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Original article

Molecular mechanisms determining severity in patients with Pierson syndrome

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54

55 **Abstract**

56 Null variants in *LAMB2* cause Pierson syndrome (PS), a severe congenital nephrotic
57 syndrome with ocular and neurological defects. Patients' kidney specimens show
58 complete negativity for laminin β 2 expression on glomerular basement membrane
59 (GBM). In contrast, missense variants outside the laminin N-terminal (LN) domain in
60 *LAMB2* lead to milder phenotypes. However, we experienced cases not showing these
61 typical genotype–phenotype correlations. In this paper, we report six PS patients: four
62 with mild phenotypes and two with severe phenotypes. We conducted molecular studies
63 including protein expression and transcript analyses. The results revealed that three of
64 the four cases with milder phenotypes had missense variants located outside the LN
65 domain and one of the two severe PS cases had a homozygous missense variant located
66 in the LN domain; these variant positions could explain their phenotypes. However, one
67 mild case possessed a splicing site variant (c.3797+5G>A) that should be associated
68 with a severe phenotype. Upon transcript analysis, this variant generated some
69 differently sized transcripts, including completely normal transcript, which could have
70 conferred the milder phenotype. In one severe case, we detected the single-nucleotide
71 substitution of c.4616G>A located outside the LN domain, which should be associated
72 with a milder phenotype. However, we detected aberrant splicing caused by the creation
73 of a novel splice site by this single-base substitution. These are novel mechanisms
74 leading to an atypical genotype–phenotype correlation. In addition, all four cases with
75 milder phenotypes showed laminin β 2 expression on GBM. We identified novel
76 mechanisms leading to atypical genotype–phenotype correlation in PS.

77

78 **Introduction**

79 Pierson syndrome (PS; OMIM #609049) is typically characterized as a congenital
80 nephrotic syndrome progressing to early-onset end-stage renal disease (ESRD), usually
81 in the first year of life, ocular abnormalities such as microcoria, and retardation of
82 psychomotor development ¹⁻⁴. It is a rare autosomal recessive disease, the incidence of
83 which remains unknown, despite PS being a major cause of congenital nephrotic
84 syndrome or steroid-resistant nephrotic syndrome (SRNS) ^{5,6}. In 1963, some cases with
85 these symptoms were reported by Pierson et al. ⁷, and in 2004 PS was described as
86 being caused by mutations in the *LAMB2* gene encoding laminin $\beta 2$ ³.

87 Laminins are cross-shaped heterotrimeric glycoproteins composed of α , β , and γ chains,
88 which are the major constituents of basement membranes together with type 4 collagens,
89 nidogens, and sulfated proteoglycans ^{8,9}. Laminin trimerization occurs in the
90 endoplasmic reticulum, and the heterotrimers polymerize with each other through the
91 laminin N-terminal (LN) domain of each laminin chain, and then laminin plays
92 important roles in cell adhesion, migration, and differentiation ⁸. Laminin-521
93 composed of $\alpha 5$, $\beta 2$, and $\gamma 1$ chains is a major laminin heterotrimer, which is contained
94 in the glomerular basement membrane (GBM), basal lamina of intraocular muscles, and
95 the synaptic basement membrane of neuromuscular junctions ⁹.

96 Although cases with *LAMB2* variants typically show severe phenotypes as mentioned
97 above, recently some cases showing milder phenotypes with *LAMB2* variants have been
98 reported ¹⁰⁻¹⁵. Matejas et al. systematically investigated the genotype–phenotype
99 correlations in 51 previously reported cases ¹⁶. They found that PS patients possessing
100 pathogenic missense variants on at least one allele tend to show milder phenotypes of
101 later-onset nephrotic syndrome and development of ESRD than patients possessing

truncating mutations on both alleles. As an exception, cases with *LAMB2* missense variants located in the region encoding the LN domain displayed severe phenotypes because the LN domain is an important site for laminin polymerization and protein processing; these variants thus led to impaired laminin $\beta 2$ ^{17, 18}. These reports provided robust evidence for the existence of genotype–phenotype correlation in PS. However, some cases not consistent with this correlation have also been reported. For example, Choi et al. reported patients with mild phenotypes having truncating mutations in both alleles¹⁰, and Hasselbacher et al. described a severe PS patient with a missense variant located outside of the LN domain in both alleles¹⁷. It is still unknown why these patients showed such irregular phenotypes.

To shed light on this issue, we here report six cases, namely, four with milder phenotypes (including two siblings) without end-stage renal failure until 1 year old, ocular abnormalities, or psychomotor retardations, and two with severe phenotypes with congenital nephrotic syndrome leading to end-stage renal failure early in life (within 1 year after birth). All *LAMB2* variants were detected by targeted sequencing of podocyte-related genes. The phenotypes in four of the patients were explained by their genotypes in accordance with previous reports, but two cases showed atypical phenotypes. Therefore, we investigated the molecular mechanisms determining the severity of their conditions.

Material and Methods

Ethical considerations

All procedures were reviewed and approved by the Institutional Review Board of Kobe University School of Medicine. Informed consent was obtained from all patients or their parents.

Patients

In total, 140 cases with congenital nephrotic syndrome/infantile nephrotic syndrome/steroid-resistant nephrotic syndrome were screened by targeted sequencing. Among them, seven cases (including two siblings) had compound heterozygous or homozygous pathogenic variants in the *LAMB2* gene and were diagnosed with PS. In this study, we report six of them, as one was excluded due to a refusal to consent to publication of the details of that case. Cases 1–4 showed mild phenotypes for PS, as presented below and in Table 1. Renal biopsies were conducted in cases 1–5, and electron microscopy (EM) findings are shown in Table 1 and Supplemental Fig. 1.

Targeted sequencing

Comprehensive analysis was conducted for 45 podocyte-related genes (Supplementary Table 1), including genes causative of congenital nephrotic syndrome, infantile nephrotic syndrome, and SRNS, using next-generation sequencing (NGS) and Miseq (Illumina, San Diego, CA) for all patients. Genomic DNA was extracted from peripheral blood leukocytes from the patients using the Quick Gene Mini 80 system (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). The sample library for NGS analysis was prepared using HaloPlex Target Enrichment kit 500 kb for Illumina (Agilent Technologies, Santa Clara, CA, USA), in accordance with the manufacturer's workflow. Briefly, 225 ng of genomic DNA was used for a restricted reaction, and

hybridized at 54°C for 16 h with next-generation sequencing probes. All indexed DNA samples were amplified by polymerase chain reaction and sequenced using the Miseq platform. The results were analyzed using SureCall 3.0 (Agilent Technologies).

Sanger sequencing

The *LAMB2* gene variants detected by targeted sequencing were confirmed by Sanger sequencing. Mutational sites of *LAMB2* in each patient were amplified by PCR. PCR-amplified products were purified using a MiniElute PCR Purification kit (QIAGEN Inc., Chatsworth, CA, USA) and subjected to direct sequencing using a dye terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) and an automatic DNA sequencer (ABI Prism 3130; PerkinElmer Applied Biosystems, Forester City, CA). For variant descriptions, NM_002292 was used as a reference sequence of *LAMB2*.

RNA isolation from blood leukocytes and urine-derived cells

Total RNA was isolated from blood leukocytes using an RNA stabilization agent (RNAlater; Invitrogen, Carlsbad, CA, USA) and a RiboPure Blood Kit (Invitrogen), while for urine-derived cells RNeasy Mini Kit (QIAGEN) was used. Urine-derived cells were cultured in accordance with a previously reported protocol¹⁹. Briefly, the urine was centrifuged at 1400 rpm and the sediment was washed with phosphate-buffered saline. After culturing the washed sediment on a gelatin-coated dish (Iwaki; Asahi Techno Glass Co., Tokyo, Japan) using the optimal medium (CC-3125, EGMTM-MV: Microvascular Endothelial Cell Growth Medium BulletKitTM; Lonza, Walkersville, MD, USA) with penicillin (100 U/ml) and streptomycin (1 mg/ml) for approximately 1–3

weeks, urine-derived cells were obtained. These included many renal progenitor cells having the ability to differentiate into tubules and podocytes.

Transcript analysis

Total RNA was reverse-transcribed into cDNA using Ecody Premix (double-primed; Clontech Laboratories, Inc., Mountain View, CA, USA), and PCR was performed using the following primer pairs of *LAMB2* in case 3: forward primer: 5'-CCACCTGCAACGAGTTCACAG-3', and reverse: 5'-GCTGTCATAGGCACCCAGGAA-3'. Furthermore, to detect more transcript products, we performed nested PCR using other pairs of *LAMB2* primer: first forward primer: 5'-GAGCTGTCACCGCTGCACAT-3', and reverse: 5'-GCCCCACACACCAGCTCATT-3', and second forward primer: 5'-CTGCAAGTTGATGGCTTGAA-3', and reverse: 5'-TGCCTTCCAATTCCTGTAGC-3'. After 40 cycles of amplification, PCR products were subcloned into pT7 vector (Novagen, Inc., Madison, WI) and the inserted products were sequenced using a dye terminator cycle sequencing kit and an automatic DNA sequencer as mentioned above. In case 6, the following primer pairs were used: forward primer: 5'-TGGACAAGGCTAATGCTTCC-3', and reverse: 5'-CTATTTCTGCCCCGTTTCAA-3'. Sequencing was performed in the same way as described above.

Immunohistochemical analyses of laminin $\beta 2$

Double staining for laminin $\beta 2$ and type 4 collagen $\alpha 5$ chain was performed using frozen kidney tissue. Each sample was fixed with acetone for 10 min, blocked with 10%

goat serum (Vector Laboratories, Burlingame, CA, USA), and incubated with primary monoclonal antibody of laminin $\beta 2$ chain (sc-20777, H-300) (Santa Cruz Biotechnology, Dallas, CA, USA) and type 4 collagen $\alpha 5$ chain (H53) (Shigei Medical Research Institute, Okayama, Japan) overnight. All samples bound with each primary antibody were detected using specific secondary antibodies, namely, Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-rat IgG, for 90 min. Finally, nuclei of each sample were stained with DAPI for 5 min. All samples were washed with PBS three times between each procedure.

Results

Genetic examination

Targeted sequencing by NGS and Sanger sequencing revealed that all six cases had homozygous or compound heterozygous variants in *LAMB2* (Fig. 1, Table 1); no other pathogenic variants were detected in other genes in all cases. Cases 1–2 had a known missense variant located outside of the LN domain²⁰ and a novel deletion. In case 3, a novel splice site variant and duplication were found. Cases 4 and 5 had novel homozygous variants. The variant in case 5 was located in the LN domain, while that in case 4 was outside of it, which were both predicted to be disease-causing by three different software programs (PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>; SIFT, <http://sift.jcvi.org/>; and Mutation Taster, <http://www.mutationtaster.org/>). Case 6 had a novel deletion and a novel pathogenic missense variant.

Transcript analysis

The phenotypes in Cases 3 and 6 were atypical, in that they did not match the previously reported genotype–phenotype correlation. Therefore, we conducted transcript analyses for these cases.

To study the pathogenicity of splice variant c.3797+5G>A in Case 3, we performed transcript analysis on both leukocytes and urine-derived cells. Agarose gel electrophoresis of cDNA extracted from these cells revealed one larger and two smaller transcripts in addition to the normal-sized band (exons 24A–C in Fig. 2). Exon 24A included a 96-bp insertion, featuring the whole of intron 24. Exons 24B and 24C included 133- and 319-bp deletions due to the generation of new splicing donor sites (Fig. 2, Supplemental Fig. 2). These three transcripts included a premature stop codon (Supplementary Fig. 2). The splicing pattern was also completely identical in leukocytes and urine-derived cells.

Although these three transcripts and the other variant (c.5073_5076dupCCAG) all showed truncation, the phenotypes in case 3 were milder than those in typical PS. Therefore, we hypothesized that the splice variant c.3797+5G>A produced not only abnormal transcripts but also a wild-type transcript. Although the wild-type transcript in Figure 2 (second panel from the top in Fig. 2) was generally thought to be generated by an allele containing another variant c.5073_5076dupCCAG, we demonstrated that this normal transcript is also synthesized from an allele containing c.3797+5G>A. To investigate the normally spliced transcript production by c.3797+5G>A in intron 24, we conducted nested RT-PCR for the broader area spanning from exon 21 to exon 32 (Fig. 3). Although the proportion of wild-type transcripts isolated by the subcloning method was small, we found a completely normal transcript with insertion of the whole of exon 24 and without 4-bp CCAG duplication in exon 30, which means that the splicing

variant of c.3797+5G>A also created the completely normal transcript, in addition to the three abnormal transcripts. The small amount of completely normal transcript led to Case 3 having an atypically milder phenotype.

Upon in silico analysis using splice prediction software (Human Splicing Finder; <http://www.umd.be/HSF3/>), the consensus value scores of these three aberrant donor sites were 92.0, 82.96, and 83.0, respectively. Although the score of the original donor site decreased from 81.59 to 69.43, the mutant score was still high and showed only a small decrease (Supplemental Table 2). The interpretation of this result is described in the discussion section.

We focused on the pathogenicity of the missense variant in case 6 because this case showed a severe clinical course despite this variant being outside of the LN domain. The splice prediction software predicted the creation of a new splicing acceptor site by this missense variant. Therefore, we also conducted transcript analysis for this case. We detected a new transcript that led to a 44-bp deletion in exon 28 (Fig. 4, Supplemental Fig. 3), and it was revealed that case 6 had a compound heterozygous truncating mutation.

Laminin β 2 expression

To investigate the mechanism behind our cases exhibiting a variety of phenotypes, we conducted immunostaining analysis of laminin β 2 (Fig. 5). Compared with the findings in the control, immunohistochemical analysis of laminin β 2 indicated a characteristic linear expression pattern on GBM equivalent to that for type 4 collagen α 5 in cases 1–4. Conversely, case 5 showed completely negative expression of laminin β 2. Although Case 4 showed a severer phenotype than Cases 1–3, the laminin β 2 expression level was

almost the same in these four cases. These findings may have been due to this immunohistochemical analysis having insufficient sensitivity to differentiate these severity levels.

Discussion

PS shows various phenotypes, which are related to the *LAMB2* genotype in most cases. However, in some patients, this correlation does not hold. Here, we have presented a series of six PS cases, including four with mild phenotypes and two with severe ones. Cases 4 and 6 showed clear deviation from the previously reported genotype–phenotype correlation, although the phenotypes of the other four cases matched the genotypes in line with previous reports. Therefore, we conducted transcript analysis and immunostaining of laminin $\beta 2$ and revealed a new mechanism determining the phenotypes of these two cases.

Case 3 showed an extremely mild phenotype for PS, with no nephrotic syndrome, eye anomaly, or mental retardation. This was despite having a 4-bp duplication (c.5073_5076dupCCAG) leading to frameshift and a splice site mutation (c.3797+5G>A). Transcript analysis using cDNA isolated from leukocytes and urine-derived cells revealed that the splicing variant (c.3797+5G>A) actually produced four different splicing variants, including one normal transcript. In the splice prediction software algorithm, with +5 splicing variants, original splice sites are expected to be broken when splice variants reduce the consensus values of original splice sites by 10% or more and the values fall below 65²¹. If the original splice site breaks, the site with the highest consensus value is recognized as a novel splicing site. As a result, only one alternatively spliced transcript would be produced. However, in Case 3, we found four

different splicing variants, including one normal splicing pattern. This indicated that the splice variant affected authentic splice site activation, but the original splice site was not completely broken and generated a normal transcript. This was because the consensus value of the authentic site was still higher than 65 and the rate of change was only 14.9%, which was reported not to have sufficient power to disrupt the splice site²¹. Our results also indicated that three spliced transcripts were produced because the strengths of activation among the three aberrant splice sites were similar. These splicing patterns were considered to be very rare²² and these findings reveal a novel mechanism leading to a milder phenotype of PS.

Case 6 with a two-base deletion (c.4907_4908del) and a single-nucleotide substitution located outside of the LN domain (c.4616G>A) exhibited a severe phenotype typical of PS. Transcript analysis revealed that the single point mutation (c.4616G>A) was not a missense variant but a variant leading to aberrant splicing with a 44-bp deletion at the transcript level. It is well known that exonic single-base substitutions can sometimes cause the creation or activation of cryptic splice sites²³. The variants causing aberrant splicing by single-nucleotide mutation in exons have a strong impact on protein synthesis, but this is often mistaken for simple missense mutations²⁴. The use of a prediction tool is beneficial to predict aberrant splicing in these cases²¹.

In our study, immunostaining of laminin β 2 also proved to be useful for predicting the severity of the phenotypes in PS. Among five patients who underwent renal biopsy, laminin β 2 was expressed on basement membrane only in cases with milder phenotypes and is not exhibited at all in typical PS. Although typical cases of PS with truncating variants show complete deficit of laminin β 2 on the GBM, as shown in Case 5⁹, there is only one case report showing a mild phenotype with laminin β 2 expression on GBM¹³.

In the present study, all PS cases with milder phenotypes (Case 1-4) showed residual laminin $\beta 2$ on GBM, even in Case 4 who showed severer phenotype of developing ESRD at 3 years old comparing to Case 1-3. In all cases, genetic analysis clearly explained their milder phenotypes. A correlation between the clinical manifestation and the expression of protein in immunostaining was reported in Alport syndrome^{25, 26}, and immunohistochemistry was also shown to be useful for predicting the severity of phenotypes in PS.

Pathological features of the basement membrane, such as its thinness or lamellation like in Alport syndrome, were observed in all cases regardless of the severity of the symptoms. Similar findings of changes in the basement membrane have been reported in several other PS cases with milder phenotypes^{11-13, 17}. It is difficult to diagnose PS without extrarenal symptoms, so these electron microscopic findings can provide an important clue for estimating the causative gene.

NGS using a target panel enabled us to diagnose all cases. The availability of a comprehensive gene screening system is useful in atypical cases with milder phenotypes for PS. Moreover, early and correct diagnosis of inherited kidney disease is important to avoid unnecessary treatments such as the use of immunosuppressants.

In conclusion, we have reported six PS patients, including two cases with atypical phenotypes not matching the normal genotype–phenotype correlation of PS. We conducted molecular studies including protein expression analysis and transcript analysis. In the transcript analysis, we revealed novel mechanisms leading to atypical genotype–phenotype correlation in PS cases. In addition, by applying immunofluorescent analysis, we found that all cases with milder phenotypes showed laminin $\beta 2$ expression on GBM. These results can explain a variety of phenotypes in PS

and show the value of molecular analysis for revealing the mechanisms behind atypical phenotypes.

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Conflict of Interest None

353 References

- 354 1. Togawa H, Nakanishi K, Mukaiyama H, Hama T, Shima Y, Nakano M, et al.
 355 First Japanese case of Pierson syndrome with mutations in LAMB2. *Pediatr Int*.
 356 2013;55:229-31.
- 357 2. Wuhl E, Kogan J, Zurowska A, Matejas V, Vandevoorde RG, Aigner T, et al.
 358 Neurodevelopmental deficits in Pierson (microcoria-congenital nephrosis)
 359 syndrome. *Am J Med Genet A*. 2007;143:311-9.
- 360 3. Zenker M, Aigner T, Wendler O, Tralau T, Muntefering H, Fenski R, et al.
 361 Human laminin beta2 deficiency causes congenital nephrosis with mesangial
 362 sclerosis and distinct eye abnormalities. *Hum Mol Genet*. 2004;13:2625-32.
- 363 4. Zenker M, Pierson M, Jonveaux P, Reis A. Demonstration of two novel LAMB2
 364 mutations in the original Pierson syndrome family reported 42 years ago. *Am J*
 365 *Med Genet A*. 2005;138:73-4.
- 366 5. Sadowski CE, Lovric S, Ashraf S, Pabst WL, Gee HY, Kohl S, et al. A
 367 single-gene cause in 29.5% of cases of steroid-resistant nephrotic syndrome. *J*
 368 *Am Soc Nephrol*. 2015;26:1279-89.
- 369 6. Trautmann A, Bodria M, Ozaltin F, Gheisari A, Melk A, Azocar M, et al.
 370 Spectrum of steroid-resistant and congenital nephrotic syndrome in children: the
 371 PodoNet registry cohort. *Clin J Am Soc Nephrol*. 2015;10:592-600.
- 372 7. Pierson M CJ, Hervouuet F, Rauber G. An unusual congenital and familial
 373 congenital malformative combination involving the eye and kidney. *J Genet*
 374 *Hum*. 1963;12:184-213.
- 375 8. Domogatskaya A, Rodin S, Tryggvason K. Functional diversity of laminins.
 376 *Annu Rev Cell Dev Biol*. 2012;28:523-53.
- 377 9. Zenker M, Aigner T, Wendler O, Tralau T, Muntefering H, Fenski R, et al.
 378 Human laminin beta2 deficiency causes congenital nephrosis with mesangial
 379 sclerosis and distinct eye abnormalities. *Hum Mol Genet*. 2004;13:2625-32.
- 380 10. Choi HJ, Lee BH, Kang JH, Jeong HJ, Moon KC, Ha IS, et al. Variable
 381 phenotype of Pierson syndrome. *Pediatr Nephrol*. 2008;23:995-1000.
- 382 11. Falix FA, Bennebroek CA, van der Zwaag B, Lapid-Gortzak R, Florquin S,
 383 Oosterveld MJ. A novel mutation of laminin beta2 (LAMB2) in two siblings
 384 with renal failure. *Eur J Pediatr*. 2017;176:515-19.
- 385 12. Kagan M, Cohen AH, Matejas V, Vlangos C, Zenker M. A milder variant of
 386 Pierson syndrome. *Pediatr Nephrol*. 2008;23:323-7.
- 387 13. Lehnhardt A, Lama A, Amann K, Matejas V, Zenker M, Kemper MJ. Pierson
 388 syndrome in an adolescent girl with nephrotic range proteinuria but a normal
 389 GFR. *Pediatr Nephrol*. 2012;27:865-8.
- 390 14. Matejas V, Al-Gazali L, Amirlak I, Zenker M. A syndrome comprising
 391 childhood-onset glomerular kidney disease and ocular abnormalities with
 392 progressive loss of vision is caused by mutated LAMB2. *Nephrol Dial*
 393 *Transplant*. 2006;21:3283-6.
- 394 15. Mohney BG, Pulido JS, Lindor NM, Hogan MC, Consugar MB, Peters J, et al.
 395 A novel mutation of LAMB2 in a multigenerational mennonite family reveals a
 396 new phenotypic variant of Pierson syndrome. *Ophthalmology*.
 397 2011;118:1137-44.

16. Matejas V, Hinkes B, Alkandari F, Al-Gazali L, Annexstad E, Aytac MB, et al. Mutations in the human laminin beta2 (LAMB2) gene and the associated phenotypic spectrum. *Hum Mutat.* 2010;31:992-1002.
17. Hasselbacher K, Wiggins RC, Matejas V, Hinkes BG, Mucha B, Hoskins BE, et al. Recessive missense mutations in LAMB2 expand the clinical spectrum of LAMB2-associated disorders. *Kidney Int.* 2006;70:1008-12.
18. Chen YM, Kikkawa Y, Miner JH. A missense LAMB2 mutation causes congenital nephrotic syndrome by impairing laminin secretion. *J Am Soc Nephrol.* 2011;22:849-58.
19. Lazzeri E, Ronconi E, Angelotti ML, Peired A, Mazzinghi B, Becherucci F, et al. Human Urine-Derived Renal Progenitors for Personalized Modeling of Genetic Kidney Disorders. *J Am Soc Nephrol.* 2015;26:1961-74.
20. Ogino D, Hashimoto T, Hattori M, Sugawara N, Akioka Y, Tamiya G, et al. Corrigendum: Analysis of the genes responsible for steroid-resistant nephrotic syndrome and/or focal segmental glomerulosclerosis in Japanese patients by whole-exome sequencing analysis. *J Hum Genet.* 2016;61:771-2.
21. Desmet FO, Hamroun D, Lalande M, Collod-Beroud G, Claustres M, Beroud C. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res.* 2009;37:e67.
22. Shimmoto M, Fukuhara Y, Itoh K, Oshima A, Sakuraba H, Suzuki Y. Protective protein gene mutations in galactosialidosis. *J Clin Invest.* 1993;91:2393-8.
23. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet.* 2002;3:285-98.
24. Woolfe A, Mullikin JC, Elnitski L. Genomic features defining exonic variants that modulate splicing. *Genome Biol.* 2010;11:R20.
25. Oka M, Nozu K, Kaito H, Fu XJ, Nakanishi K, Hashimura Y, et al. Natural history of genetically proven autosomal recessive Alport syndrome. *Pediatr Nephrol.* 2014;29:1535-44.
26. Hashimura Y, Nozu K, Kaito H, Nakanishi K, Fu XJ, Ohtsubo H, et al. Milder clinical aspects of X-linked Alport syndrome in men positive for the collagen IV alpha5 chain. *Kidney Int.* 2014;85:1208-13.

Figure legends

Fig. 1. Sanger sequence chromatograms of *LAMB2* and family trees in cases 1–6.

(A) A truncating variant (c.225delC) and nontruncating variant (c.2095G>C) were identified in case 1 and case 2 (case 2 is the sister of case 1). Their asymptomatic older brother has only one variant. (B) A splice site variant (c.3797+5G>A) and truncating variant (c.5073_5076dupCCAG) were detected in case 3. (C, D) Homozygous nontruncating variants (c.821T>C and c.482T>C) were found in cases 4 and 5, respectively. (E) Compound heterozygous variants of a nontruncating variant (c.4616G>A) and a truncating variant (c.4907_4908del) were detected in case 6.

Fig. 2. Transcript analyses of a novel *LAMB2* splice site variant in case 3.

To perform RT-PCR, overlapping primers were used (black arrows). The underlined letters are aberrant donor sites. Agarose gel electrophoresis of RT-PCR products of both leukocytes and urine-derived cells generated three different transcripts in addition to the normal transcript (dashed line box), exon 24A, exon 24B, and exon 24C, leading to a 96-bp insertion (whole of intron 24), 133-bp deletion, and 319-bp deletion.

Fig. 3. Additional transcript analyses using long-range, nested RT-PCR in case 3.

The first and second primers were designed to overlap exon-exon junctions (black arrows). Agarose gel electrophoresis of the *LAMB2* RT-PCR amplicons of both leukocytes and urine-derived cells showed two differently sized transcripts. One of these transcripts included the normal transcript without a 4-bp deletion (dashed line box). This means that the splicing variant of c.3797+5G>A created the completely normal transcript in addition to the three abnormal transcripts.

457

458 **Fig. 4. Transcript analyses of a point mutation in exon 28 of *LAMB2* in control and**
459 **case 6.** Locations of the pair of primers, mutation (c.4616G>A), and aberrant acceptor
460 site are indicated by a black arrow, triangle, and underline, respectively. A transcript
461 derived from blood cDNA in the patient with a smaller size than normal was detected
462 by electrophoresis, which was proven to feature a 44-bp deletion due to abnormal
463 splicing.

464

465 **Fig. 5. Immunohistochemistry of type 4 collagen $\alpha 5$ and laminin $\beta 2$ on GBM in**
466 **healthy control and our cases.** In the control and cases 1–4, laminin $\beta 2$ exhibited a
467 characteristic linear expression pattern on GBM equivalent to that with type 4 collagen
468 $\alpha 5$. Case 5 showed complete negativity for laminin $\beta 2$ expression.

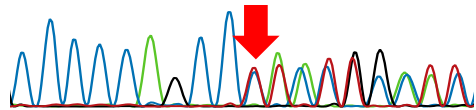
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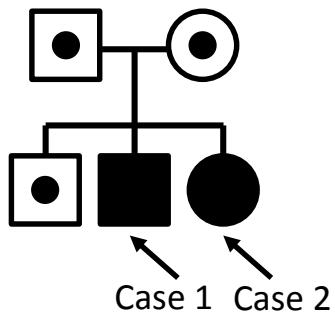
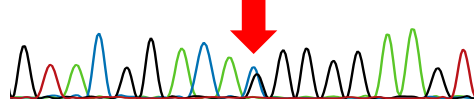
Fig.1

A

Exon 2
CCCCCAGCCTAATGGATT

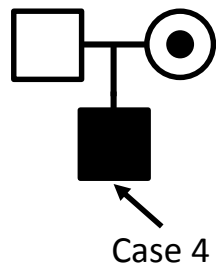
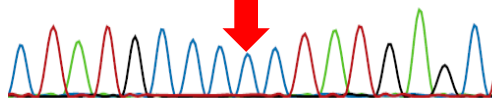


Exon 16
GTACGGACAGGGGGAAGT



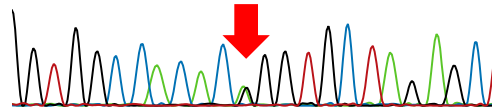
C

Exon 7
CTATGCCCTCTATGAGC

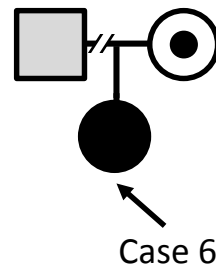
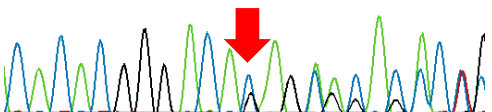


E

Exon 28
GTGGCCACACGGGTGCTAGAGC

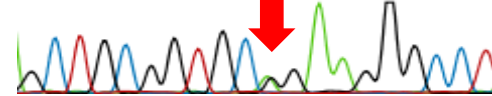


Exon 29
CACACGGGACACAGAGCAGACCC

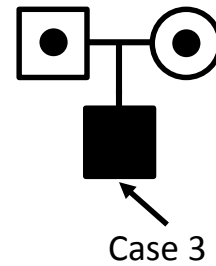
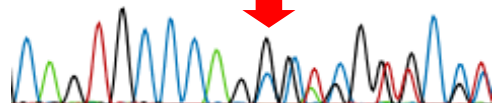


B

Exon 24
GCTGCGGTGCGGAAGGGCCT



Exon 30
CAGTGCCAGGGTCGTGCCT



D

Exon 5
GCTGCCATGCTGGTGGAAACGC

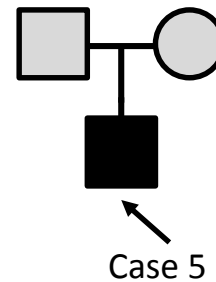
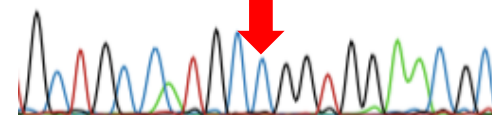


Fig.2

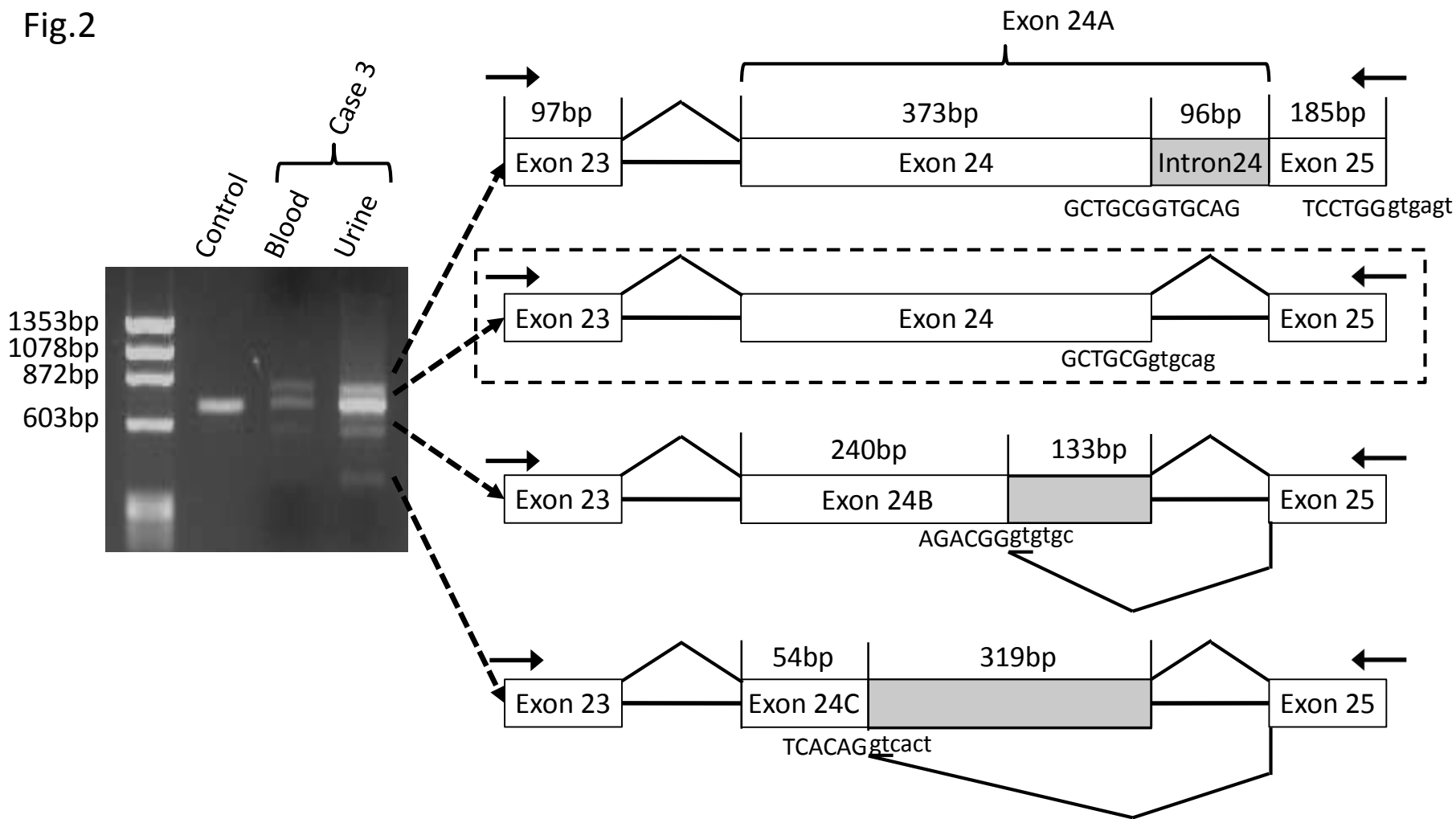


Fig.3

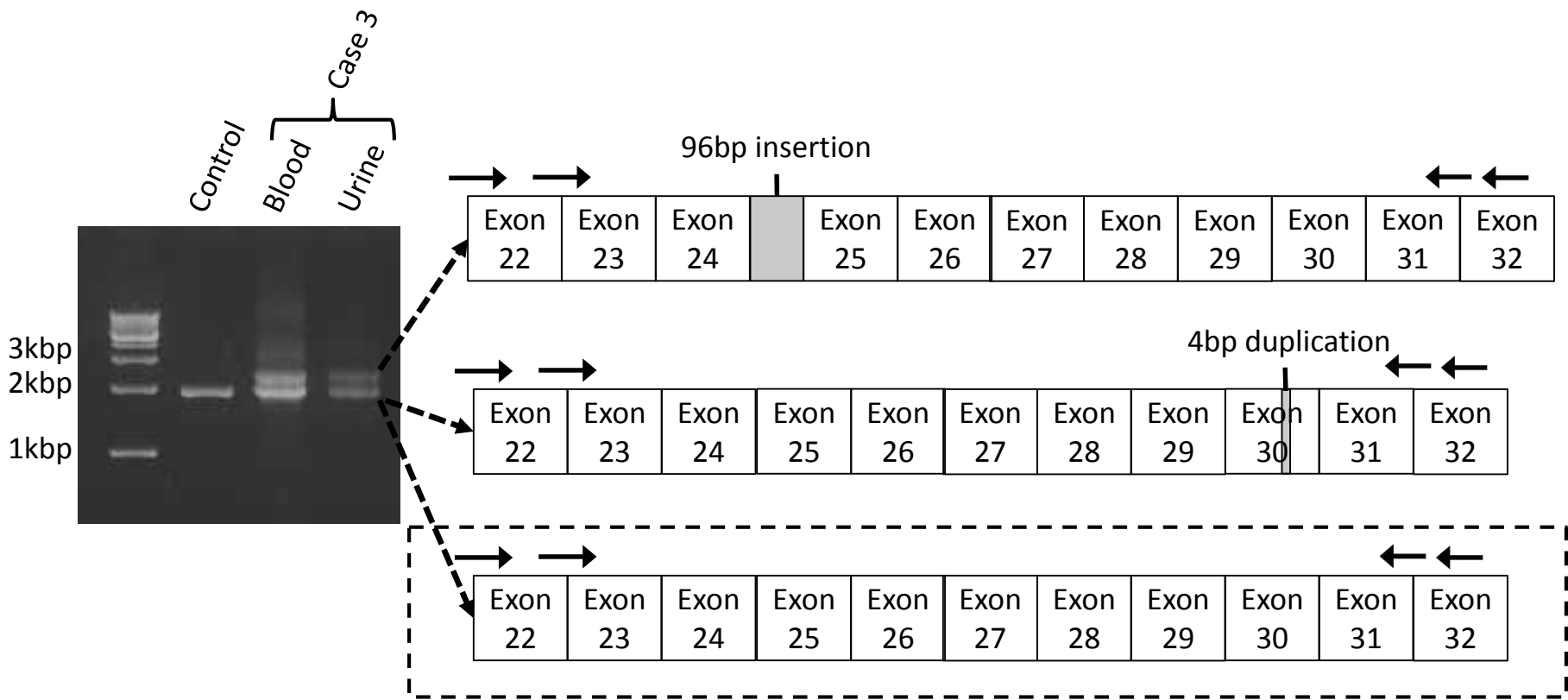


Fig.4

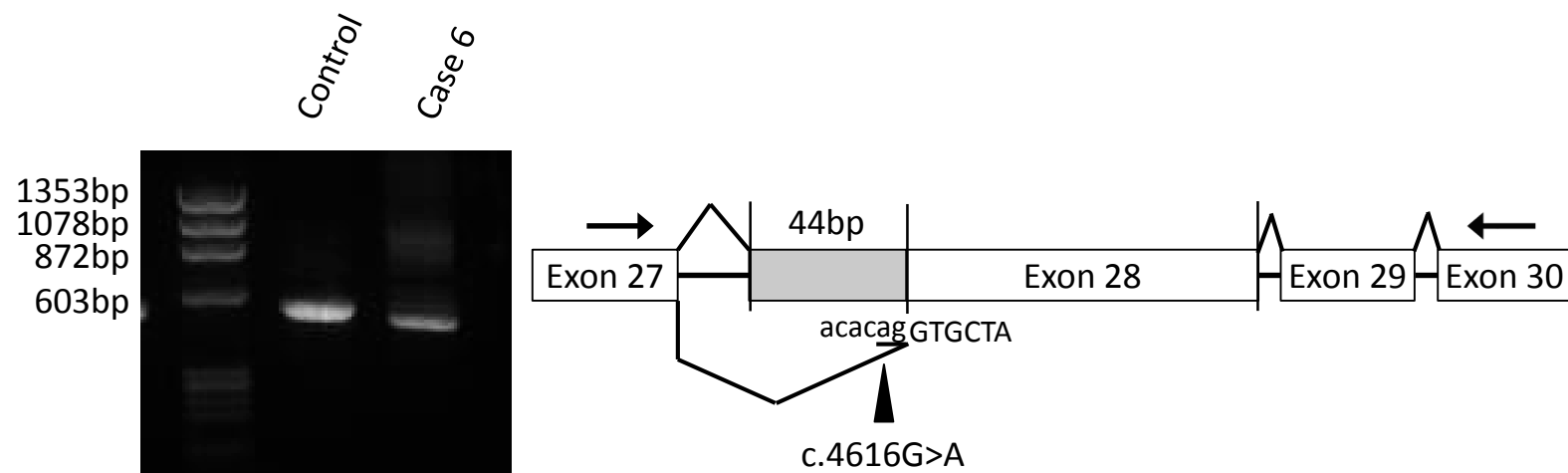
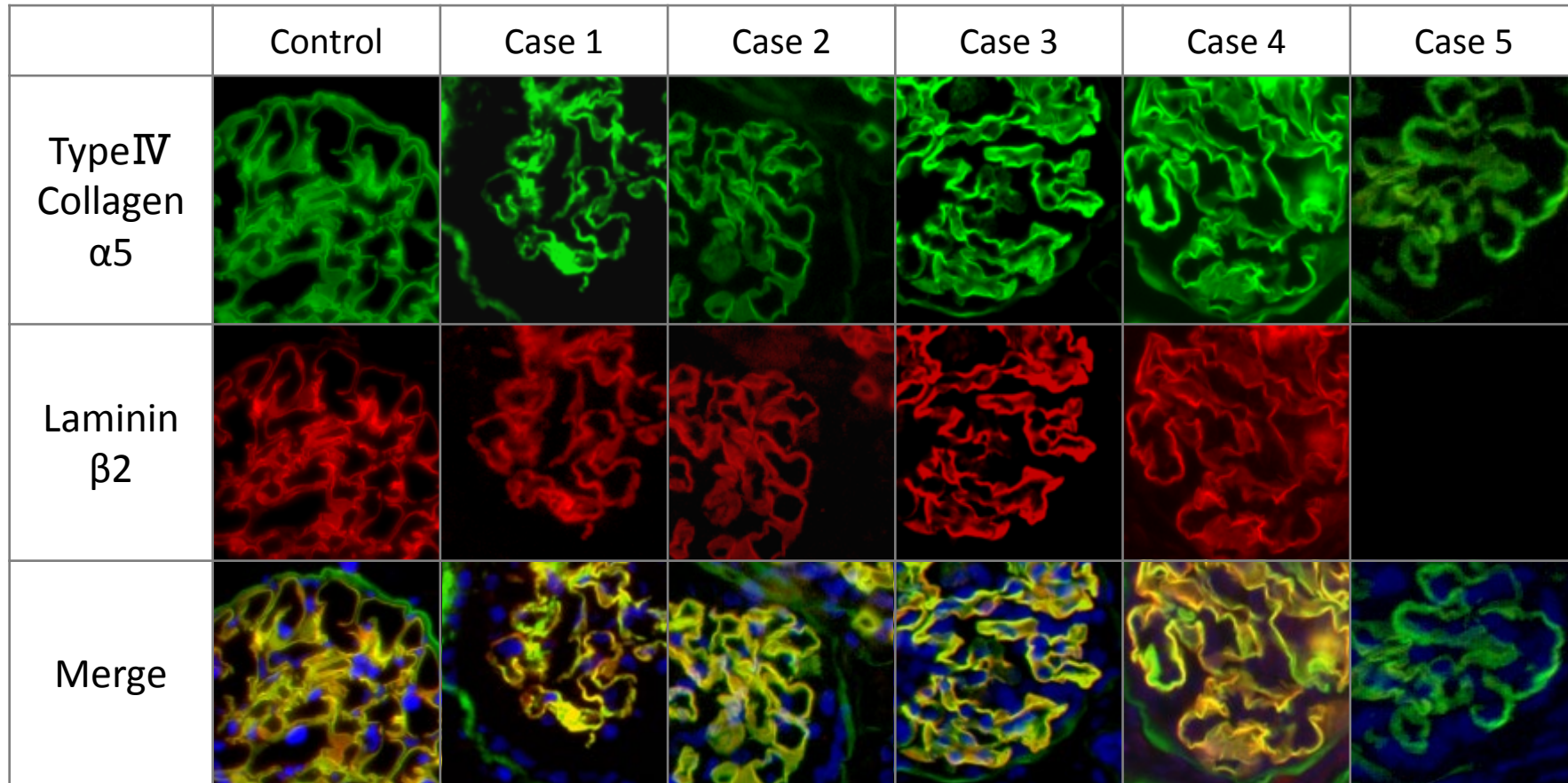


Fig.5



1 Table 1. Characteristics of four cases with heterozygous or homozygous *LAMB2*
 2 variants

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
ID	Neph4-1	Neph4-2	A278	Neph89	Neph23	Neph133
Age of follow-up	7 years	3 year	5 years	4 years	11 months	7 year
Sex	Male	Female	Male	Male	Female	Female
Serum creatinine (mg/dl)	0.27	0.17	0.19	4.17	-	-
Proteinuria (g/gCr)	0.07	0.30	0.11	62.6	-	-
Hematuria	+	++	++	++	ND	-
Age at NS onset	6 months	1 month	–	2 years	Day 0	1 month
Age at ESRD	–	–	–	3 years	3 months	2 months
Ocular abnormalities	–	–	–	–	–	+
Psychomotor retardation	–	–	–	–	+	+
Biopsy	MP	MP	TBM	MP	Obsole-	N/A

findings	TBM BWC	TBM BWC	BWC	TBM FPE	science	
Gene variants	c.225delC, p.Tyr76Thrfs*36 c.2095G>C, p.Gly699Arg		c.3797+5G> A c.5073_5076 dupCCAG, p.Gln1692Valfs*21	c.821T>C, p.Leu274Pro	c.482T>C, p.Leu161Pro	c.4616G>A, p.Arg1539Gln c.4907_4908 delAG, p.Glu1636Alafs*22
Treatment	Steroid, ARB	ARB	ARB	RRT	RRT	RRT

3

4 ND, not determined; NS, nephrotic syndrome

5 MP, mesangial proliferation; TBM, thin basement membrane

6 BWC, basket-weave change; FPE, foot process effacement

7 DMS, diffuse mesangial sclerosis; N/A, not available

8 RRT, renal replacement therapy