



Identification of a *De Novo* Heterozygous Missense *FLNB* Mutation in Lethal Atelosteogenesis Type I by Exome Sequencing

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Background: Atelosteogenesis type I (AO-I) is a rare lethal skeletal dysplastic disorder characterized by severe short-limbed dwarfism and dislocated hips, knees, and elbows. AO-I is caused by mutations in the filamin B (*FLNB*) gene; however, several other genes can cause AO-like lethal skeletal dysplasias.

Methods: In order to screen all possible genes associated with AO-like lethal skeletal dysplasias simultaneously, we performed whole-exome sequencing in a female newborn having clinical features of AO-I.

Results: Exome sequencing identified a novel missense variant (c.517G>A; p.Ala173Thr) in exon 2 of the *FLNB* gene in the patient. Sanger sequencing validated this variant, and genetic analysis of the patient's parents suggested a *de novo* occurrence of the variant.

Conclusions: This study shows that exome sequencing can be a useful tool for the identification of causative mutations in lethal skeletal dysplasia patients.

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Key Words: Atelosteogenesis type I, *FLNB*, Mutation, Exome sequencing

INTRODUCTION

Lethal skeletal dysplasias commonly include thanatophoric dysplasia, achondrogenesis, osteogenesis imperfecta type 2, congenital hypophosphatasia, and atelosteogenesis (AO), and can be distinguished by four key features: bone mineralization, fractures, macrocranium, and trunk length [1]. AO is a rare lethal skeletal dysplasia. It represents a heterogeneous group of disorders with the following features: incomplete ossification of long bones and markedly short, hypoplastic distal femora and humeri resulting in rhizomelic dwarfism [2]. Rare types of lethal skeletal dysplasias, including AO, are difficult to diagnose accu-

rately owing to their clinical heterogeneity [3, 4].

AO is subdivided into types I (AO-I), II (AO-II), and III (AO-III). AO-I and AO-III are associated with *FLNB* mutations [5], while AO-II is caused by *SLC26A2* (*DTDST*) mutations [6]. The *FLNB*-related disorders constitute a spectrum of phenotypes ranging from spondylacropotarsal synostosis syndrome and Larsen syndrome at the mild end, to AO-I, AO-III, and boomerang dysplasia at the severe end [7, 8]. Besides *FLNB*, many genes, including *ALPL*, *ARSE*, *COL1A1/2*, *COL2A1*, *COL11A1/2*, *CRTAP*, *DYNC2H1*, *EBP*, *EVC*, *EVC2*, *FGFR3*, *IFT80*, *IFT122*, *LEPRE1*, *NEK1*, *PEX7*, *PPIB*, *SLC26A2*, *SLC35D1*, *SOX*, *TRIP11*, and *WDR35*, have been identified to be involved in AO-like lethal

skeletal dysplasias [9].

Several recent studies have described how exome sequencing has allowed the identification of causative mutations in many heterogeneous and complicated disorders [10, 11]. Confirming a definitive molecular diagnosis is often difficult and costly in skeletal dysplasia because patients may be in a critical condition, in addition to having a phenotypically complex presentation. This clinical and genetic diversity reflects the number of causative genes, and molecular diagnosis of such disorders by conventional Sanger sequencing, usually an expensive and time-consuming gene-by-gene screening process, is sometimes impractical [10]. Exome sequencing allows simultaneous analysis of all coding sequences of genes. The advantage of exome sequencing is that it does not require a prior knowledge of the genes responsible for a disorder. In this study, we performed exome sequencing of a newborn patient with clinical features of AO-I and successfully identified a *de novo* *FLNB* mutation.

METHODS

1. Patient

A female baby was born as the first of twins of non-consanguineous healthy parents at 27 weeks of gestation via emergent cesarean section due to fetal distress. Both her parents were 31 yr old, and her mother's obstetric history was gravida 1, para 0. She was impregnated with twins through *in vitro* fertilization due to infertility. Prenatal ultrasonography at 25 weeks of gestation revealed that one fetus had polyhydramnios and multiple skeletal anomalies consistent with skeletal dysplasia, such as very poorly ossified femora and humeri with severe shortening of all extremities.

The patient had respiratory difficulty from birth and required mechanical ventilation support. It was very difficult to advance the endotracheal tube into the trachea after passing through the larynx. A pulmonary surfactant was instilled to treat the respiratory distress syndrome of prematurity. Oxygenation and ventilation were not effective even though high-frequency oscillatory ventilation was applied. She died of respiratory failure at three hours after birth, possibly secondary of laryngeal stenosis and pulmonary hypoplasia.

The clinical features of the patient indicated AO. However, other AO-like skeletal dysplasias could not be excluded. As conventional gene-by-gene sequencing is too costly and time-consuming, exome sequencing, allowing simultaneous analysis of multiple genes, was performed as a post-mortem genetic study with the written informed consent of the parents.

2. Whole-exome sequencing

Genomic DNA was extracted from peripheral blood leukocytes by using a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Exome sequencing was performed by DNA Link (DNA Link Inc., Seoul, Korea), and the bioinformatics services were provided by Samsung SDS (Samsung SDS, Ltd., Seoul, Korea). The SureSelect Human All Exon 50 Mb kit (Agilent Technologies, Santa Clara, CA, USA) was used for in-solution enrichment of coding exons and flanking intronic sequences, following the manufacturer's standard protocol. Adapted sequences for the Illumina HiSeq2000 sequencing system (Illumina Inc., San Diego, CA, USA) were ligated, and the enriched DNA samples were subjected to standard sample preparation for the HiSeq2000 instrument. The Burrows-Wheeler alignment (BWA) was used to align sequence reads to the human reference genome (hg19), and variants were called using the GATK software package [12-14]. The single nucleotide polymorphism (SNP) and short indel candidates were identified at nucleotide resolution. These variants were annotated by ANNOVAR (version 2011 Jun 18) [15] to filter SNPs reported in the dbSNP database (build 135) [16] and the 1000 Genomes Project (<http://1000genomes.org>).

Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and SIFT (Sorting Intolerant From Tolerant) (<http://sift.jcvi.org/>) were used to predict the consequences of the missense variants on protein function. The candidate variant identified by exome sequencing was confirmed by using conventional sequencing.

RESULTS

1. Patient

The patient's weight was 685 g (<10th percentile), height was 22 cm (<10th percentile), and head circumference was 24 cm (10-25th percentile) at birth. This is in contrast to the second infant twin, who had a weight of 925 g (25-50th percentile), height of 36 cm (50th percentile), and head circumference of 26 cm (50-75th percentile). The affected infant also had a depressed nasal bridge, hypertelorism, micrognathia, and low-set ears at birth. Her thoracic cage was small, and the abdomen was protuberant. She had markedly short limbs, with talipes equinovarus deformities and spatulated short fingers. Radiographs showed incomplete ossification and hypoplasia of the vertebrae, humeri, femora, tarsals, phalanges, and pelvis. Radiographs also revealed multiple skeletal abnormalities. The clavicles were relatively elongated, the thorax was small and bell-shaped, and several vertebrae had scoliosis and coronal clefts. The humeri were

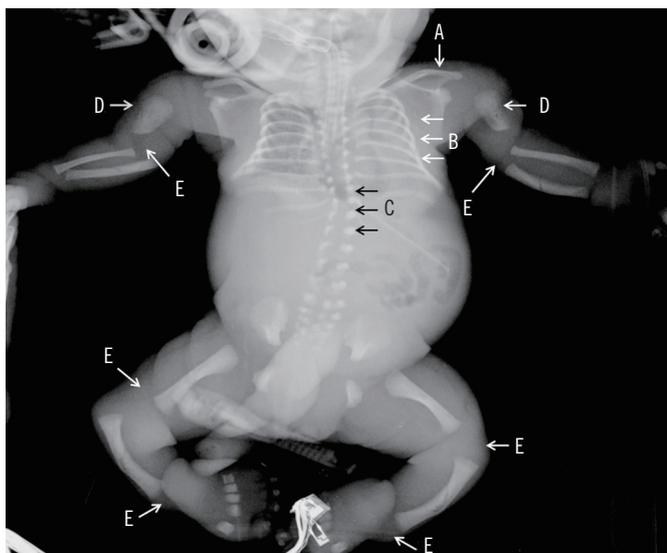


Fig. 1. Radiograph showing incomplete ossification and hypoplasia of the vertebrae, humeri, femora, tarsals, phalanges, and pelvis. The clavicles are relatively elongated (A), the thorax is small and bell-shaped (B), and the vertebrae have scoliosis and coronal clefts (C). The humeri are severely shortened and distally hypoplastic (D), the fibulae are completely absent, and both ankle joints, knee joints, and elbow joints are dislocated (E).

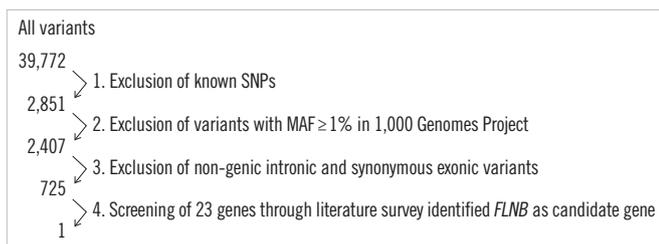


Fig. 2. Data analysis algorithm applied to the exome sequencing data. A data analysis algorithm was used to filter all the single nucleotide polymorphisms (SNPs) detected using exome sequencing, with numerous variants excluded at every filtering step. Abbreviation: MAF, minor allele frequency.

severely shortened and distally hypoplastic. The ulnae, radii, and tibiae were bowed. Both fibulae were completely absent. The ankles, knees, and elbows were dislocated (Fig. 1).

1. Data analysis

A mean coverage of 47.1 was achieved, and 86.6% of the targeted bases were read >10 times by exome capture and sequencing. Of the 39,772 identified SNPs, the pathogenic variant was prioritized by using the following steps: After the exclusion of known dbSNP variants and variants with minor allele frequency (MAF) \geq 1% in the 1000 Genomes Project, 2,407 variants remained, from which the non-genic intronic variants and synonymous exonic variants were excluded. For the remaining 725

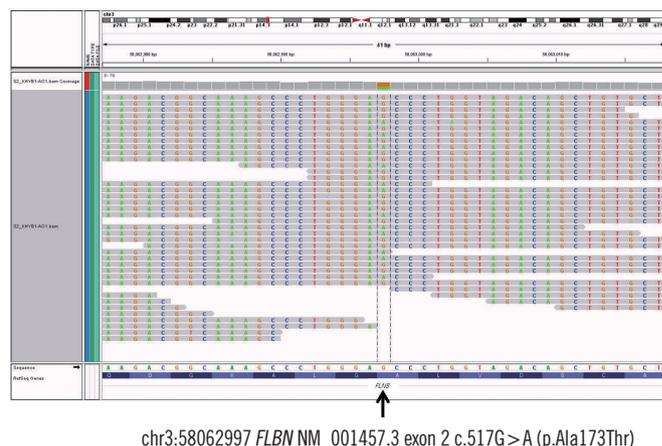


Fig. 3. Integrative Genomics Viewer snapshot of the novel *FLNB* mutation (NM_001457.3: c.517G>A; p.Ala173Thr) identified by exome sequencing.

variants, 23 genes identified through literature survey, including PubMed, UpToDate, and GeneReviews, were screened for determining the relevance of the remained variants (Fig. 2). Consequently, we found one non-synonymous variant (NM_001457.3: c.517G>A; p.Ala173Thr) in the *FLNB* gene (Fig. 3) that was also not reported in the Human Gene Mutation Database. This variant was predicted as 'probably damaging' by Polyphen-2, with a score of 0.998, and as 'not tolerated' by SIFT, with a probability of 0.01, and was regarded as the best candidate for further evaluation.

3. Validation of the variant

Sanger sequencing of the *FLNB* exon 2 of the patient, both her parents, and her twin sister was performed, which revealed that the c.517G>A (p.Ala173Thr) variant was present only in the patient, indicating that the mutation occurred *de novo*. When a control group of 100 alleles from 50 healthy subjects was examined by using exon 2-targeted sequence analysis, no carriers were found with the c.517G>A variant.

DISCUSSION

Mammals have three filamin genes: *FLNA*, *FLNB*, and *FLNC*. Filamin (Fln) A, B, and C comprise a family of three actin-binding proteins, each sharing the common features of an N-terminal actin-binding domain followed by an immunoglobulin-like repeats domain that contains the receptor-binding region at the C-terminus [17]. The proteins are highly homologous, can interact to form both homodimers and heterodimers, and likely share similar functions across various organ systems. FlnA is predomi-

nantly found in the brain and blood vessels, FlnB in bones, and FlnC in muscles. Insight into the function served by these proteins, however, has been limited to observations based on human diseases, their expression patterns, and isolated cellular and molecular culture studies, largely owing to the fact that null *FLNA* and *FLNC* mice are embryonic lethal [18, 19].

FLNB mutation causes a range of skeletal disorders, from mild forms such as spondylocarpotarsal syndrome and Larsen syndrome, to severe forms such as AO-I, AO-III, and boomerang dysplasia [7]. The most significant determinant of lethality is the degree of pulmonary hypoplasia. Atelosteogenesis refers to 'incomplete ossification,' and is characterized by defective endochondral ossification resulting in rhizomelic dwarfism [20]. AO-I is lethal during fetal life or early neonatal period [5]. Boomerang dysplasia is lethal and shows more severe defects in ossification than AO, and is characterized by the complete absence of ossification in certain limbs and vertebrae, and hypoplastic boomerang-shaped femora [4, 21]. Pulmonary hypoplasia, severe laryngeal narrowing, severe tracheal narrowing, or tracheomalacia cause certain kinds of respiratory failure and early neonatal death [22]. Luewan et al. [23] reported that the combination of pulmonary hypoplasia and tracheobronchomalacia is a major cause of death in *FLNB*-related disorders. Larsen syndrome, AO-I, AO-III, and boomerang dysplasia are inherited in an autosomal dominant manner, and spondylocarpotarsal syndrome is inherited in an autosomal recessive manner [7].

As in the present case, most lethal *FLNB*-related disorders are caused by *de novo* mutations; thus, the risk of recurrence in subsequent pregnancies is very rare. The majority of the reported mutations are in exons 2-5, and mutations associated with the most severe phenotypes almost invariably occur in exons 2-5 [21]. The actin-binding domain of FlnB consists of two calponin homology domains (CH1 and CH2) in its N-terminus [21]. Most of the causative mutations of AO-I are located in exons 2 and 3, which encode CH2 [21]. The novel c.517G>A (p.Ala173Thr) variant in the present case occurs in exon 2 and is predicted to interrupt actin binding.

The c.517G>A (p.Ala173Thr) variant has not been reported in the Human Gene Mutation Database; however, a G to T substitution at 518th nucleotide, resulting in an alanine to valine substitution at the 173th amino acid, has been registered as a causal mutation of atelosteogenesis. A patient with the p.Ala173Val mutation was reported to have vertebral abnormalities and carpal and pharyngeal abnormalities. Mutations in *FLNB* produce diverse phenotypes depending on their nature and location [24]. A different nucleotide substitution within a codon

known to harbor a causative mutation, such as the one reported here, is likely to affect the phenotype in a similar manner. Therefore, the location of the present variant, the control study, and in silico analyses suggest that the novel variant in the present case is a pathologic mutation of AO-I.

The diagnosis of *FLNB*-related disorders is based on clinical features, which might not always be conclusive, especially for patients in critical condition. Since exome sequencing allows the simultaneous analysis of all coding sequences of genes without a priori knowledge of their contribution to diseases, it can be a useful, time-saving, and cost-effective tool for providing extended sequence information, not only for the previously known disease-related genes, but also to identify novel disease-associated genes.

In conclusion, we presented a patient with AO-I who had a novel *de novo* heterozygous missense *FLNB* mutation (c.517G>A; p.Ala173Thr). We also showed that exome sequencing analysis can be a useful tool to identify causative mutations in patients with lethal skeletal dysplasias.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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