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BRIEF COMMUNICATION

Identification of novel *LFNG* mutations in spondylocostal dysostosis

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Abstract

Spondylocostal dysostosis (SCDO) is a heterogeneous group of skeletal disorders characterized by multiple segmentation defects involving vertebrae and ribs. Seven disease genes have been reported as causal genes for SCDO: *DLL3*, *MESP2*, *TBX6*, *HES7*, *RIPPLY2*, *DMRT2*, and *LFNG*. Here we report a Japanese SCDO case with multiple severe vertebral anomalies from cervical to sacral spine. The patient was a compound heterozygote for c.372delG (p.K124Nfs*) and c.601G>A (p.D201N) variants of *LFNG*, which encodes a glycosyltransferase (*O*-fucosylpeptide 3-beta-*N*-acetylglucosaminyltransferase). The missense variant was in the DxD motif, an active-site motif of the glycosyltransferase, and its loss of the enzyme function was confirmed by an in vitro enzyme assay. This is the second report of *LFNG* mutations in SCDO.

Introduction

Spondylocostal dysostosis (SCDO) is a heterogeneous group of rare congenital disorders characterized by multiple segmentation defects of vertebrae (SDV) and malformation and/or defect of ribs. SDV of SCDO presented with hemivertebrae, butterfly vertebrae, fusion, absence, and block and mixed vertebrae. SCDO patients have short neck, short trunk, and scoliosis, and sometimes present with thoracic insufficiency syndrome caused by reduced size of thorax [1, 2].

Seven types of SCDO have been identified based on their phenotypes and disease genes: SCDO1 (OMIM#277300) due to *DLL3* mutations [3], SCDO2 (OMIM#608681) due to *MESP2* mutations [4], SCDO3 (OMIM#609813) due to a *LFNG* mutation [5], SCDO4 (OMIM#613868) due to *HES7* mutations [6], SCDO5 (OMIM#122600) due to *TBX6* mutations [7], and SCDO6 (OMIM#616566) due to *RIPPLY2* mutations [8]. Recently, a case of SCDO due to a homozygous *DMRT2* mutation has been reported [9]. All but SCDO5 are autosomal recessive traits. Autosomal dominant and autosomal recessive inheritance have been reported in *TBX6* mutations [7, 10]. *TBX6* mutations have also been reported in congenital scoliosis, a relatively common congenital disorder of the spine which has overlapping phenotypes with SCDO [7, 11].

Here, we report on a case of SCDO caused by compound heterozygous *LFNG* mutations. This is the second SCDO case with *LFNG* mutations. Both mutations we identified were novel and resulted in loss of LFNG function.

A 9-month-old Japanese boy was referred to us because of spinal deformity. His family history was unremarkable. Both parents had no apparent deformities. His height was about −2.5 SD. He had multiple vertebral anomalies from cervical to sacral vertebrae as well as defect and fusion of ribs (Fig. 1). There was no scoliosis and dysplasia of tubular bones. He had no respiratory problem, hand abnormalities, and other comorbidities, except for inguinal herniation.

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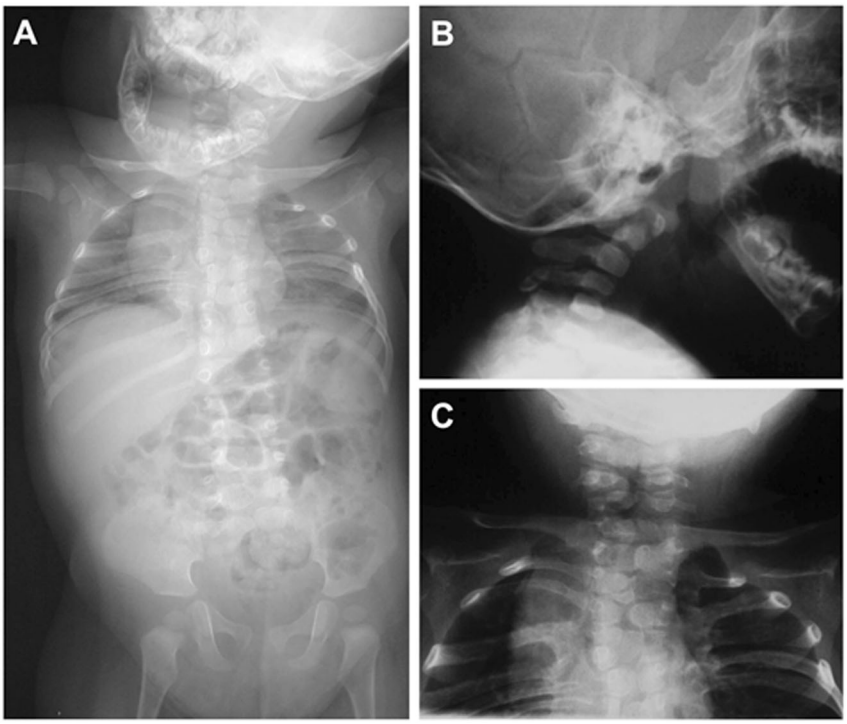

Fig. 1 Radiographs of the spondylocostal dysostosis patient with *LFNG* mutations. **a** Multiple anomalies of the vertebrae and ribs. Pelvis and long tubular bones are normal. **b** Lateral view of the cervical spine showing mal-alignment (kyphosis) and hypoplastic odontoid process. **c** Antero-posterior view of cervico-thoracic region of the spine. Pebble-beach appearance of the vertebral bodies

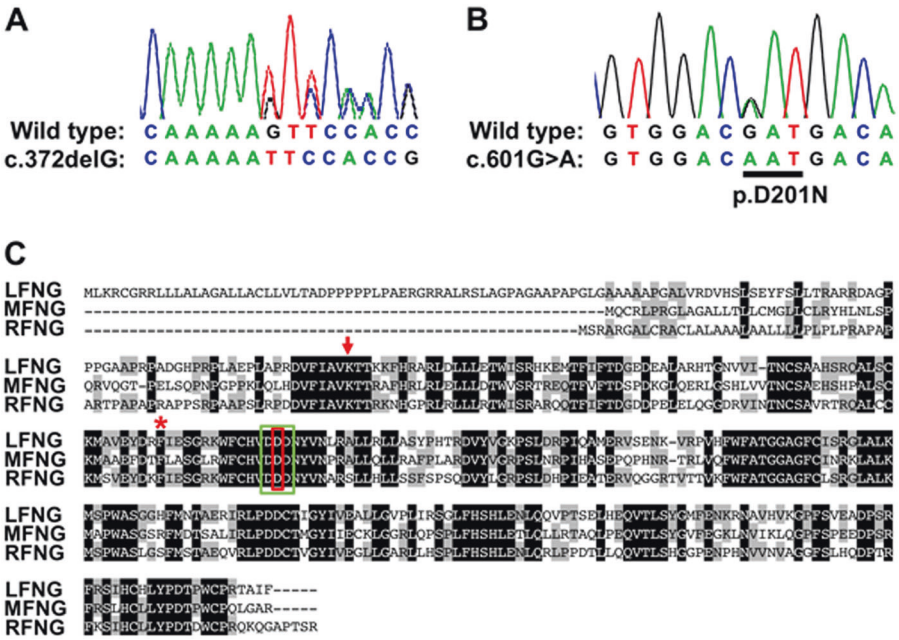

Fig. 2 *LFNG* mutations in the spondylocostal dysostosis patient. Direct sequence of the genomic DNA of the patient for **a** c.372delG and **b** c.601G>A. **c** The amino acid alignment between fringe families. Black bar, completely conserved region; gray bar, conserved region between two fringe families; blue line, the previously reported mutation position; green line, the DxD motif. Red arrow head, red asterisk and red line are K124, F188 and D201 of LFNG, respectively

Based on the radiographic features, he was diagnosed as having SCDO, most likely SCDO3.

We sought the mutation of the patient. Informed consent was obtained from the patient's parents. The study was approved by the ethical committee of all participating hospitals and RIKEN. Genomic DNAs were extracted from peripheral blood leukocytes of the patient and nails of his parents. We examined SCDO genes by direct sequencing using a 3730xl DNA analyzer (Applied Biosystems). The primer sequences are available on request. Genetyx (Genetyx, Tokyo, Japan) was used for aligning sequencing chromatographs to reference sequences (NM_001040167.1). The patient had two likely pathogenic variants in *LNFG:* c.372delG (p.K124Nfs*) in exon 1 and c.601G>A (p.D201N) in exon 4 (Fig. 2a, b). The former came from the mother and the latter from the father. We did

Table 1 Evaluation of two *LNFG* variants identified in the present study

Variant		Frequency				Prediction		
Nucleotide change	Amino acid change	ExAC	esp6500	iJGVD	HGVD	SIFT	Ployphen−2	Mutation Taster
c.372delG	p.K124Nfs*21	0	0	0	0	–	–	–
c.601G>A	p.D201N	0	0	0	0	Damaging	Probably damaging	Disease causing

not identify any likely disease-causing variants in other SCDO genes.

The two variants (c.372delG and c.601G>A) were absent from ExAC (Exome Aggregation Consortium, http://exac.broadinstitute.org), esp6500 (NHLBI Exome Sequencing Project, http://evs.gs.washington.edu/EVS), iJGVD (Integrative Japanese Genome Variation Database, https://ijgvd.megabank.tohoku.ac.jp), and HGVD (Human Genetic Variation database, http://www.hgvd.genome.med.kyoto-u.ac.jp/index.html) databases (Table 1). c.372delG (p.K124Nfs*21) was considered to cause nonsense-mediated mRNA decay (NMD). c.301G>A (p.D201N) was predicted to be pathogenic by bioinformatics prediction tools (Table 1). *LFNG* is a member of the Fringe gene family which includes *MFNG* and *RFNG*. They encode evolutionarily conserved glycosyltransferases that modify Notch and its ligands in the Golgi and act to determine boundaries during somitogenesis [12, 13]. p.D201 was located in the DxD motif which is highly conserved within the Fringe family (Fig. 2c) and in all known Fringe proteins from *Drosophila melanogaster* to human [5, 14, 15]. The motif is the catalytic site of many nucleoside disphosphate-binding glycosyltransferases [16, 17]. A previous study reported that a single amino acid alteration in the DxD motif disrupted all Fringe activities [18].

To confirm the pathogenicity of c.301G>A (p.D201N) variant, we assayed the GlcNAc-transferase activity of the p.D201N mutant protein in vitro. An expression vector of human *LFNG* was constructed as described previously [19] with slight modifications. Site-specific mutagenesis to produce p.D201N-LFNG was performed by using Inverse PCR-based site-directed mutagenesis (TOYOBO). The expression plasmids were transfected into HEK293T cells using FuGENE HD (Promega, Madison, WI). The conditioned media and cell lysates were collected 3 days after transfection and analyzed by western blotting after an electroporesis on a 10% sodium dodecyl sulfate–polyacrylamide gel using peroxidase-conjugated anti-DYKDDDDK antibody (Wako, Osaka, Japan) (Fig. 3a). The amount of recombinant LFNG protein was estimated by the standard curve from luminescent intensity of 3xFLAG-tagged bovine alkaline phosphatase (Sigma) using ImageQuant TL (GE Healthcare). GlcNAc-transferase activity was examined as described previously [19]. The mutant LFNG showed a significantly reduced GlcNAc-transferase activity compared to that of the wild-type LFNG (Fig. 3b), indicating that c.301G>A (p.D201N) variant lost its enzyme function.

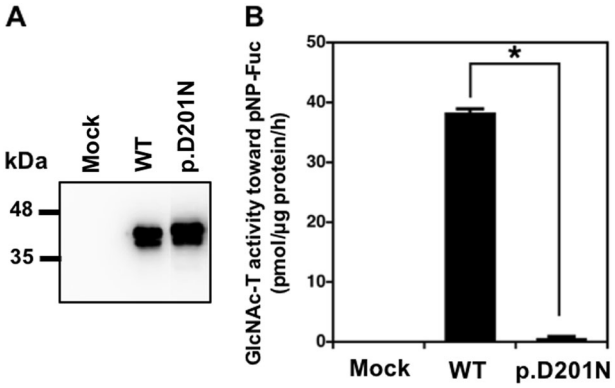

Fig. 3 The functional analysis of the mutant LFNG (p.D201N). **a** Western blotting of FLAG-tagged wild-type (WT) and p.D201N-LFNG proteins. **b** GlcNAc-transferase (GlcNAc-T) activity of the recombinant LFNGs in the cell lysate. Values are the means ± standard errors ($n = 3$). p.D201N-LFNG showed significantly decreased enzyme activity. *$P < 0.0001$ versus the WT-LFNG by Student's *t*-test

To our knowledge, only one *LFNG* mutation has been reported previously [5]: Sparrow et al. [5] found a homozygous missense mutation, c.564C>A (p.F188L) in exon 3 of *LFNG* in a Lebanese patient with SCDO. The patient had extensive congenital vertebral anomalies and hand anomalies. Both parents heterozygous for the mutant allele had no spine and hand anomalies. p.F188 was a highly conserved residue (Fig. 2c). The mutant LFNG was not localized to the correct compartment of the cell, was unable to modulate Notch signaling in a cell-based assay, and was enzymatically inactive. The loss of LFNG function is likely pathogenesis of SCDO. *Lfng*-null mice show vertebral and ribs abnormalities due to the disruption of somitogenesis [20].

In the present study, we identified two novel mutations, c.372delG (p.K124Nfs*) and c.601G>A (p.D201N), which were compound heterozygous in a Japanese SCDO patient. Both mutations were also considered to cause loss of LFNG function, although their causal mechanisms were not mislocalization of the mutant LFNG protein, but loss of protein by NMD (p.K124Nfs*) and loss of the enzyme activity (p.D201N). The patient had severe vertebral anomalies from cervical to sacral spines, which are similar to previously reported SCDO with *LFNG* mutation. It is reported that

SCDO3 had more severely disorganized spine than SCD1 and SCD2 [3–5]. Unlike the previous case [5], the present case did not have hand anomalies. Further accumulations of the cases with *LFNG* mutations are necessary to clarify the range of SCDO3 phenotype.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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