis with emphasis on CSGALNACT1. (e) In vitro GalNAc-transferase activities of p.Asp432Tyr-CSGalNACT1 and of p.Asn264Ser-CSGalNACT1 from cell lysates were significantly decreased towards chondroitin as the acceptor compared to that of wild-type (WT)-CSGalNACT1. Values are the means ± standard error (n = 3). *p < .0005 and **p < .0001 versus WT were calculated by the analysis of variance (ANOVA) Dunnett test. Expression levels of the recombinant CSGalNAcT-1 proteins were estimated by comparing the chemiluminescence intensity using the standard curve generated with concentration-defined 3×FLAG-bovine alkaline phosphatase. Two clones of variants p.Asn264Ser are shown. (f) CS/DS-stub antibodies showed a significantly reduced binding to patients’ fibroblasts as compared to the control subject, indicating that the number of CS/DS chains were reduced in the fibroblast cells from P1 and P2. *p < .0005 versus control (Cont) were calculated by the ANOVA Dunnett test.
**TABLE 1**  Clinical and genetic findings in four individuals with biallelic CSGALNACT1 variants

| Patient (Reference) | P1 (Vodopiutz et al., 2017) | P2 | P3 | P4 (Meyer et al., 2019) |
|---------------------|-----------------------------|----|----|------------------------|
| Sex                 | F                           | M  | F  | M                      |
| Ethnicity           | Northern European           | Northern European | Northern European/Jewish | Turkish |
| Parental consanguinity | −  −                      | −  −          | +  +                      |
| Current age         | 5 years 7 months            | 5 years 10 months | 10 years 5 months       | 12 years 3 months |
| Clinical characteristics | short femurs and midface hypoplasia | short femurs | Single umbilical artery, ventriculomegaly, high maternal serum AFP level | short femurs |
| Increased nuchal translucency | ND                        | +  | −  | −                      |
| Birth measures       | HC 34.0 cm, P54, SD 0.1 W 2.826 g, P16, SD −0.97 H 46.5 cm, P8, SD −1.57 Apgar scores 9/10/10 | HC 36.0 cm, P89, SD 1.2 W 3.610 g, P79, SD 0.76 H 48.0 cm, P15, SD −1.24 Apgar scores 9/10/10 | HC 31.88 cm, P89, SD −1.69 W 3.056 g, P < 1, SD −2.6 H 43 cm, P < 3, SD −3.3 Apgar scores 7/8/ND | HC 35.5 cm, P75, SD 0.69 W 3.100 g, P46, SD −0.1 H 50.0 cm, P15, SD −1.24 Apgar scores not reported |
| Age and measurements at last follow-up | 3 years 11 months HC 49.5 cm, P55, SD −0.14 H 95 cm, P13, SD −1.23 W 14.8 kg, P15, SD 0.79 SH ND SH/LL ND LL ND | 5 years 7 months HC 56.7 cm, P > 97, SD 3.3 H 100.2 cm, P < 3, SD 3.03 W 19.0 kg, P40, SD −0.26 SH 59.3 cm, SD −1.69 SH/LL 1.45, SD 3.36 LL 40.9 cm, SD −3.62 | 10 years 5 months HC 51 cm, P10, SD −1.44 H 132.4 cm, P3, SD −1.77 W 23.8 kg, P < 3, SD −4.26 SH 77 cm, SD 0.59 SH/LL ND LL ND | 12 years 3 months HC 54 cm, P34, SD −0.41 H 128 cm, P < 3, SD −3.14 W 32 kg, P6, SD −1.53 SH ND SH/LL ND LL ND |
| Neonatal respiratory distress | −  | +  | +  | −  |
| Apnoes/ALTE          | −  | +  | −  | −  |
| Disproportionate stature | +  | +  | −  | +  |
| Short stature        | −, 8–15, SD −1.23 | +, 3P, SD −3.03 | +, P3, SD −1.77 | P<3, SD −3.14 |
| Brachydactyly        | +  | +  | −  | −  |
| Macroecephaly        | −  | +  | +  | −  |
| Single palmar crease | −  | +  | −  | −  |
| Facial dysmorphism   | Midface hypoplasia, frontal bossing, flat nasal bridge, anteverted nares at birth, beaked nose, prominent nasal | Downslanting and short palpebral fissures, | −  | −  |

(Continues)
| Patient (Reference) | P1 (Vodopiutz et al., 2017) | P2 | P3 | P4 (Meyer et al., 2019) |
|---------------------|-----------------------------|----|----|------------------------|
| Midface hypoplasia, frontal bossing, flat nasal bridge, short nose, anteverted nares at birth, prominent nasal tip, and microretrogna phia | tip, downslanting palpebral fissures, and dysplastic external ears | microretrogna phia, beaked nose, prominent nasal tip, and dysplastic external ears |
| Joint laxity | + | + | - | + |
| Joint dislocations | - | - | + | - |
| Pectus excavatum | - | + | - | - |
| Scoliosis | - | + | + | - |
| Hyperlordosis | + | + | - | + |
| Other skeletal features | pes planus | 2/3 toe syndactyly, clinodactyly, and pes planus | 2/3 toe syndactyly, camptodactyly of fifth DIP joint, clinodactyly of second finger, limited extension at elbows and knees, and history of overlapping fingers |
| Muscular hypotonia in early infancy | + | + | + | - |
| Mild motor delay | + | + | + | - |
| Language delay | - | + | + | - |
| Mild attention deficit hyperactivity disorder | ND | + | + | - |
| Eye | - | Hyperopia | Hyperopia, mild optic nerve anomaly, strabismus, and retinal pigment mottling |
| Miscellaneous | - | Ankyloglossia and nevus flammeus over both eyes | Clinical features of mosaic trisomy 8 Tethered cord, chilblain lesions, deafness conductive left side, Nevus flammeus over both eyes, deep plantar creases, bilateral deeply set eyes, feeding difficulties, widely spaced nipples, panhypopituitarism, and velopharyngeal insufficiency |
| Radiological characteristics | Advanced carpotarsal bone age in infancy | + | + Vanished at age 4.5 years | + vanished at age 9 years |
| Patient (Reference) | P1 (Vodopiutz et al., 2017) | P2 | P3 | P4 (Meyer et al., 2019) |
|---------------------|---------------------------|----|----|-------------------------|
| Neonatal monkey wrench appearance femoral neck | + | + | ND | ND |
| Flat acetabular roofs | + | + | ND | + |
| Short long bones | + | + | – | – |
| Epiphyseal dysplasia | + | + | ND | – |
| Vertebral abnormalities | Coronal and sagittal clefting of vertebrae | Coronal clefting of vertebrae | Oar shaped ribs | – |
| Desbuquois dysplasia type 1; characteristic hand anomalies | – | – | – | – |
| Miscellaneous | – | Narrow chest | Radial head luxation and dysplasia | – |
| Bone densitometry | ND | Normal at 4.5 years | ND | ND |
| Brain MRI | Normal | Mild ventriculomegaly, cavium vergae, and mild bilateral volume loss in hippocampal region | Mild ventriculomegaly, agenesis of corpus callosum, periventricular grey matter heterotopia, absent septum pellucidum, and hypoplastic of inferior cerebellar vermis | ND |
| EEG | ND | Normal | Normal | ND |
| NCV | ND | Normal at age 2 months | ND | ND |
| Echocardiography | ND | ASD | VSD, history of patent ductus arteriosus, patent foramen ovale, abnormality of the tricuspid valve, tricuspid valve regurgitation | ND |
| CDG screening | Normal | Normal | Normal | ND |
| Genetics | | | | |
| CSGALNACT1 variants (ACMG classification) | Compound-heterozygous c.1151C>G p.Pro384Arg (PS3, PS4, PM2, PM3: pathogenic) NC_000008.10:g.19269401_19324691del 55-kbp deletion, exons 5-8 (PVS1, PS3, PM2, PM3: Pathogenic) | Compound-heterozygous c.1294G>T p.Asp432Tyr (PS3, PS4, PM2, PM3: pathogenic) NC_000008.10:g.19340642_19425619delinsA 85-kbp deletion, exon 4 (PVS1, PS3, PS4, PM2, PM3, PM4: Pathogenic) | Homozygous c.791A>G p.Asn264Ser (PS3, PS4, PM2, PM3: Pathogenic) | Homozygous c.372del (PVS1, PM2, PM3: Pathogenic) |
| In silico algorithms for | | | | |
| PolyPhen-2 | p.Pro384Arg | p.Asp432Tyr | p.Asn264Ser | p.(His125Thrfs*9) |
| PROVEAN | Probably damaging (1.0) | Probably damaging (1.0) | Probably damaging (1.0) | NA |
| Mutation Taster | Deleterious (~8.298) | Deleterious (~7.798) | Deleterious (~3.600) | Disease-causing |

(Continues)
knees (Figure 2c). Mild language delay, specifically in expressive language, and ADHD were noted. MRI of the brain and spine at 2 days of life showed mild ventriculomegaly, agenesis of corpus callosum, periventricular gray matter heterotopia, absent septum pellucidum, hypoplastic inferior cerebellar vermis, and tethered cord. Electroencephalography and metabolic testing were normal. She has been treated with growth hormone for panhypopituitarism since age 7 years. She has tricuspid valve regurgitation and a small ventricular septal defect. Notably, she appears to have blended phenotypes of two different disorders as strabismus, camptodactyly, limited joint mobility, contractures, deep plantar and palmar creases, and corpus callosum aplasia are typical findings in mosaic trisomy 8 (for a review see https://rarediseases.info.nih.gov/diseases/5359/mosaic-trisomy-8; Figures 1m–o and 2c and Table 1).

3.4 | Identified CSGalNAC-T1 missense variants confer loss-of-function in vitro

The function of CSGalNAC-T1 in GAG synthesis is depicted in Figure 3d. We expressed p.Asp432Tyr, p.Asn264Ser, and WT encoding CSGalNAC-T1 in HEK293T cells in soluble form by replacing the first 36 amino acids of CSGalNAC-T1 with a cleavable preprotrypsin signal sequence as well as a 3×FLAG epitope to facilitate enzyme purification and elimination of endogenous CSGalNAC-T1. The presence of the WT CSGalNAC-T1, but not the mutant enzymes, was detected in the conditioned medium by immunoblotting (Figure S3). In contrast, the protein expression levels in the cell fraction were comparable between the WT and the mutant enzymes (Figures 3e and S3).

GalNAc-transferase activities of p.Asp432Tyr-CSGalNAC-T1 and of p.Asn264Ser-CSGalNAC-T1 from the cell lysates toward chondroitin (Figure 3e) and toward pNP-GlcUA (Figure S3) were significantly decreased compared to WT-CSGalNAC-T1 (p < .0001 and p < .0005, respectively). These data indicate that both CSGALNACT1 variants lead to a loss of enzyme function.

3.5 | GAG chain quantity and composition analyses in patients’ fibroblasts

To examine how the reduced activity of CSGalNAC-T1 affects the biosynthesis of CS/DS side chains of PGs, we determined the relative numbers of CS/DS chains in fibroblast cells from patients P1, P2, and the control. No fibroblasts were available from P3. CS/DS-stub antibodies showed a significantly reduced binding to the patients’ cells compared to the control subject (p < .0005; Figure 3f).

To examine the level of GAGs in patients’ cells, we carried out quantification of disaccharides from CS/DS chains by a combination of enzymatic digestion with an anion-exchange HPLC (Figures S4–6 and Tables S1–3). We observed increased levels of the CS moiety in the GAG fractions prepared from the conditioned media from both patients P1 and P2 compared to that of a healthy control (Figure S3). The amount of CS in the cell preparations increased in patient P2, whereas it decreased in patient P1 (Figure S4). In addition, we
observed a marked increase in the amounts of DS for the GAG fractions prepared from both conditioned media and cell preparations of both of the patients’ cell cultures compared to the control fibroblasts (Figure S5 and Table S2).

These observations suggest an increase in the amounts of CS/DS synthesized by the patients’ fibroblasts, consistent with previous results (Vodopiutz et al., 2017), although the numbers of CS/DS chains from the fibroblast cultures of the patients were reduced compared to control.

The amount of HS from the cell preparations of patients P1 and P2 increased compared to that from the control (Figure S6) and the amount of HS in conditioned medium of cells from patient 2 increased, whereas it decreased in patient P1, compared to that from the control fibroblasts (Figure S6 and Table S3).

4 | DISCUSSION

Aggregate data from our previous work (Vodopiutz et al., 2017), a current case report (Meyer et al., 2019), and this study show biallelic CSGALNACT1 variants in four unrelated individuals who present with a mild skeletal dysplasia with an advanced bone age. Three of four patients (P1, P2, and P4) display nonproportionate short stature, hyperlordosis, pes planus, and mild joint laxity. Such a phenotype appears confounded by mosaic trisomy 8 in patient P3 who displays short stature. Language delay and ADHD, as well as variable facial dysmorphism, were seen in patients P2 and P3; whether any of these symptoms are also due to the biallelic CSGALNACT1 variants is currently unclear. CSGALNACT1 encodes CSGalNAcT-1 which regulates the initiation of CS/DS chain formation by enzymatically catalyzing the transfer of a GalNAc residue from UDP-GalNAc onto the linker region in the O-Xyl glycosylation pathway (Mizumoto, 2018; Mizumoto et al., 2015b; Uyama et al., 2002). In addition to detecting two large, inactivating CSGALNACT1 deletions in patients, we showed that all three identified CSGALNACT1 missense variants are loss-of-function variants by demonstrating significantly reduced CSGalNAcT-1 activities in vitro. A consequent decrease in CS/DS chain initiation activity was reflected in reduced numbers of CS/DS chains on core proteins in fibroblasts from patients P1 and P2, whereas the total amount of the CS/DS disaccharides was, unexpectedly, increased compared to control fibroblasts. The reduced CSGalNAcT-1 function thus leads to a reduced number of elongated CS/DS chains in fibroblasts, which might produce the connective tissue abnormalities in CSGALNACT1-CDG.

We were not able to study GAG alterations in cartilage, as no such patient material was available. However, we speculate that a decreased CSGalNAcT-1 activity would cause reduced numbers and/or decreased amounts of CS chains in cartilage, to produce the skeletal symptoms in patients. Our hypothesis is supported by studies of Csgalnact1 knockout mice that exhibit reduced body length with significantly shortened long bones (humerus and tibia) when compared to WT mice, whereas the head circumference is of normal size. Additionally, Csgalnact1<sup>−/−</sup> mice display mild neurocognitive impairment (Yoshioka et al., 2017) and a reduction in CS content in cartilage and brain compared with WT mice, but have normal fertility and lifespan (Ida-Yonemochi et al., 2018; Sato et al., 2011; Watanabe et al., 2010; Yamada, Nanakana, Kitagawa, Takeuchi, & Jinno, 2018). These mouse studies suggest that CSGalNAcT-1 is necessary for normal levels of endochondral ossification, and the decrease in CS amount in the cartilage growth plate causes a rapid degradation of the prominent CS-PG, aggrecan. CS-PGs are known for their roles in cartilage, connective tissue, and brain development and function (Sato et al., 2011; Shimbo et al., 2017; Watanabe et al., 2010; Yamada et al., 2018).

The clinical features in CSGALNACT1-CDG patients are milder than those caused by enzyme deficiencies, which are located upstream and downstream of CSGalNAcT-1 in GAG synthesis (Baasanjav et al., 2011; Bui et al., 2014; Dundar et al., 2009; Malfait et al., 2013; Nakajima et al., 2013; Unger et al., 2010). This might be explained by the fact that CSGalNAcT-1 deficiency can be widely compensated for by the functionally redundant enzyme CSGalNAcT-2. Our demonstration of a reduced total number but potentially elongated CS/DS chains in fibroblasts of CSGALNACT1-CDG patients is consistent with the original biochemical characterization of CSGalNAcT-1: CSGalNAcT-1 predominantly initiates CS chain synthesis by transfer of the first GalNAc residue, whereas CSGalNAcT-2 is supposed to elongate CS chains by transferring the second and subsequent GalNAc residues (Sato et al., 2003; Uyama et al., 2002, 2003). Recent experiments in a mouse model suggested that even additional CS synthases other than Csgalnact1 and Csgalnact2 might exist to initiate CS chain synthesis (Shimbo et al., 2017). The observed increase in HS levels in patients’ fibroblasts (P1 and P2) is consistent with findings in Csgalnact1 knockout mice where upregulation of HS chain synthesis has been demonstrated as well (Takeuchi et al., 2013), caused by accumulation of the tetrasaccharide linker, which functions as an acceptor substrate for the HS glycosyltransferases. It remains unclear whether an imbalance of CS/DS and HS chains contributes to the pathogenesis in CSGALNACT1-CDG.

Advanced bone age and monkey wrench appearance of the femur are radiographic features which are rare in other skeletal dysplasias but common in GAG-biosynthesis disorders due to ACAN, XylT1, CANT1, and IMPAD1 variants. ACAN encodes the CS-PG aggrecan, which is an important component of the cartilage matrix (Gkourogianni et al., 2017; Nilsson et al., 2014; Statin et al., 2010; Tompson et al., 2009). XylT1 encodes for enzyme directly involved in synthesis and modification of PGs, similar to CSGALNACT1 (Bui et al., 2014; Schreml et al., 2014), whereas CANT1 and IMPAD1 dysfunctions are supposed to negatively affect overall GAG synthesis by causing feedback inhibitions of several glycosyltransferases and sulfotransferases through increased UDP and adenosine-3′,5′-bisphosphate levels in the Golgi apparatus, respectively (Huber et al., 2009; Vissers et al., 2011). Advanced bone age in CSGALNACT1-CDG might result from defective glycosylation of aggrecan and other CS-PGs in the cartilage matrix. Testing for PG biosynthesis defects is recommended in patients with skeletal dysplasia and advanced bone age or monkey
wrench appearance of the femur, especially if further symptoms of PG biosynthesis defects are present such as joint laxity, or neurodevelopmental delay. Vanishing of advanced bone age and monkey wrench appearance with age in CSGALNAC1-CDG highlights the importance of a complete neonatal radiographic workup in patients with unknown skeletal dysplasia.

Joint laxity is a common symptom in GAG biosynthesis disorders, and was noted in P1, P2, and P4 but was not present in P3 with an additional trisomy 8 mosaicism. Limited joint mobility is a characteristic feature of mosaic trisomy 8 and might counteract joint hypermobility in P3. A trisomy 8 mosaicism. Limited joint mobility is a characteristic feature of patients with unknown skeletal dysplasia.

Lights the importance of a complete neonatal radiographic workup in neurodevelopmental delay. Vanishing of advanced bone age and PG biosynthesis defects are present such as joint laxity, or wrench appearance of the femur, especially if further symptoms of PG biosynthesis defects are present such as joint laxity, or neurodevelopmental delay. Vanishing of advanced bone age and monkey wrench appearance with age in CSGALNAC1-CDG highlights the importance of a complete neonatal radiographic workup in patients with unknown skeletal dysplasia.

In summary, the three unrelated individuals reported by us and an independent recent case indicate that biallelic CSGALNAC1 variants result in a mild skeletal dysplasia with advanced bone age. Our findings also expand the clinical spectrum of O-Xyl glycosylation pathway defects.

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CONFLICT OF INTERESTS

N. K. is a distinguished Jean and George Brumley Professor and a paid consultant for and holds significant stock of Rescindo Therapeutics, Inc. Remaining authors declare that there are no conflict of interests.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.