How to Cite this article: Yukimasa Taniguchi, China Nagano, Kiyotoshi Sekiguchi, Atsushi Tashiro, Noriko Sugawara, Haruhide Sakaguchi, Chisato Umeda, Yuya Aoto, Shinya Ishiko, Rini Rossanti, Nana Sakakibara, Tomoko Horinouchi, Tomohiko Yamamura, Atsushi Kondo, Sadayuki Nagai, Hiroaki Nagase, Kazumoto Iijima, Jeffrey Miner, and Kandai Nozu, Clear evidence of LAMA5 gene biallelic truncating variants causing infantile nephrotic syndrome, Kidney360, Publish Ahead of Print, 10.34067/KID.0004952021

Article Type: Original Investigation

Clear evidence of LAMA5 gene biallelic truncating variants causing infantile nephrotic syndrome

DOI: 10.34067/KID.0004952021


Key Points:
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We report 3 patients with LAMA5 biallelic truncating variants manifesting infantile nephrotic syndrome and in vitro heterotrimer assays

We report one SRNS case with biallelic LAMA5 missense variants

Abstract:
Background: Pathogenic variants in single genes encoding podocyte-associated proteins have been implicated in about 30% of steroid resistant nephrotic syndrome (SRNS) patients in children. However, LAMA5 gene biallelic variants have been identified in only 7 patients so far, and most are missense variants of unknown significance. Furthermore, no functional analysis had been conducted for all but one of these variants. Here, we report three patients with LAMA5 gene biallelic truncating variants manifesting infantile nephrotic syndrome and one SRNS case with biallelic LAMA5 missense variants. Methods: We conducted comprehensive gene screening of Japanese patients with severe proteinuria. Using targeted next-generation sequencing, 62 podocyte-related genes were screened in 407 unrelated patients with proteinuria. For the newly discovered LAMA5 variants, we conducted in vitro heterotrimer formation assays. Results: Biallelic truncating variants in the LAMA5 gene (NM_005560) were detected in 3 patients from 2 families. All patients presented with proteinuria within 6 months of age. Patients 1 and 2 were siblings possessing a nonsense variant (c.9232C>T, p.(Arg3078*)) and a splice site variant (c.1282+1G>A) that led to exon 9 skipping and a frameshift. Patient 3 had a remarkable irregular contour of the glomerular basement membrane. She was subsequently found to have a nonsense variant (c.8185C>T, p.(Arg2720*)) and the same splice site variant in patients 1 and 2. By in vitro heterotrimer formation assays, both truncating variants produced smaller laminin α5 proteins that nevertheless formed trimers with laminin β1 and γ1 chains. Patient 4 showed SRNS at the age of eight and carried compound heterozygous missense variants (c.1493C>T, p.(Ala498Val)) and (c.8399G>A, p.(Arg2800His)). Conclusions: Our patients showed clear evidence of biallelic LAMA5 truncating variants causing infantile nephrotic syndrome. We also discerned the clinical and pathological characteristics observed in LAMA5-related nephropathy. LAMA5 variant screening should be performed in congenital/infantile nephrotic syndrome patients.

Funding: Grants-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and: China Nagano, 18K15713; Collaborative Research Program of Institute for Protein Research, Osaka University: Yukimasa Taniguchi, CR-21-06; Foundation for the National Institutes of Health (FNIH): Jeffrey H. Miner, R01DK078314, R01DK058366


Data Availability Statement:

Clinical Trials Registration:

Registration Number:

Registration Date:

The information on this cover page is based on the most recent submission data from the authors. It may vary from the final published article. Any fields remaining blank are not applicable for this manuscript.
Clear evidence of LAMA5 gene biallelic truncating variants causing infantile nephrotic syndrome

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Key Points

- *LAMA5* gene biallelic variants have been identified in only 7 patients so far and no functional analysis had been conducted for all but one
- We report 3 patients with *LAMA5* biallelic truncating variants manifesting infantile nephrotic syndrome and in vitro heterotrimer assays
- We report one SRNS case with biallelic *LAMA5* missense variants

Abstract

Background

Pathogenic variants in single genes encoding podocyte-associated proteins have been implicated in about 30% of steroid resistant nephrotic syndrome (SRNS) patients in children. However, *LAMA5* gene biallelic variants have been identified in only 7 patients so far, and most are missense variants of unknown significance. Furthermore, no functional analysis had been conducted for all but one of these variants. Here, we report three patients with *LAMA5* gene biallelic truncating variants manifesting infantile nephrotic syndrome and one SRNS case with biallelic *LAMA5* missense variants.

Methods

We conducted comprehensive gene screening of Japanese patients with severe proteinuria. Using targeted next-generation sequencing, 62 podocyte-related genes were screened in 407 unrelated patients with proteinuria. For the newly discovered *LAMA5* variants, we conducted in vitro heterotrimer formation assays.

Results
Biallelic truncating variants in the *LAMA5* gene (NM_005560) were detected in 3 patients from 2 families. All patients presented with proteinuria within 6 months of age. Patients 1 and 2 were siblings possessing a nonsense variant (c.9232C>T, p.(Arg3078*)) and a splice site variant (c.1282+1G>A) that led to exon 9 skipping and a frameshift. Patient 3 had a remarkable irregular contour of the glomerular basement membrane. She was subsequently found to have a nonsense variant (c.8185C>T, p.(Arg2720*)) and the same splice site variant in patients 1 and 2. By in vitro heterotrimer formation assays, both truncating variants produced smaller laminin α5 proteins that nevertheless formed trimers with laminin β1 and γ1 chains. Patient 4 showed SRNS at the age of eight and carried compound heterozygous missense variants (c.1493C>T, p.(Ala498Val) and c.8399G>A, p.(Arg2800His)).

**Conclusions**

Our patients showed clear evidence of biallelic *LAMA5* truncating variants causing infantile nephrotic syndrome. We also discerned the clinical and pathological characteristics observed in *LAMA5*-related nephropathy. *LAMA5* variant screening should be performed in congenital/infantile nephrotic syndrome patients.
Introduction

Nephrotic syndrome is characterized by heavy proteinuria, hypoalbuminemia, edema and hyperlipidemia. Most children with nephrotic syndrome have steroid sensitive nephrotic syndrome. However, 10-20% of nephrotic syndrome patients have steroid resistant nephrotic syndrome (SRNS) and are at risk to progress to end stage kidney disease (ESKD) \(^1\).

Focal segmental glomerulosclerosis (FSGS) is the most frequently observed pathological finding in SRNS and often develops into ESKD in children. Hereditary FSGS is a genetically heterogeneous condition that is associated with more than 60 podocyte-related genes. Identifying genetic bases for SRNS/FSGS in patients could allow discontinuation of immunosuppressive therapy and provide further information about prognosis.

Currently, whole-exome sequencing has revealed new genes associated with SRNS, thus expanding the genetic heterogeneity of the disease. Chatterjee et al reported a case with biallelic \(LAMA5\) missense variants associated with FSGS for the first time \(^2\). But those are variants of unknown significance (VUS), and there are no reports of any in vitro functional or in vivo pathological analyses. Likewise, Braun et al. identified homozygous VUS in \(LAMA5\) in 5 pediatric nephrotic syndrome patients from 3 families using whole exome sequencing \(^3\). They also have not reported further in vitro functional or in vivo pathological analyses. Therefore, the role of \(LAMA5\) pathogenic variants in causing SRNS/FSGS is still unclear. The \(LAMA5\) variant \(p\).\(Arg286Leu\) is clearly pathogenic and causes complex syndromic developmental defects along with FSGS and ESKD \(^4\).

The glomerular basement membrane (GBM) is an important component of the kidney’s glomerular filtration barrier. The GBM contains members of the four major classes of
basement membrane proteins: type IV collagen, laminin, heparan sulfate proteoglycan, and nidogen. The GBM's major laminin isoform is laminin-521 (LM-521), a cross-shaped heterotrimeric glycoprotein composed of the laminin α5, β2, and γ1 chains. The LAMA5 gene encodes laminin α5, which is a widely expressed chain found in many embryonic and adult basement membranes.

We have characterized in detail three infantile nephrotic syndrome patients with biallelic LAMA5 truncating variants and one SRNS case with a LAMA5 missense variant. These patients provided clear evidence for the first time that biallelic truncating variants in LAMA5 can cause early-onset nephrotic syndrome.
Materials and methods

Study participants
Following informed consent, we obtained clinical data and blood samples from individuals with severe proteinuria in Japan. Study approval was obtained from the Institutional Review Board of Kobe University Graduate School of Medicine (IRB approval number 301). Patients were enrolled between January 2016 and January 2021. We performed targeted next generation sequencing in 407 families.

Genetic analysis
Genomic DNA was extracted from peripheral blood using the Quick Gene Mini 80 system (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and used for targeted sequencing and Sanger sequencing. Targeted sequencing using next-generation sequencing (NGS) was conducted for genes responsible for inherited renal disease (listed in Supplementary Table 1). The sample library for NGS analysis was prepared using SureSelect XT2 custom capture library 0.5-2.9Mb (Agilent Technologies, Santa Clara, CA, USA), in accordance with the manufacturer’s workflow. Briefly, 150 ng of genomic DNA was used for a restricted reaction and hybridized using SureSelect XT Low Input reagents. All indexed DNA samples were amplified by polymerase chain reaction and sequenced using the Miseq platform (Illumina, San Diego, CA, USA). The results were analyzed using SureCall 3.0 (Agilent Technologies).

Transcript analysis
Total RNA was isolated from urine-derived cells using RNeasy Mini Kit (QIAGEN Hilden, Germany). Urine-derived cells were cultured in accordance with a previously reported protocol. Total RNA was reverse-transcribed into cDNA using Ecodry Premix (Double Primed) (Takara Bio Inc., Kusatsu, Japan), and PCR was performed. The primers used are shown in Supplementary Table 2.

**Immunofluorescence analysis of laminin α5**

Double immunostaining for laminin α5 and laminin β2 or type IV collagen α5 chain were performed using frozen kidney tissues. Each sample was fixed with acetone for 10 min, followed by heat-induced epitope retrieval. After blocking with 10% goat serum (Vector Laboratories, Burlingame, CA, USA), samples were incubated with primary monoclonal antibodies (mAbs) against the laminin α5 chain (AMAb91124) (Atlas Antibodies AB, Stockholm, Sweden), laminin β2 chain (sc-20777, H-300) (Santa Cruz Biotechnology, Dallas, CA, USA) or type IV collagen α5 chain (H53) (Shigei Medical Research Institute, Okayama, Japan) overnight. Bound mAbs were detected using specific secondary antibodies, Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-rat IgG, for 90 min. Finally, samples were incubated with DAPI for 5 min to stain nuclei.

**Construction of Expression Vectors**

Expression vectors for human laminin α5 (LMα5-pcDNA3.1), β1 (LMβ1-pCEP4), and FLAG-tagged γ1 (LMγ1FLAG-pcDNA3.1) were prepared as described previously. An expression vector for a human laminin α5 chain mutant with mouse immunoglobulin κ-chain (Ig-κ) leader sequence and 10×His-tag was prepared as followed. A cDNA encoding the Ig-κ leader sequence and 10×His-tag sequence
(LMα5His-pcDNA3.1) were prepared by extension PCR using LMα5-pcDNA3.1 as a template. The primers used are shown in Supplementary Table 3.

The resultant cDNA fragment was inserted into the HindIII and SacII sites of LMα5-pcDNA3.1 using GeneArt™ Seamless Cloning and Assembly Kit (Thermo SCIENTIFIC). Expression vectors for two human LAMA5 truncation mutants, Arg2720* and Arg3078*, were prepared by extension PCR using a LMα5His-pcDNA3.1 as a template. Resultant cDNA fragments were inserted into the BbrCI and AscI sites of LMα5His-pcDNA3.1 using GeneArt™ Seamless Cloning and Assembly Kit. The DNA sequence of the resulting vectors were confirmed using an ABI PRISM 3130 Genetic Analyzer.

**In Vitro Secretion of wild-type LM511 and its Chain-termination Mutants**

Wild-type LM511 and its mutants were transiently expressed in the FreeStyle™ 293 Expression System (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, 293-F cells were simultaneously transfected with expression vectors for laminin α5His, β1, and γ1FLAG, and grown for 3 days. To inhibit the proteolytic cleavage between laminin globular (LG) domain 3 and 4, 200 µg/mL heparin was added to the culture medium at 4 hours after transfection. At 72-75 hours after transfection, the cells were collected and washed with D-PBS without divalent cations. Cell pellets were lysed with lysis buffer (50 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1%(w/v) sodium dodecyl sulfate, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton™ X-100, protease inhibitor cocktail (FUJIFILM Wako Pure Chemical Corporation, Osaka Japan). The conditioned media and cell lysates were clarified by centrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (SDS-PAGE) under reducing conditions, followed by immunoblotting with mAbs against horseradish peroxidase (HRP)-conjugated PentaHis (for laminin α5 chain, QIAGEN). Bound antibodies were visualized using ECL Western Blotting Detection Reagents (Cytiva). Band densities were quantified using image J packaged in Fiji.

To confirm the heterotrimerization of LMα5 chain-termination mutants in LM511, the conditioned media were incubated with anti-human laminin α5 mAb 5D6 and Protein G Sepharose 4 Fast Flow (Cytiva, Tokyo, Japan) or anti-FLAG M2 agarose (Sigma) overnight at 4°C. After centrifugation, resultant immunoprecipitates were separated by SDS-PAGE under reducing conditions, followed by immunoblotting with mAbs HRP-conjugated PentaHis (for laminin α5), DG10 (for laminin β1), C12SW and anti-FLAG mAb M2 (for laminin γ1). After reaction with HRP-conjugated donkey anti-mouse IgG pAb (Jackson ImmunoResearch, PA, USA), the antigen-antibody complexes were visualized using ECL Western Blotting Detection Reagents (Cytiva).
**Results**

**Patients**

A total of 407 unrelated patients (216 men/boys and 191 women/girls) were included for genetic analysis by targeted sequencing using NGS. *LAMA5* (NM_005560.4) gene variants were detected in four patients in three families. The genetic and concise clinical information for patients 1 and 2 were already published in our previous report\(^\text{15}\), but no precise clinical and pathological findings were described. Table 1 summarizes the characteristics of our four patients and the seven patients from previous reports with *LAMA5* variants.

Patient 1 was a 13-year-old boy. At 3 months of age, his edema was detected by chance when he had a fever. Laboratory findings were as follows: serum total protein, 3.9 g/dl; serum albumin, 1.8 g/dl; serum Cr, 0.55 mg/dl and cholesterol, 940 mg/dl. Urinalysis showed heavy proteinuria. He was diagnosed with nephrotic syndrome. As his renal function was gradually deteriorating, peritoneal dialysis was started at the age of one-year. We conducted genetic testing using targeted sequencing of the genes known to be responsible for inherited kidney disease and detected compound heterozygous variants in *LAMA5*. The variants include a heterozygous G to A substitution at base position 1282+1 in intron 9 (IVS9+1G>A) and a heterozygous C to T substitution at base position 9232 in exon 68, which replaces the amino acid arginine with a stop codon at codon 3078 (p.Arg3078*) (Fig 1A, 1B). IVS9+1G>A led to the entire exon 9 (91bp) being skipped at the transcript level (Fig 1C), meaning that this is also a truncating variant. The patient’s parents each carried a different variant in heterozygous form.
The patient’s mother was phenotypically healthy and did not have proteinuria. The patient’s father had hypoplastic kidney, but he did not have proteinuria and did not reach ESKD.

Patient 2 was a 5-year-old girl. She is patient 1’s younger sister. At 4 months of age, edema and hypoalbuminemia were detected. Urinalysis showed heavy proteinuria, and she was diagnosed with nephrotic syndrome. She did not have any extra-renal abnormalities. Peritoneal dialysis was started at the age of three and half months. She carried the same variants as Patient 1.

Patient 3 was a 6-year-old girl in whom hypoalbuminemia was detected by chance when she had been infected with the virus at 6 months of age. She had congenital cataract and hypoplastic kidney. There were no family history of kidney diseases. She was diagnosed with infantile nephrotic syndrome and started steroid therapy. However, she did not respond to immunosuppressive therapy. At 11 months of age, she underwent a renal biopsy. Pathologically, light microscopy demonstrated diffuse mesangial sclerosis, and electron microscopy demonstrated thinning and irregular structure of the GBM (Fig 1D). She reached end stage kidney disease at three years and three months of age. We conducted targeted sequencing analysis using NGS and found compound heterozygous variants in LAMA5. The variants include a heterozygous G to A substitution at base position 1282+1 in intron 9 (the same as Patients 1 and 2), and a heterozygous C to T substitution at base position 8158 in exon 60, which replaces the amino acid arginine with a stop codon at codon 2720 (p.Arg2720*) (Fig 1E, 1F). We conducted immunostaining analysis of laminin α5. In the control, laminin α5 showed the characteristic linear GBM pattern; however, Patient 3 showed weakly positive staining for laminin α5, although laminin β2 and collagen α5 (IV) showed normal patterns (Fig 2A, 2B). This suggests that
mutant laminin α5 could form laminin-521 heterotrimers, though levels in the GBM appear reduced.

Patient 4 was a 9-year-old girl. At 8 years of age, she was referred to the hospital because of proteinuria, which had been identified in a school-based urine screening program. Urinalysis showed heavy proteinuria. She did not have any extra-renal abnormalities. She was diagnosed with nephrotic syndrome and started steroid therapy. She underwent a renal biopsy because of SRNS. Pathologically, light microscopy demonstrated focal segmental glomerulosclerosis cellular variant, and electron microscopy demonstrated thinning and irregular structure of the GBM (Fig 3A). We conducted targeted sequencing analysis using NGS. She had compound heterozygous missense variants in LAMA5. The variants include a heterozygous C to T substitution at base position 1493 in exon 12, which replaces the amino acid alanine with valine at codon 498 (p.Ala498Val) and a heterozygous G to A substitution at base position 8399 in exon 62, which replaces the amino acid arginine with histidine at codon 2800 (p.Arg2800His) (Fig 3B, 3C). We conducted immunostaining analysis of laminin α5. In the control, laminin α5 shows a characteristic linear GBM pattern; Patient 4 showed the same degree of expression of laminin α5 as control (Fig 3D, 3E). The patient’s parents each carried a different variant in heterozygous form. They were phenotypically healthy and did not have proteinuria.

**In vitro analysis**

To investigate the potential pathogenicity of the two nonsense variants, LMα5-Arg2720* and -Arg3078* (Fig 4A), we assayed their synthesis and secretion in the context of the LM-511 trimer. We expressed either wild-type or mutant tagged human
LMα5 along with tagged LMβ1 and γ1 to produce either wild-type or mutant LM-511 trimers by cotransfecting 293-F cells with three expression vectors:
LMα5His-pcDNA3.1, LMβ1- pCEP4, and LMγ1FLAG-pcDNA3.1. Conditioned media were separated by SDS-PAGE, followed by immunoblotting using the mAb against the 5xHis tag at the amino-terminus of LMα5. This analysis showed that both truncating mutants, LMα5-Arg2720* and -Arg3078*, exhibited a decreased trimer secretion level by about 40% and 30%, respectively, compared with wild-type (Fig. 4B and C).
Consistent with these results, the signal intensities in cell lysates were increased by 4-5 fold for the mutants compared with wild-type (Fig. 4B and C).

To further explore whether secreted LMα5-Arg2720* and -Arg3078* mutants can trimerize with laminin β1 and γ1, we performed immunoprecipitation using mAbs against the human laminin α5 chain (5D6) and the FLAG tag at the amino-terminus of the laminin γ1 chain. Immunoblotting analyses showed that laminin β1 and γ1 were detected in mAb 5D6 immunoprecipitates (Fig. 4D left panel). When the γ1 chain was immunoprecipitated with anti-FLAG mAb, laminin α5 and β1 chains were detected in the precipitates (Fig. 4D right panel). These results indicate that both α5-Arg2720* and -Arg3078* mutants are able to assemble with laminin β1 and γ1 to yield a trimer, but compared with wild-type, secretion efficiencies of the mutant LM-511s were decreased by an unidentified intracellular quality control mechanism. This is in good agreement with our in vivo findings that the level of laminin α5 was decreased in glomeruli of Patient 3.
Discussion

This paper presents three infantile nephrotic syndrome patients with \textit{LAMA5} gene biallelic truncating variants and one SRNS case with \textit{LAMA5} biallelic missense variants. For our patients, we performed in vitro heterotrimer formation assays and demonstrated defects in the function of truncating variants. This is the first report to show clear evidence that \textit{LAMA5} pathogenic variants can lead to nephrotic syndrome in the absence of major developmental anomalies. Our patients suggest the need for awareness of \textit{LAMA5} gene screening in patients with early-onset proteinuria.

Three of our patients (Patients 1, 2 and 3) possess biallelic truncating variants. One was a nonsense variant, and the other was a consensus donor splice site variant that led to skipping of exon 9 (91bp) and a frameshift. Therefore, both should be considered disease causing pathogenic variants. Although one of our patients (Patient 4) had only missense variants, pathologically and clinically these variants should also be considered disease causing. Until now, several pathogenic and likely pathogenic variants, including splice sites and frameshifts, have been reported in Clinvar database. Limited to variants for which phenotypic information was available, only seven proteinuric patients with biallelic \textit{LAMA5} variants were reported, and all of them were missense variants. The first case was an adult female with proteinuria beginning at age 27, as reported by Chatterjee et al \cite{2}. Recently, using whole exome sequencing, homozygous missense variants in \textit{LAMA5} were identified in five pediatric nephrotic syndrome patients in three families \cite{3}. All five patients had nephrotic syndrome with onset before 4 years of age. One of them did not respond to immunosuppressive treatment and reached ESKD at age 6, but on the other hand, other patients responded to immunosuppressive treatment. This indicates significant phenotypic heterogeneity in this disease, which resembles somewhat the
situation for \textit{LAMB2} gene variants\textsuperscript{16}.

Our three patients with truncations (Patients 1, 2 and 3) showed more severe phenotypes than the patients with missense variants, but not lethality. \textit{Lama5} null mice die at late fetal stages with multiple development defects\textsuperscript{17}, and variant of \textit{Lama5} specifically in podocytes causes nephrotic syndrome\textsuperscript{8}. One reported homozygous variant in human \textit{LAMA5} (Arg286Leu) that impairs laminin polymer formation causes a complex syndromic disorder in addition to FSGS and ESKD\textsuperscript{4}, and the overall features (including syndactyly) are consistent with the phenotypes of the \textit{Lama5} null mouse. That Patients 1, 2 and 3 with biallelic truncating variants survived the fetal period and manifested either no or mild extra-renal symptoms suggests that the presence of truncated LM\textsubscript{α5} proteins maintains significant LM-521 functions. Our in vitro biochemical analysis using recombinant truncated proteins explained this phenotype.

Laminins are a group of cross-shaped heterotrimeric proteins, each consisting of α, β and γ subunits joined together through a coiled coil domain\textsuperscript{16}. Three mutants we characterized were able to form trimers because the coiled-coil domain was preserved in each truncating variant. Based on the results of our in vitro trimer formation assay, mutant LM-521 trimers should form, although the laminin α5 proteins would be truncated due to the nonsense variants. Although secretion of the mutant trimers into Patient 3’s GBM was observed, the level was low. This weak accumulation of incomplete laminin α5 likely saved the lives of our patients.

Renal pathological findings showed irregular architecture of the GBM with similarity to Alport or Pierson syndrome in Patients 3 and 4. These were the novel findings of \textit{LAMA5}-related nephrosis. Variant of the mouse \textit{Lama5} gene results in varying degrees of proteinuria and rates of progression to nephrotic syndrome\textsuperscript{8}. The GBM of proteinuric
mice appeared thickened with a moth-eaten appearance. These mouse pathological findings were in concordance with our patients. We also performed immunofluorescence analysis that showed weak deposition of laminin α5, but it was not negative in Patient 3. This result was somewhat surprising to us, because we did not expect biallelic truncating variants to produce laminin α5 able to form laminin-521 trimers. But our in vitro trimer formation assay clearly proved that the two truncated laminin α5 variants can trimerize. Patient 4 showed a laminin α5 expression level similar to the control. This result is in concordance with the clinical course of preserved renal function in patient 4. In limitation, we did not quantify the fluorescent intensity.

The age of onset in Patients 1-3 was under one year. In congenital and infantile nephrotic syndrome patients, monogenetic disease is typically suspected. In the PodoNet study, genetic disease was identified in 23.6% of patients with SRNS; the most commonly mutated genes were NPHS2, WT1, and NPHS1. In Japan, genetic components were identified in 30% of proteinuric patients; the most commonly mutated genes were WT1, NPHS1 and INF2. However, comprehensive genetic testing was performed before the publication of a report of pathogenic homozygous LAMA5 variants in pediatric nephrotic syndrome in 2019. Among the patients diagnosed as congenital nephrotic syndrome or infantile nephrotic syndrome for which the causative gene has not been identified, LAMA5 biallelic truncated variants, such as in three of our four patients, may be the hidden cause. It is therefore necessary to consider LAMA5 as one of the causative genes of congenital/infantile nephrotic syndrome.

We have reported three patients of infantile nephrotic syndrome with LAMA5 biallelic truncating variants and one SRNS case with LAMA5 missense variants. Our patients showed clear evidence that LAMA5 biallelic variants can cause SRNS. We think it is
likely that nephrotic syndrome patients with these types of \textit{LAMA5} variants are so rare because only specific types of \textit{LAMA5} variants are compatible with life.

\textbf{Disclosures:}


**Funding:**

This study was supported by Grants-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (subject ID: 18K15713 to China Nagano). This work was performed in part under the Collaborative Research Program of Institute for Protein Research, Osaka University, CR-21-06. J.H.M. was supported by grants from the National Institutes of Health (R01DK078314 and R01DK058366).

**Author Contributions:**

Supplemental Material:

Supplementary Table 1: Genes targeted by next-generation sequencing analysis
Supplementary Table 2: The primers for PCR
Supplementary Table 3: The primer sets for extension PCR
Supplementary Figure 1: Direct sequencing for transcript analysis
References


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25
Figure Legends

Fig. 1. Genetic and pathological analyses of Patients 1, 2 and 3 reveal variants in *LAMA5* and GBM defects in Patient 3.

A, B  *LAMA5* gene sequencing analysis of Patients 1 and 2 and their parents. A splice site variant (c.1282+1G>A) and a nonsense variant (c.9232C>T) were detected in Patients 1 and 2 (the sister). The nonsense variant is linked to a nearby variant (c.9235C>T). Each parent carried a different one of these variants in heterozygous form. Red arrows indicate the heterozygous variants in the chromatograms.

C  RT-PCR analysis of *LAMA5* RNA from Patient 2’s urinary cells using primers in exons flanking the c.1282+1G>A variant reveals a full length and a shorter amplicon. Sanger sequencing (Supplementary Fig. 1) indicates that the shorter product lacks exon 9, indicating exon skipping resulting in a frameshift.

D  Electron microscopic analysis of Patient 3’s kidney biopsy reveals irregular appearance of the GBM (arrowheads) and podocyte foot process effacement.

E, F  Genetic analysis of Patient 3 and her mother. The same splice site variant found in Patients 1 and 2 (c.1282+1G>A) and a novel nonsense variant (c.8185C>T) were detected in Patient 3. The mother carried the nonsense variant.

Fig. 2. Immunofluorescence analysis reveals reduced levels of laminin α5 in the GBM of Patient 3.

A  Immunostaining for laminin α5 and laminin β2 in control and Patient 3 shows the characteristic linear GBM patterns in the control, but Patient 3 showed a very weak linear pattern for laminin α5.
B  Immunostaining for laminin α5 and collagen IV α5 in control and Patient 3 shows the characteristic linear GBM patterns in the control, but Patient 3 showed a very weak linear pattern for laminin α5.

**Fig. 3. Genetic and pathological analyses of Patient 4 and her parents reveals**

*LAMA5* **missense variants and a GBM defect.**

A  Electron microscopy shows an irregular appearance of the GBM.

B, C  Genetic analysis of *LAMA5* reveals compound heterozygous missense variants c.1493C>T (p.Ala498Val) and c.8399G>A (p.Arg2800His). Each parent carried a different one of these variants in heterozygous form. Red arrows indicate the heterozygous variants in the chromatograms.

D  Immunofluorescence analysis reveals normal levels of laminin α5 in the GBM of Patient 4. Immunostaining for laminin α5 and laminin β2 in control and Patient 4 shows the characteristic strong, linear GBM patterns.

E  Immunostaining for laminin α5 and collagen IV α5 in control and Patient 4 shows the characteristic strong, linear GBM patterns.

**Fig. 4. Secretion efficiencies of wild-type LM-511 and its laminin α5 chain-termination mutants.**

A  Domain structure of the laminin α5 chain. The sites of truncation in Patients 1 and 2 (R3078) and in Patient 3 (R2720) are positioned in laminin globular (LG) domain 2 and in the carboxyl-terminal region of the laminin coiled-coil (LCC) domain, respectively.
(B) The amount of wild-type and mutant LM-511 expressed in and secreted from cells. Equal amounts of conditioned media and cell lysates were applied to SDS-PAGE under reducing conditions. Laminin $\alpha_5$ chains are detected with HRP-conjugated anti-5×His tag mAb *(upper panel)*. Proteins blotted on polyvinylidene difluoride membranes were visualized with Coomassie brilliant blue (CBB) and used as loading controls *(lower panel)*.

(C) Band densities in B were quantified using ImageJ software. Data was expressed as average ± standard deviation.

(D) $\alpha_5$-R2720* and -R3078* mutants heterotrimerize with laminin $\beta_1$ and $\gamma_1$. 6×His tagged laminin $\alpha_5$ and the two chain-termination mutants were coexpressed with laminin $\beta_1$ and FLAG tagged $\gamma_1$ chains. Conditioned media were immunoprecipitated with anti-laminin $\alpha_5$ mAb *(left panel)* or anti-FLAG M2 mAb agarose *(right panel)*. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions, followed by immunoblotting with mAbs against 5×His tag, $\beta_1$, and $\gamma_1$. Conditioned media were used as input samples.
**Fig. 1**

**A**
c.1282+1G>A

**Patient 1**

**Mother**

**Father**

**Patient 2**

**B**
c.9232C>T

**Patient 1**

**Mother**

**Father**

**Patient 2**

**C**
Co Pt 2

**91bp**

**D**
Patient 3

**E**
c.1282+1G>A

**Patient 3**

**Mother**

**F**
c.8158C>T

**Patient 3**

**Mother**
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**Fig. 2B**

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Fig. 3

A

Patient 4

B

c.1493C>T  

Control  

Patient 4  

Mother  

Father

C

c.8399G>A

Control  

Patient 4  

Mother  

Father

D

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Supplementary data

Supplementary Table 1: Genes targeted by next-generation sequencing analysis

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121 | GON7 | Galloway-Mowat |
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123 | OSGEP | Galloway-Mowat |
124 | TPRKB | Galloway-Mowat |
125 | TP53RK | Galloway-Mowat |
126 | NUP133 | Galloway-Mowat |
127 | WDR4 | Galloway-Mowat |
128 | WDR73 | Galloway-Mowat |
### Supplementary Table 2: The primers for PCR

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### Supplementary Table 3: The primer sets for extension PCR

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Supplementary Figure 1
Direct sequencing for transcript analysis

① The single band shown in the control (Co) and the upper band shown in patient (Pt) corresponded to exon 8, exon 9 and 10; the splicing pattern was normal.

② The lower band shown in Pt corresponded to exon 8 and exon 10; exon 9 skipping occurred.
Supplementary data

Supplementary Table 1: Genes targeted by next-generation sequencing analysis

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