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# Hypomorphic Mutations in *TONSL* Cause SPONASTRIME Dysplasia

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**Abstract**

SPONASTRIME dysplasia is a rare recessive skeletal dysplasia characterized by short stature, facial dysmorphism, and aberrant radiographic findings of the spine and long bone metaphysis. No causative genetic alterations for SPONASTRIME dysplasia have yet been determined. Using whole exome sequencing, we identified biallelic *TONSL* mutations in 10 of 13 individuals with SPONASTRIME dysplasia. TONSL is a multi-domain scaffold protein that interacts with DNA replication and repair factors, and plays critical roles in resistance to replication stress and maintenance of genome integrity. We show here that cellular defects in dermal fibroblasts from individuals are complemented by expressing wild-type *TONSL*. In addition, *in vitro* cell-based assays and *in silico* analyses of TONSL structure support the pathogenicity of those *TONSL* variants. Intriguingly, a knock-in *Tonsl* mouse model leads to embryonic lethality, implying the physiological importance of TONSL. Overall, these findings indicate that genetic variants resulting in reduced function of TONSL cause SPONASTRIME dysplasia and highlight the importance of the TONSL in embryonic development and postnatal growth.

**Introduction**

SPONASTRIME dysplasia (MIM: 271510), the denotation of which originate from SPOndylar and NAsal alterations with STRIated MEtaphyses,[1] is a very rare but distinct entity that can be categorized as a spondyloepimetaphyseal dysplasia, transmitted as an autosomal recessive trait. To date, only 15 individuals with this disorder have been reported[1-10] based on the diagnostic criteria of a unique combination of clinical and radiological findings, as suggested by Langer et al.[5] The major clinical features are mild to moderate short-limb type dwarfism, relatively large head with a prominent forehead, as well as epicanthic folds observed in infancy or early childhood. However, the clinical criteria are nonspecific and radiological features must be present for a diagnosis. Diagnostic criteria based on the radiological features focus on the changes in the lumbar vertebrae and metaphyseal changes in the long bones. Metaphyseal irregularities and striations, as proposed by the disease's name, are also important diagnostic criteria, but are not seen as consistently as the lumbar

vertebral changes.[5] To date, causative genetic mutations for SPONASTRIME dysplasia have not been determined, and thus a genetic test for this disease is not yet clinically available.

Tonsoku-like DNA repair protein (TONSL) is a multi-domain scaffold protein that interacts with DNA replication and repair factors including anti-silencing function 1 (ASF1) , minichromosome maintenance complex component helicases (MCM helicases), H3 histone, H4 histone, and MMS22-like protein (MMS22L).[11-15] TONSL consists of 1,378 amino acids with annotated domains such as eight tetratricopeptide repeats (TPR), three Ankyrin (ANK) repeats, an ubiquitin-like domain (UBL), and seven leucine-rich repeats (LRR).[13] It was reported that the TONSL-MMS22L heterodimer plays a key role in homologous recombination required for repairing spontaneous replication-associated DNA lesions. At the cellular level, depleting TONSL causes pronounced defects in the rate of cell proliferation and enhances the sensitivity to camptothecin (CPT), a topoisomerase 1 inhibitor that induces DNA breakage at replication forks.[12,13] Specifically, TONSL-MMS22L is recruited to the sites of stalled replication forks during normal S phase by replication protein A (RPA1, RPA2 and RPA3) bound to single-strand DNA (ssDNA), and promotes RAD51 loading for strand invasion.[16] Therefore, it was demonstrated that small interfering RNA (siRNA)-mediated TONSL knockdown leads to loss of damage-induced RAD51 foci formation in cells treated with genotoxic agents.[11,12,16]

In this study, in order to identify the causative genetic alteration for SPONASTRIME dysplasia, we recruited 13 individuals, including a previously reported case,[10] from four different ethnicities, who satisfied the diagnostic criteria of the disease. By performing whole exome sequencing and Sanger sequencing, we identified autosomal recessive hypomorphic and loss-of-function (LoF) mutations in the *TONSL* (MIM: 604546) gene of individuals with SPONASTRIME dysplasia. In further studies, using the dermal fibroblasts from individuals, *in vitro* cell-based assays, *in silico* struture simulation, and *in vivo* knock-in mouse model, we demonstrated the pathogenicity of *TONSL* variants, suggesting that defects in replication-associated DNA damage repair and the resultant inefficient cell proliferation due to *TONSL* mutations might be the underlying pathogenic mechanism for SPONASTRIME dysplasia.

## Material and Methods

**Subjects.** Written informed consent was obtained from the individuals or their parents. The Institutional Review Boards of the Seoul National University Hospital, Seoul, Republic of Korea and Samsung Medical Center, Seoul, Republic of Korea approved the studies.

**Whole exome sequencing (WES) and whole genome sequencing (WGS).** To identify genomic variants that cause SPONASTRIME, we performed WES. Additionally, WGS was conducted in cases where only a single pathogenic *TONSL* allele was identified by WES (P01-1 and P01-2)**.** Basic statistics of the WES data are summarized in Table S2. Based on the inheritance pattern of the individuals, we hypothesized that the disease follows an autosomal recessive fashion. Thus, we eliminated variants that did not satisfy the following criteria: (1) Variants showing allele frequency < 1% in ESP6500 and 1000 Genome Project; (2) Variants not found in our in-house database; (3) Protein altering variants; and (4) High quality of reads (read number > 20, QS > 30, or minor allele frequency > 20%). The resulting list of variants is displayed in Table S3. For the structural variants from WGS data, Manta (0.20.2) was used with default settings and Control-FREEC (6.4) for identifying copy number variants. Window size was set as 10,000 and read counts were normalized based on GC-content bias for Control-FREEC. CNV type was classified based on genome ploidy value 2, where values below 2 denoted loss and values above 2 denoted gain.

**Amino acid conservation and base-level functionality analyses.** To evaluate functionality of nine missense variants in *TONSL*, orthologous sequences from 61 mammalian species were downloaded from the UCSC browser and aligned with the human TONSL. The CADD and GERP scores across *TONSL* coding sequences were downloaded from dbNSFP.[17]

**Long-range PCR.** Long-range PCR (LR-PCR) was conducted to analyze the exon 23 deletion of *TONSL* in P01-1, P01-2 and the mother using the following primers: TONSL-exon22-F: 5'-GAAGAGACTGCCAAGCCAAG-3' and TONSL-exon24-R: 5'-TACCATTTCTGTGGCCCTTC-3'.

**Sanger sequencing.** Sequencing of *TONSL* candidate variants found from WES or WGS analysis were conducted using standard PCR and Sanger sequencing methods (primer sequences available upon request). Sequence data were aligned to the reference sequence Sequencher software (Gene

Codes Corporation, Ann Arbor, MI, USA).

**Reverse transcription-PCR and cloning.** To investigate the splicing changes caused by the splicing site and deep intronic mutations in P11, reverse transcription-PCR and cloning of the amplicon was performed. The mRNA was harvested from the circulating leukocytes of proband and parents using QIAamp RNA Blood Mini Kit (Qiagen, Germany). The cDNA were transcribed using Transcriptor First Strand cDNA Synthesis Kit and then PCR amplification was carried out using the primers TONSL4F 5'-TATGACCACTGCCAGTCGAG-3' and TONSL11R 5'-TGAGCTCCCGTAGTCTGGTT-3', which encompass both paternal and maternal mutations. After PCR based cloning using an All in One™ PCR Cloning Kit (Biofact, Korea), 30 colonies were picked for PCR and sequencing analyses using the same primers.

**Cell culture, cell immortalization, mutagenesis and TONSL cell line establishment.** Dermal fibroblasts from individuals were grown in high glucose and no glutamine DMEM (Gibco, 10313) supplemented with 15% fetal bovine serum (Gibco), glutamine (Gibco, 35050-061), MEM non-essential amino acid (Gibco, 11140-050), penicillin and streptomycin (Gibco, 15140-122) and grown in 5% $CO_2$ and 3% $O_2$ at 37°C. BJ foreskin fibroblasts used as a normal control and were obtained from ATCC. HeLa, U2OS and 293T cells were grown in high glucose DMEM (Gibco, 11965) supplemented with 10% fetal bovine serum, penicillin and streptomycin (Gibco, 15140-122) and grown in 5% $CO_2$ at 37°C. Dermal fibroblasts from individuals were transformed by human papilloma virus E6 and E7 protein and immortalized by catalytic subunit of human telomerase (hTERT) through retroviral transduction. HPV16 E6E7 genes (a gift from Howley Lab, Harvard Medical School, Boston, Massachusetts, USA) were subcloned into pMSCVneo (Clontech) and used to transform the dermal fibroblasts. pWZL-hTERT was used to immortalize the dermal fibroblasts from individuals. The gene coding wild-type TONSL was amplified from a cDNA library prepared from U2OS cells by polymerase chain reaction (PCR) with the following primers TONSL-F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAGCCTGGAGCGCGAGC-3') and TONSL-R (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGAGGCGCCGAAAGAAGAGC-3'). The

PCR product was cloned into a Gateway BP vector, pDONR223, using BP clonase. The pDONR223 clone was sequenced and then recombined into pHAGE vectors using LR clonase (Thermo Fisher Scientific). Using the pDONR223-TONSL template, we generated the point mutation plasmids used in this study using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Genomics). The primers used to generate TONSL mutations are listed in Table S5.

**Fiber analysis.** For replication fork stalling, P03 cells were pulse-labelled with 25 μM 5-chloro-20-deoxyuridine (CldU: Sigma-Aldrich C6891), washed with PBS, pulse-labelled with 250 μM 5-iodo-20-deoxyuridine (IdU; Sigma-Aldrich I7125), and harvested. Cells were washed and resuspended to $5\times10^5$ cells/ml in PBS. The cells were lysed in spreading buffer (200 mM Tris-HCl, pH 7.5, 50 mM EDTA, 0.5% SDS) on glass slides. DNA fibers were spread by gravity, then fixed with methanol:acetic acid (3:1), denatured with 2.5M HCl. CldU was detected using rat anti-BrdU antibody (clone BU1/75, ICR1; Abcam, ab6326; 1:750) and IdU with mouse anti-BrdU antibody (clone B44; BD Biosciences, 347583; 1:750). Slides were fixed using 4% paraformaldehyde, then stained with Alexa Fluor 594or 488 conjugated secondary antibodies (Life Technologies). Images were acquired using a Nikon Eclipse Ni microscope with 60× oil-immersion objectives and NIS-Elements software (Nikon Instruments). ImageJ software (US National Institutes of Health; NIH) was used to analyze replication fork structures (>1000 fork structures) and CldU/IdU track lengths (>300 ongoing forks). For CPT treatment (DNA damage repair) fiber analysis, cells were treated with 100 μM CldU for 30 minutes, then 250 μM 5-iodo-20-deoxyuridine (IdU; Sigma-Aldrich I7125) for 30 minutes with 2.5 μM/50 nM CPT or DMSO. DNA plug was prepared by ~$2\text{-}4.5\times10^5$ cell/plug using by low-melting agarose (Bio-Rad 161-3112), followed by lysis with 20 mg/ml Proteinase K (Roche 03115828001) for two days at 50°C. To stretch the DNA fibers, 22 mm×22 mm silanized coverslips (Genomic Vision) were dipped into the DNA solution for 13 minutes and pulled out a constant speed (300 μM/s) using a Molecular Combing System (Genomic Vision MCS-001). The coverslips were baked for 4 hours at 60℃ and incubated with acid for denaturation. CldU- and IdU-labeled tracts were detected by incubating for 2 hours at room temperature (RT) with rat anti-BrdU antibody (dilution 1:100 detects BrdU and CldU; Abcam 6326) and mouse anti-BrdU antibody (1:10, detects BrdU and

IdU; Becton Dickinson347580). Slides were fixed in 4% paraformaldehyde/PBS and incubated for 1 hour at RT with Alexa Fluor 488-conjugated goat anti-rat antibody (dilution 1:100, A21208; Molecular Probes/Thermo Fisher) or Alexa Fluor 568-conjugated goat anti-mouse antibody (dilution 1:100, A21124; Molecular Probes/Thermo Fisher). Finally, they were mounted with ProLong Gold Antifade Reagent (Molecular Probes) and stored at -20℃. DNA fibers were observed with Carl Zeiss. Axio Observer 7 & ApoTome 2 (Motorized Fluorescence Microscope with Grid Projection) 63 objective. For each experiment, a total of 200 DNA fibers were analyzed, where the number of DNA fibers was measured with ImageJ.

**siRNA transfection, cell proliferation and CPT sensitivity assay.** The siRNA targeting *TONSL* coding regions are listed in Table S6. Here, $3\times10^5$ cells were plated in 6-well plates and transfected siRNA in reverse transfection manner. Cells were transfected a second time after 24 hours. Then 48 hours after the second transfection, 4,000 cells were plated in 4 wells (12 well plates, SPL) for cell counting, and 1,000 cells were plated in 4 wells (96 well plates, Corning #3603) for Hoechst staining. The cells in the 12-well plates were counted using Z1 Coulter Particle Counter (Beckman Coulter) and the nuclei were counted using Cytation 3 (BioTek) for Hoechst stained cells in the 96-well plates. Then, 1,000 cells were plated in 96 well plates in 4 wells and were treated with increasing concentrations of CPT 24 hours after plating. The cells were counted 5 days after treatment. Cells were then stained with Hoechst and counted using Gen 5 (BioTek). For cell lines from individuals, 40,000 cells were plated in 6-well plates in triplicates, and after 24 hours, increasing concentration of CPT was treated. Four days after drug treatment, cells were passed 1:4 to 6 well plates and counted 4 days after using Z1 Coulter Particle Counter (Beckman Coulter).[18]

**Immunoblot.** Cell lysate was prepared by boiling cells in 95 °C for 5 minutes in 2X SDS sample buffer (RBC). For resolution of the proteins, 7.5% precast gels (Bio-Rad) were used, and immunoblotting was performed with anti-HA (Biolegend; MMS-101R, Lot B224726), anti-TONSL (Bethyl: A303-843A, Lot #1), anti-GAPDH (Santa Cruz: SC-25778, Lot K0615), anti-phosphorylated CHEK1 (Cell Signaling: 2348P, Lot #11), anti-phosphorylated CHEK2 (Cell Signaling: 2661P, Lot #11) and anti-α-Tubulin (Abfrontier: LF-PA0146, Lot MJL01-02) antibodies to detect each protein.

**Immunofluorescence (BrdU/Rad51).** For immunofluorescence, $3\times10^5$ cells were plated in 6-well plates (SPL) with cover glass. For HeLa and U2OS cells, siRNA was treated in reverse and forward transfection manner and plated 48 hours after the second transfection. After 24 hours, cells were either treated with CPT (500 μM) overnight or BrdU (20 μg/mL) for 4 hours, then fixed with 3.7% formaldehyde in PBS. For BrdU incorporation, the Invitrogen protocol was followed. Cells were washed in PBS and fixed in 3.7% formaldehyde in PBS for 15 minutes. After washing cells in PBS, cells were permeabilized in 0.1%Triton X-100 buffer for 20 minutes, then in 1N HCl for 10 minutes on ice. Cells were then incubated in 2N HCl for 10 minutes in room temperature (RT), then in phosphate/citric acid buffer for 10 minutes. Cells were washed in permeabilization buffer twice, then incubated with Alexa Fluor® 488 conjugated anti-BrdU primary antibody overnight at RT. For all others, cells were fixed in 3.7% formaldehyde for 15 minutes, then permeabilized with 0.1% Triton X-100 for 20 minutes with PBS washing in between. After washing with PBS twice, the cells were blocked with PBG (0.2% [w/v] cold fish gelatin, 0.5% BSA in PBS) for 1 hour at room temperature. Then, the cells were incubated with 1:7000 of anti-Rad51 antibody (Abcam, Rb:ab133534, Lot #: GR219215-36), and 1:1000 anti-γ-H2A.X antibody (Cell Signaling, #9718S, Lot #13) in PBG overnight at 4°C. The following day, cells were washed three times with PBG and incubated with fluorescent-conjugated secondary antibody for 30 minutes. Secondary antibodies were purchased from Abcam (Ms Alexa Fluor® 594: ab150112; Rb Alexa Fluor® 594: ab150064; Ms Alexa Fluor® 488: ab150109 and Rb Alexa Fluor® 488: ab150061). After washing with PBG three times, the coverslips were embedded in Vectashield (Vector Laboratories) supplemented with DAPI. Nikon A1 confocal microscope was used equipped with a CFI-Apochromat 60X NA-1.4 oil objective, the A1-DUG GaAsP multi detector unit, and the NIS-Element C-ER software. For the BrdU assay, the ratio was calculated by dividing the number of BrdU incorporated cells by the number of total cells counted.

**Animals and ethics statement.** All mice were purchased from Taconic Biosciences (Dae Han Biolink Co., Ltd., Chungbuk, Republic of Korea) and housed at the specific pathogen-free (SPF) facility of the Yonsei Laboratory Animal Research Center. Animal experimental procedures were conducted in

accordance with the Korean Food and Drug Administration (KFDA) guidelines, reviewed, and approved by the Institutional Animal Care and Use Committees (IACUC) at Yonsei University (Permit Number: 201506-322-02).

**Preparation of CRISPR/Cas9 mRNA and donor DNA.** Cas9 mRNA was synthesized using a mMESSAGE mMACHINE® T7 Ultra kit (Ambion) and diluted to working concentration in endonuclease-free injection buffer (0.25 mM EDTA, 10 mM Tris, pH7.4) immediately before microinjection. A plasmid encoding the *S. pyogenes* Cas9 (SpCas9) protein[19] was obtained from ToolGen, Inc. (Seoul, Republic of Korea). The crRNAs were designed by searching for "NGG" or "CCN (the reverse complement sequence of NGG)" sequences near the point mutation target sites. The crRNA sequences used in this study were: crRNA1 5′-GGTCCAGCCCCCTCCCATCC-3′, crRNA2 5′-GAACCCGGATGGGAGGGGGC-3′, crRNA3 5′-CCGGGTTCGAGTTCAAATTC-3′, and crRNA4 5′-CCTGAATTTGAACTCGAACC-3′. The 106-bp synthesized single stranded oligonucleotide (ssDNA) used as donor DNA was p.Arg924Trp ssDNA 5′-TAGAAACTTCTGTCTTCTGACTGTCCCCTCCCTCTGTCTTTCCTGCTAGCTTCTGGTCCAGC CTCCTCCTATCTGGGTTCGGGTTCAGATTCAGGATAACCTTTTCCTCATCCCCGTTCCC-3′. The tracrRNA, crRNAs targeting the *Tonsl* gene, and ssDNA donor for homology-directed repair were obtained from Integrated DNA Technologies (IDT).

**Microinjection.** Microinjection of one-cell embryos was performed as previously described.[20] In brief, 3–4 week old C57BL/6 female mice were superovulated by intra-peritoneal (i.p.) injections of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma) and 5 IU human chorionic gonadotropin (hCG, Sigma) at 48 hour intervals. The fertilized eggs were then collected from the superovulated females crossed with stud males, and were microinjected with a mixture of Cas9 mRNA (50 or 100 ng/μL), four crRNAs (10, 25, or 50 ng/μL each), tracrRNA (tracrRNA mixed with crRNA at 1:1 molar ratio), and donor ssDNA (200 ng/μL). Microinjections were performed in the cytoplasm of one-cell embryos using a piezo-driven manipulator (Prime Tech), followed by embryo transfer into the oviducts of pseudo-pregnant ICR mice to produce a living mouse.

**Founder screening and genotyping PCR.** To screen founders carrying the p.Arg924Trp mutation in

the *Tonsl* gene, PAGE-PCR assay was performed as previously described[21] using genomic DNA obtained from newborns that were produced from the microinjected embryos. In brief, the genomic regions spanning the crRNA target site were amplified by PCR. After simply denaturing and annealing the PCR products, the resulting products were analyzed by acrylamide gel electrophoresis. Then, the candidates were cloned in T-Blunt PCR Cloning Vector (SolGent Co., Ltd., Republic of Korea), and were validated by direct sequencing analysis (Cosmobiotech Co., Ltd., Republic of Korea). The primer sequences used in PAGE-PCR were: F 5'-TGAATGCAGAGCCTGCAGAGA-3' and R 5-TCTAGGGAGCAGAGTGCCAAG-3'. For genotyping PCR, DNA was extracted from tails or yolk sacs. Primers F, 5'-AAGCAGTCTTCAGCATGGGACT-3' and WT R, 5'-AACTCGAACCCGGATG-3' were used to identify the *Tonsl* WT allele. Primers F and KI R 5'-ACCCAGATAGGAGGAG-3' were used to identify the *Tonsl* p.Arg924Trp allele. The primer WT R is annealed specifically to the sequence of the *Tonsl* WT allele, whereas KI R to the sequence of the Tonsl p.Arg924Trp allele.

**Embryo collection.** Gestation was dated by the detection of a vaginal plug (as E0.5) or following *in-vitro* fertilization (IVF) and embryo transfer. Embryos were either fixed in 10% neutral buffered formalin (Sigma-Aldrich) or snap-frozen in liquid nitrogen. Stereomicroscope images were generated from specimens of fixed embryos.

## Results

### *Diagnosis and clinical information of individuals with SPONASTRIME dysplasia*

We recruited 13 individuals, including a previously reported case,[10] satisfying the diagnostic criteria of the disease from four different ethnicities – Korean, Indian, Finnish and Brazilian (African black and non-Latin European). Their clinical features are presented on Table 1 and case reports (see Supplemental note). The median of height standard deviation scores (SDS) of all the individuals of this study was -4.9 (ranging from -0.9 to -10.0). They were short with height SDS down to -10, but a mildly affected individual (P03) had a height SDS of only -0.9. All the individuals recruited shared

similar facial dysmorphism (Figure 1A). Other clinical findings shared by more than one individual included short dental root, airway narrowing, cataracts and joint laxity. Radiographic features were characterized by distinct changes in vertebrae and metaphyses of the long bones. It is worth noting that the radiographic features changed and became more conspicuous with age (Figure 1B and Table S1).

***Identification of TONSL mutations in individuals with SPONASTRIME dysplasia***

To identify the pathogenic mutation causing the disease, whole exome sequencing (WES) of individuals and available family members was conducted in eight individuals from seven families (five Koreans, one Brazilian, and one Finnish; P01-1 to P07) (Tables S2 and S3). Written informed consent was obtained from each individual or their parents, and the study was approved by the Institutional Review Board. Based on the hypothesis that this disease is inherited in an autosomal recessive manner, we retrieved rare variants (<1% frequency in the public database) that were specifically harbored by the probands in homozygous or compound heterozygous status from the eight individuals. Among the individual-specific variants that followed a recessive pattern, variants in *TONSL* were present in four individuals (Table 2). The remaining four individuals harbored single variants in the gene and required further analysis to verify whether they also fit into the recessive model. For example, subsequent whole genome sequencing analysis of the sibling probands (P01-1 and P01-2) identified an exon 23 deletion in the maternal allele, which was validated by polymerase chain reaction (PCR). Next, an additional cohort from India was recruited and subjected to singleton WES (P08 to P10), which led to the discovery of biallelic variants in *TONSL*. Finally, we screened two additional individuals (one Korean and one Indian) for such *TONSL* variants by Sanger sequencing and identified biallelic mutations in one (P11) and a single mutation in the other (P12). P11 has a splicing site variant and a deep intronic variant whose subsequent changes in mRNA were confirmed by reverse transcription-PCR. Only one mutation was identified in the *TONSL* gene in the three probands, P05, P06 and P12 (Table 2). Because the parents harboring the same mutation were unaffected, it is highly likely that the individuals had undetermined variants in the remaining allele of *TONSL*, such as a cryptic structural variation or a noncoding variation. All the *TONSL* mutations

identified were confirmed by Sanger sequencing (Figure S1A-B). No significant variation was found in other skeletal dysplasia-related genes in affected individuals. Ten out of thirteen individuals (76.9 %) carried one LoF allele and one missense allele (Table 2). Except for nonsense mutations, which may lead to nonsense-mediated mRNA decay, *TONSL* missense mutations were found in various locations throughout the protein-coding region (Figure 2A), mostly within functionally annotated domains (8 out of 9). The nine missense variants displayed complete or near-complete evolutionary conservation across vertebrate species (Figure 2B) and were found in low frequency in the healthy population (5/9 not found in the ExAC database, all nine $<1.0 \times 10^{-4}$). To predict the functionality of the missense variants, the CADD and GERP scores were compared with the rest of the amino acid residues of the protein and displayed significant differences (Figure 2C and Figure S2A-D). *TONSL* is tolerant to LoF variants (ExAC pLI = 0.00), but no individual in this study carried LoF variants in both alleles, implying a critical but minimal requirement of TONSL function for survival.

***Complementation of defects in dermal fibroblasts from individuals by expression of wild-type TONSL***

In an attempt to validate that TONSL variants are causal for the SPONASTRIME phenotype, we established primary dermal fibroblasts from two individuals, P03 and P04. Immunoblot analysis showed a pronounced decrease in TONSL levels compared to those in normal human fibroblasts, BJ cells (Figure 3A). siRNA treatment abrogated endogenous TONSL protein levels (Figure 3A and Figure S5A). To further validate the causative nature of TONSL variants, we transduced an empty vector (EV) and HA-tagged wild-type (WT) TONSL into P03 and P04 cells (Figure 3B) and performed functional complementation assays. Expressing WT *TONSL* successfully rescued the enhanced sensitivity to CPT (Figure 3C) and DNA damage-induced RAD51 foci formation in both cell lines (Figure 3D and E). As controls for the CPT sensitivity assay, we used BJ cells, and the FANCP(also known as SLX4) deficient dermal fibroblasts, the FANCP cells, derived from an individual with Fanconi anemia. The FANCP cells were found to be sensitive to CPT, which was

rescued by introducing wild-type SLX4 protein through lentiviral transduction.[18,22] CPT sensitivity assay of P03 and P04 cells were designed similar to that of the previous FANCP cell assay by using EV and wild-type TONSL complemented cells. In addition to the normal control BJ cells, EV as well as wild-type SLX4 FANCP cells were used for comparing CPT sensitivity. In addition, to confirm the impaired DNA replication caused by TONSL variants found in individuals, BrdU incorporation assay was performed and quantified by immunofluorescence. As expected, the BrdU incorporation ratios in P03/EV and P04/EV cells were less than that in BJ cells, whereas the complemented cells showed increased BrdU incorporation (Figure 3F). In a study by Duro et al., it was reported that although *TONSL* knock-down cells are sensitive to CPT, they are not sensitive to hydroxyurea (HU) compared to control cells.[6] In order to test if the fibroblasts from individuals with mutant *TONSL* display similar characteristics, we treated P03 and P04 cells with HU. Consistent with previous reports, both P03 and P04 cells were not sensitive to HU (Figure S3). As mentioned earlier, TONSL is involved in homologous recombination, and its impairment results in decreased cell proliferation and increased sensitivity to CPT.[12, 13] Because homologous recombination is an important part of DNA damage repair and thus DNA replication, it is possible that TONSL variants may increase the occurrence of stalled replication fork due to DNA damage repair defects. In order to evaluate the impairment of replication restart and DNA damage repair of the mutant *TONSL*, we performed fiber analysis with fibroblast from P03 and normal control cell (Figure S4). We used nucleotide analogs CldU and IdU to track the first label origin and the second label origin newly synthesized DNA. Ongoing forks have both CldU and IdU tracks, whereas stalled forks can be monitored by CldU-only labeled tracks. The percentage of each tracks from the total of all CldU incorporated tracks was then calculated to compare the changes in ongoing or stalled replication forks. Compared to normal human fibroblast, the percentage of ongoing replication fork was slightly decreased in P03 cells, and the number of stalled forks was significantly increased in P03 cells compared to normal cells (Figure S4). Next, we performed fiber analysis for CPT sensitivity using P03 and P04 cells with EV and WT-TONSL and BJ cells as controls. When treated with 50 nM of CPT, P03/EV and P04/EV cells showed decreases in replication length compared to BJ cells, which were rescued by WT-TONSL (Figure 3G). Taken

together, these data display strong evidence that the TONSL variants found in individuals impair DNA replication and repair capacity, all of which were rescued by WT-TONSL, demonstrating the pathogenic nature of *TONSL* mutations in the disease phenotype.

***Validation of pathogenic TONSL mutations in in vitro cell-based assay***

To systematically evaluate the pathogenicity of *TONSL* variants found in other individuals whose primary fibroblasts were not available, we established an *in vitro* cell-based assay using HeLa cell lines. We focused on missense *TONSL* mutations since nonsense mutations may be subject to nonsense-mediated mRNA decay. First, we tested if we could recapitulate the results of previous reports showing that TONSL depletion leads to enhanced sensitivity to CPT.[11,12] In order to accomplish this, we established an assay system using HeLa cell line, where mutant *TONSL* is expressed but the endogenous wild-type is selectively knocked down. The strategy was to establish individual HeLa cell lines that stably express each mutant *TONSL* by lentiviral transduction, then treating cells with siRNA targeting *TONSL* 3'-untranslated region (UTR), which selectively knocks down only the endogenous *TONSL* mRNA. If successful, the HA-tagged *TONSL* is expressed, and any cellular defects due to *TONSL* mutation can be observed without the interference of the endogenous *TONSL*. WT-TONSL and EV cell lines were established as positive and negative controls. To first test the siRNA's, HeLa cells were treated with siRNA targeting the coding region and the 3' UTR. As shown in Figure S5A-B, depleting TONSL by targeting the coding or the 3' UTR region in HeLa cells resulted in hypersensitivity to CPT. Then, HeLa cells stably expressing the individual EV, HA-tagged *WT-TONSL* and the HA-tagged *TONSL* missense mutant were successfully established (Figure S5C). The siRNA targeting the *TONSL* 3′ UTR selectively knocked down endogenous *TONSL*, but not HA-tagged WT or mutant *TONSL* (Figure 4A). After confirming the selective knockdown of endogenous *TONSL* and decreased TONSL protein levels, EV, WT-TONSL and TONSL variant cells were treated with siRNA targeting *TONSL* 3' UTR, and were then grown for 5 days to monitor cell proliferation. Overall, a noticeable proliferation defect was observed in TONSL variant HeLa cells compared to WT-TONSL cells (Figure 4B). Of these, proliferation of cells with P02_p.Gly973Arg, P01-1;P01-

2_p.Arg934Trp, P05_p.Asp364His, and P08_p.Ser174Asn variants were significantly inhibited to a similar level as that of siTONSL-treated HeLa-EV cells (Figure 4B). This result shows that all examined TONSL variants directly impact cell proliferation, but to varying degrees. At the same time, we evaluated phosphorylated Checkpoint (CHEK1) and Checkpoint kinase 2 (CHEK2) after endogenous TONSL depletion in the individual TONSL variant HeLa cells. We found that the check-point is activated in cells with proliferation defects (Figure 4C), suggesting that the functional impairment of TONSL leads to genome instability, which results in cell cycle arrest and inhibition of cell division. To further investigate the role of each TONSL variants in repairing replication-associated DNA damage, a CPT sensitivity assay was performed. Consistent with the proliferation results, the CPT sensitivity of the TONSL variant cells was comparable to that of siTONSL-treated HeLa-EV cells, which lack TONSL (Figure 4D). In addition, we modeled the wild-type and TONSL variants based on the existing available structure to determine the basis of aberrant function of the TONSL variants (Figure S6). p.Glu539Lys (P03) and p.Arg558Gln (P04) altered the polar interactions between amino acids. Exon 23 deletion from individuals P01-1 and P01-2 changed the curvature of LRR domain significantly, and the p.664delLeu (P09) altered the direction of the C-terminal of the helix (Figures S6A-G). These changes deformed the overall structures, which may influence binding affinity between variant TONSL and its binding partners (Figure S6). From the studies using *in vitro* cell-based assay and *in silico* structural analyses, we conclude that the identified TONSL variants compromise TONSL functional activity, which potentially leads to human diseases.

***Embryonic lethality of Tonsl knock-in mouse model***

To better understand the pathophysiology and clinical correlations, we generated a CRISPR/Cas9-mediated Tonsl knock-in (KI) mouse carrying a p.Arg924Trp substitution in the *Tonsl* gene, akin to its human counterpart TONSL p.Arg934Trp variant found in individuals P01-1 and P01-2 (Figure 5A-C and S7A). Heterozygous $Tonsl^{+/Arg924Trp}$ (HT) mice were fertile and showed normal gross morphology. Intriguingly, no offspring littermates (n = 38) carrying the biallelic p.Arg924Trp variant were obtained from heterozygous intercrosses, while their WT and HT littermates were born

in a 1:2.8 ratio (Figure 5D), suggesting that the homozygotes were not viable. For development analysis, we collected embryos from embryonic days 10.5 to 12.5 (E10.5–E12.5) and observed a 1:1.4:0.6 ratio of WT:HT:KI embryos (Table S4). However, all homozygous *Tonsl*$^{Arg924Trp/Arg924Trp}$ embryos showed early embryonic lethality with fetal growth restriction at around stage E10.5 (Figure 5E and S7B-D). The yolk sacs of homozygous *Tonsl*$^{Arg924Trp/Arg924Trp}$ embryos lacked visible blood vessels and were smaller than others (Figure S7E). Consistent with *in vitro* cell-based assays of the human TONSL p.Arg934Trp variant, these results suggest that the Tonsl p.Arg924Trp variant is not functional, and that the lack of this functional protein is likely the major reason for embryonic lethality. Taken together, these findings demonstrated the physiological importance of functional Tonsl protein in embryonic development and the pathogenic nature of the TONSL p.Arg934Trp variant.

## Discussion

In this study, we report the identification of *TONSL* mutations causative for 13 individuals with SPONASTRIME dysplasia. We demonstrate that the endogenous TONSL protein level of dermal fibroblast cells from individuals presented with SPONASTRIME dysplasia is significantly lower than that of wild-type control cells. Furthermore, we found that those cells from individuals exhibit enhanced sensitivity to CPT, reduced DNA damage-induced RAD51 foci formation, and impaired replication capacity, all of which are successfully rescued by WT-TONSL through lentiviral transduction. Given the importance of TONSL function in replication fork stability during normal S phase,[23] it is reasonable to speculate that pathogenic *TONSL* variants result in impaired cell proliferation, which is critical for embryonic development and postnatal growth.[24] Similarly, mutations in various genes important for preventing replication-associated DNA lesions have been identified in individuals with growth retardation.[22,24,25]

We found increased phosphorylation of CHEK1 and CHEK2 (Figure 4C) in cells with TONSL variant in the absence of endogenous TONSL or siRNA-mediated TONSL depletion even without treatment of genotoxic agents. CHEK1 is mainly phosphorylated by Ataxia-telangiectasia and

Rad3 related (ATR), and deleterious mutations in the *ATR* (MIM:601216) gene which results in Seckel syndrome (MIM: 210600), characterized by microcephaly, dwarfism, progeria, and mental retardation.[25,26] ATR responds to a variety of DNA damage, and together with ATR interacting protein (ATRIP), recognizes RPA bound to single stranded DNA, which is often the result of a stalled replication fork.[27] We observed that mutation in *TONSL* did not affect RPA foci formation in response to CPT-induced DNA damage in cells from SPONASTRIME dysplasia individuals (unpublished data), implying that TONSL functions downstream of ATR activation. Therefore, it is possible to speculate that alterations in the ATR, which function upstream of TONSL, may lead to broader defects, whereas loss of TONSL function may cause milder disease phenotypes such as SPONASTRIME dysplasia. It remains unknown why alterations in *TONSL* result specifically in SPONASTRIME dysplasia, and understanding the genotype to phenotype correlation requires further study.

Our knock-in mouse model clearly demonstrated that even a single point mutation, p.Arg924Trp, corresponding to p.Arg934Trp identified in human SPONASTRIME dysplasia, causes embryonic lethality in mouse. Although this finding in the mouse model does not directly translate to the pathogenicity of human disease, it provides evidence supporting that the TONSL p.Arg934Trp variant might be causative of SPONASTRIME dysplasia, and highlights the physiological importance of TONSL. Homozygous p.Arg924Trp variant in mice Tonsl has severe developmental impact, where fetal growth retardation was observed approximately at E10.5, resulting in embryonic lethality. However, the heterozygotes surviving to adulthood show no apparent defects. Consistent with the *in vitro* cell-based assay (Figure 4B-D), these results suggest that Tonsl proteins carrying the p.Arg924Trp variant are not functional, and that the lack of this functional protein is considered to be the main reason for embryonic lethality of Tonsl KO mouse (Brendan Lee, personal communications) and even our KI models. However, unlike in mouse, the human TONSL p.Arg934Trp variant is found in a subgroup of individuals with SPONASTRIME dysplasia. The discrepancy between human and mouse phenotypes might be due to the difference in transcript isoforms found in humans and mice. According to the GENCODE basic project,[28] two transcripts are predicted to be translated in humans,

whereas mouse only have one transcript. Among the two transcripts in humans, the shorter isoform lacks the domain containing the Arg934 residue as well as the UBL and LRR domains. Whether the shorter isoform has any function in humans has yet to be elucidated, but the possibility that it provides partial function for survival cannot be excluded. More research is needed to understand the TONSL variants, since the presence of another TONSL isoform could have prevented lethality in humans. Although further study is required to understand how the decreased function of TONSL leads to the specific phenotype of SPONASTRIME dysplasia, we believe that our findings increase our understanding of the pathogenesis of this disease.

**Description of Supplemental Information**

Supplemental Information includes clinical case reports, seven figures, six tables, supplemental methods and references.

**Declaration of Interests**

The authors declare no competing interests.

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**Accession Numbers**

The raw genome data can be downloaded at http://biodata.kr/ (Submission ID: 1711075636).

**Web Resources**

Online Mendelian Inheritance in Man: http://www.omim.org

UCSC browser: http://genome.ucsc.edu

Protein data bank: https://www.rcsb.org/

ExAC browser: http://exac.broadinstitute.org/

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## Figure Titles and Legends

**Figure 1. Characteristic facial appearance and radiographic findings of individuals with SPONASTSRIME dysplasia.** **(A)** Facial photos of individuals from three different ethnicities. They share midfacial hypoplasia, depressed nasal root, short and upturned nose, prognathism, and a relatively large head size with a prominent forehead. P05 at age 5 years, P06 at age 5 years, and P08 at age 13 years. **(B)** Lateral spine view of P02 at age 3 years and 8 months (3.8) showing taller anterior vertebral body and convex anterior endplates, and that of P01-2 at age 13 years showing biconcavity of the endplates. Hip and knee anteroposterior views of P02 at age 3.8 years showing metaphyseal irregularity and vertical striation and small, dysplastic epiphyses, and those of P01-2 showing residual avascular necrosis of the left femoral head, and mixed dense striations and lucent area at the metaphysis of the knee.

**Figure 2. *TONSL* mutations identified in individuals with SPONASTRIME dysplasia.** **(A)** Pathogenic variants in TONSL found from SPONASTRIME individuals are displayed on TONSL protein with known functional domains. Variants in red were analyzed for variant functionality. **(B)** Evolutionary conservation of the nine missense variants found in SPONASTRIME individuals. **(C)** As parameters of missense variant ("Pathogenic") functionality of individuals, CADD and GERP values were plotted along with residues that overlapped with the ExAC database ("ExAC") and residues that were not polymophic ("Other residues").

**Figure 3. Complementation of dermal fibroblasts from individuals P03 and P04 with the *TONSL* cDNA.** **(A)** TONSL protein levels and corresponding siRNA-mediated *TONSL* depletion of cells from individuals with SPONASTRIME dysplasia. BJ cells were used as a normal control. The basal TONSL protein level in cells derived from individuals was lower than that in BJ cells, and all were

successfully depleted by siRNA treatment. C: siControl, T: siTONSL. **(B)** Stable protein level of wild-type TONSL through lentiviral transduction in fibroblasts derived from both P03 and P04. EV and WT denote the empty vector and wild-type, respectively. The asterisk indicates the cross-reacting band. **(C)** Cells from individuals were sensitive to CPT, which were rescued to a level comparable to that of BJ cells by transducing WT-TONSL. The indicated cells in triplicate were exposed to different concentration of CPT ranging 0-32 nM. After 5 days, the number of the cells were counted using a coulter counter, and the total number of cells at each concentration was divided by the number of untreated cells. FANCP/EV, a Fanconi anemia-derived cell line lacking *Slx4*, and the genetically isogenic SLX4 complemented cells, FANCP/WT-SLX4 were used as controls. The error bars represent the standard deviation (SD) of three replicates. **(D)** Representative images of CPT-induced DNA damage Rad51 and γH2A.X foci. P03/EV, P03/WT-TONSL, P04/EV and P04/WT-TONSL cells were treated with CPT (50 nM) overnight, followed by fixing and staining with anti-RAD51 and anti-γH2A.X antibodies. Impaired DNA damage induced RAD51 foci formation of those cells from individuals was recovered to that of normal cells by transducing WT-TONSL. **(E)** Statistical analysis of Rad51 foci. Percentage was calculated by counting nuclei with n≥10 Rad51 foci divided by the total nuclei for non-treated cells, or divided by γH2A.X positive nuclei for CPT treated cells. The error bars represent the SD. **(F)** BrdU incorporation into DNA was reduced in P03/EV and P04/EV cells, which were rescued to a normal level by transducing WT-TONSL. The BrdU incorporation ratio was calculated by dividing the number of BrdU incorporated cells by the number of total cells counted. The error bars represent the SD of three replicates. **(G)** DNA fiber analysis of CPT-treated SPONASTRIME individual-derived cell lines. BJ cells were used as control. Schematic of the experiment is shown on the top and the representative DNA fiber is shown on bottom. The red line indicates median value; **: $p = 0.003$; ***: $p < 0.001$.

**Figure 4. *In vitro* cell-based assay of TONSL variants shows defects in cell proliferation and enhanced sensitivity to camptothecin.** **(A)** Protein level of recombinant wild-type or individual

TONSL variant in HeLa cells upon endogenous TONSL depletion using siRNA against the *TONSL* 3' UTR. The TONSL antibody is able to detect both endogenous and HA-tagged TONSL, whereas the HA antibody is not able to detect endogenous TONSL. The asterisk indicates the cross-reacting band. **(B)** Cell proliferation assay showing varied proliferation rate of cells expressing mutant *TONSL*. EV cells were treated with either non-targeting siCtrl or siTONSL targeting the 3′ UTR. The rest of the cells were treated with siTONSL targeting the 3′ UTR. The cell proliferation rate was normalized to the cell number on day 1. Error bar represents SD of three replicates. siCtrl: siControl. **(C)** TONSL variants in the absence of endogenous TONSL lead to checkpoint activation. Whole cell extracts of HeLa cells expressing mutant *TONSL* were treated with siRNA against 3' UTR and analyzed by immunoblot with DNA damage response factors, phosphorylated CHEK1 (pCHEK1) or phosphorylated CHEK2 (pCHEK2). **(D)** TONSL variant cell lines were treated with 3′ UTR siRNA as in b, then treated with increasing concentrations of CPT. Cells were stained with Hoechst and nuclei were counted 5 days after CPT treatment. Cell survival was normalized to that in vehicle-treated cells. The error bars represent SD from three replicates.

**Figure 5. Generation and analysis of homozygous Tonsl p.Arg924Trp knock-in mouse using CRISPR-Cas9. (A)** A schematic diagram showing the mouse *Tonsl* locus and the enlarged sequences of the exon 18 of the *Tonsl* gene, along with the sequences of the $Tonsl^{Arg924Trp}$ allele. Blue letters in the $Tonsl^{WT}$ allele indicate a proto-spacer adjacent motif (PAM) sequence. Red letters indicate the substitution (C to T) target nucleotide in the $Tonsl^{WT}$ and the $Tonsl^{Arg924Trp}$ alleles. The amino acid sequences from the $Tonsl^{WT}$ and the $Tonsl^{Arg924Trp}$ alleles are shown at the top of the nucleotide sequences. The substituted nucleotides for synonymous and target mutations are shown at the bottom of the $Tonsl^{Arg924Trp}$ allele sequences by black and red asterisks (*), respectively. Forward (F) and Reverse (R) PCR primers for genotyping are indicated. **(B)** Genotyping PCR for the $Tonsl^{WT}$ and the $Tonsl^{Arg924Trp}$ alleles. The upper and bottom panels show the PCR products that were amplified from the $Tonsl^{WT}$ (115 bp) and the $Tonsl^{Arg924Trp}$ (108 bp) alleles, respectively. **(C)** Chromatogram

displaying the sequence of the $Tonsl^{WT}$ and the $Tonsl^{Arg924Trp}$ locus. **(D)** Genotype distribution of offspring from heterozygous intercrosses. **(E)** Gross morphology of whole embryos at stage E11.5. $Tonsl^{+/+}$ and $Tonsl^{+/Arg924Trp}$ mouse embryos show normal development, whereas $Tonsl^{Arg924Trp/Arg924Trp}$ embryo exhibits a growth retardation with the abnormal development of eyes and limbs.

**Table 1.** Clinical features of individuals in this series

| ID | P01-1 [a] | P01-2 [a] | P02 | P03 | P04 | P05 | P06 | P07 [b] | P08 | P09 | P10 | P11 | P12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ethnicity** | Korean | Korean | Korean | Korean | Brazilian (African black and non-Latin European) | Korean | Finnish | Korean | Indian | Indian | Indian | Korean | Indian |
| **Age of initial presentation (years. months)** | 8.5 | 6 | 0.2 | 13 | 2.2 | 2.7 | At birth | 36 | 13 | 14 | 11 | 3.5 | 1.9 |
| **Gender** | F | M | M | M | M | F | F | M | M | F | F | F | F |
| **Consanguinity** | No | No | No | No | Yes | No | No | No | No | No | No | No | No |
| **Initial presentation** | short stature | short stature | short stature | radiographic abnormality of knee | short stature | short stature | short stature | neck and shoulder pain | short stature | short stature | short stature and limb deformity | short stature | short stature |
| **Height (cm) (SDS)** | 104.1 (-3.6) | 93.4 (-4.7) | 47.9 (-4.9) | 145.2 (-0.9) | 76 (-4.0) | 74 (-4.5) | 40 (-5.6) | 136 (-6.2) | 120 (-4) | 119 (-7) | 93 (-10) | 73.6 (-5.6) | 53 (-10) |
| **Age of latest measurement (years)** | 17.5 | 15 | 5.7 | | 13 | 6 | 12.6 | 37 | 13 | 14 | 11 | 4.2 | 2 |
| **Ht cm (SDS) (latest)** | 124.4 (-6.8) | 143.5 (-4.8) | 82.6 (-6.0) | | 122 (-4.6) | 98.8 (-3.4) | 85.8 (-10.4) | 136 (-6.2 ) | 120 (-4) | 119 (-7) | 93 (-10) | 80.6 (-5.8) | |
| **Facial dysmorphism** | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) |
| **Cognitive impairment** | (-) | (-) | (-) | (-) | borderline | (-) | (-) | (-) | (-) | (-) | (-) | (-) | (-) |
| **Other findings** | short dental roots | short dental roots | hypothyroidism; nystagmus; hypospadias; airway narrowing [c] | | recurrent pneumonia in the 1st year; short dental roots | hypothyroidism; recurrent pneumonia until age 5 years; precocious puberty | cataracts; cerebro-vascular aneurysm rupture; weak voice | Arnold-Chiari malformation; cataracts | | | joint laxity | joint laxity; hip subluxation; airway narrowing [c] | |

[a] These two individuals are siblings

[b] Previously reported (Jeong et al.[10])

[c] Airway narrowing was composed of glottic narrowing and diffuse tracheal narrowing.

M: male, F: female, SDS: standard deviation score

**Table 2**. *TONSL* mutations identified in 13 individuals and their predicted protein changes

| ID | Mutation 1 | | Mutation2 | | Genetic study |
|---|---|---|---|---|---|
| **P01-1** [a] | exon 23 deletion [b] | | c.2800C>T | p.Arg934Trp | quad WES |
| **P01-2** [a] | exon 23 deletion [b] | | c.2800C>T | p.Arg934Trp | quad WES |
| **P02** | c.1531C>T | p.Gln511* | c.2917G>A | p.Gly973Arg | trio WES |
| **P03** | c.735C>G/ c.737delT [c] | p.Cys245Trpfs*20 | c.1615G>A | p.Glu539Lys | trio WES |
| **P04** | c.1673G>A | p.Arg558Gln | c.1673G>A | p.Arg558Gln | trio WES |
| **P05** | c.1090G>C | p.Asp364His | Not identified | Not identified | quad WES |
| **P06** | c.578+1G>T | | Not identified | Not identified | trio WES |
| **P07** [g] | c.2907C>A | p.Tyr969* | c.3862G>C | p.Glu1288Gln | singleton WES |
| **P08** | c.1471_1472delTC | p.Ser491Argfs*65 | c.521G>A | p.Ser174Asn | singleton WES |
| **P09** | c.1459G>A / c.1989_1991del [d] | p.Glu487Lys / p.664delLeu | c.125G>A | p.Arg42His | singleton WES |
| **P10** | c.295delT | p.Ser99fs*59 | c.1459G>A | p.Glu487Lys | singleton WES |
| **P11** | c.578+1G>A | p.Thr151Argfs*42 [e] | c.1291-11_1291-14delCCTC | p.Arg431Profs*6/ p.431_441del [f] | Sanger |
| **P12** | c.1459G>A | p.Glu487Lys | Not identified | Not identified | Sanger |

[a] These two individuals are siblings

[b] g.145657122_145658684del

[c] Paternal allele harbors a substitution followed by deletion of one nucleotide.

[d] Paternal allele harbors a substitution and an in-frame deletion.

[e] RT-PCR revealed mRNA sequence change, predicting a truncated protein or nonsense-mediated mRNA decay

[f] RT-PCR and cloning its products revealed wild-type (14/25) and two different mutant mRNAs formed by retention of a part of intron 10 (5/25) and by skipping of a part of exon 11 (6/25), which predicted these two different polypeptides.

[g] All the individuals with two or three mutations were confirmed to be biallelic by testing the parents, except P07, whose parents had already passed away, by testing the siblings.

WES: whole exome sequencing