

Human laminin β 2 deficiency causes congenital nephrosis with mesangial sclerosis and distinct eye abnormalities

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Congenital nephrotic syndrome (CNS) is clinically and genetically heterogeneous, with mutations in *WT1*, *NPHS1* and *NPHS2* accounting for part of cases. We recently delineated a new autosomal recessive entity comprising CNS with diffuse mesangial sclerosis and distinct ocular anomalies with microcoria as the leading clinical feature (Pierson syndrome). On the basis of homozygosity mapping to markers on chromosome 3p14–p22, we identified homozygous or compound heterozygous mutations of *LAMB2* in patients from five unrelated families. Most disease-associated alleles were truncating mutations. Using immunohistochemistry and western blotting we could demonstrate that the respective *LAMB2* mutations lead to loss of laminin β 2 expression in kidney and other tissues studied. Laminin β 2 is known to be abundantly expressed in the glomerular basement membrane (GBM) where it is thought to play a key role in anchoring as well as differentiation of podocyte foot processes. *Lamb2* knockout mice were reported to exhibit congenital nephrosis in association with anomalies of retina and neuromuscular junctions. By studying ocular laminin β 2 expression in unaffected controls, we detected the strongest expression in the intraocular muscles corresponding well to the characteristic hypoplasia of ciliary and pupillary muscles observed in patients. Moreover, we present first clinical evidence of severe impairment of vision and neurodevelopment due to *LAMB2* defects. Our current data suggest that human laminin β 2 deficiency is consistently and specifically associated with this particular oculorenal syndrome. In addition, components of the molecular interface between GBM and podocyte foot processes come in the focus as potential candidates for isolated and syndromic CNS.

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INTRODUCTION

Congenital nephrotic syndrome (CNS) constitutes a heterogeneous group of conditions having in common the disruption of normal glomerular permselectivity (1). The glomerular filtration barrier comprises three major components: the fenestrated capillary endothelium, the glomerular basement membrane (GBM) and the podocytes which by their interdigitating foot processes form filtration slits bridged by a membrane-anchored, extracellular meshwork of proteins, the slit diaphragm (1,2). Hitherto known molecular causes of CNS are mutations of the genes encoding nephrin in Finnish type CNS (MIM 256300) and podocin in autosomal recessive steroid-resistant NS (MIM 600995). Both proteins play essential roles in the formation of the slit diaphragm. Their close functional interrelationship is further evidenced by the recent finding of digenic inheritance of CNS (3). Additionally, autosomal dominant *WT1* mutations can cause CNS and diffuse mesangial sclerosis in patients with Denys–Drash syndrome (MIM 256370). The pathogenesis of NS caused by specific *WT1* mutations has been debated but is not completely understood so far (4). A considerable proportion of patients with CNS, however, do not belong to either of these entities (1), suggesting that additional, hitherto unidentified genes play a role in its pathogenesis. Aside from isolated renal disease, CNS can occur in association with various non-renal symptoms. A definite classification of CNS has not yet been established.

In two unrelated consanguineous families with a total of 11 affected offspring, we recently delineated a new autosomal recessive entity comprising severe CNS with diffuse mesangial sclerosis and distinct eye abnormalities clinically characterized by microcoria (fixed narrowing of the pupils), but obviously representing a complex ocular maldevelopment including hypoplasia of iris and ciliary body, lenticonus posterior and corneal and retinal anomalies (Fig. 1) (5). The condition was first described by Pierson *et al.* (6) in 1963, and a small number of cases with similar manifestations has been reported, so far (7–10). However, the disorder that we proposed to term Pierson syndrome had not been recognized as a separate entity before. In all previous reports, the disorder was uniformly associated with early lethality. Herein we describe the molecular basis of this disease.

RESULTS

Linkage analysis and identification of the candidate gene

Homozygosity mapping in the two families reported earlier (5) identified a candidate region on chromosome 3p flanked by markers D3S1768 and D3S1766 (Fig. 2). Multipoint lod score analysis generated a lod of 3.5 for locus D3S2409 (Table 1). The candidate region derived from gross mapping still encompassed a segment of ~17 cM. Linkage refinement, however, was not attempted because *LAMB2* (MIM 150325), which is located within the candidate region close to D3S2409, represented a perfect candidate on the basis of the findings that *lamb2*^{-/-} mice display CNS (11).

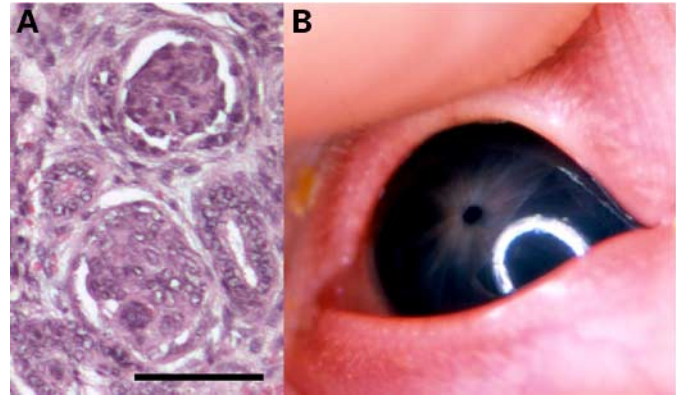


Figure 1. Main clinical features of Pierson syndrome: (A) diffuse mesangial sclerosis in a kidney biopsy (bar: 100 μ m) and (B) clinical appearance of the ocular anomalies in an affected newborn.

Mutational analysis

Patients and obligate heterozygotes from five unrelated families with at least one child affected by Pierson syndrome were subjected to mutational screening by bidirectional direct sequencing of the entire coding region, including the exon–intron boundaries of *LAMB2* (GenBank accession no. AAB34682.2). The families included two in whom linkage analysis had been performed as well as one unreported and two previously reported families (8,12) with similar renal and ocular manifestations in affected children (Table 2). In patients from four families we found homozygous or compound heterozygous *LAMB2* mutations predicting a truncation of the protein. Two disease-associated alleles were nonsense mutations at the codons 1507 and 1562 of the protein, which comprises 1798 amino acid residues. Two alleles were 1 bp deletions leading to translational stop codons at positions 1150 and 1520, and another was a 1 bp insertion creating a premature termination codon at position 1760 (Fig. 3A–C and E; Table 2). In the remaining family (family 4), both affected children had a homozygous missense mutation (Fig. 3D) predicting the substitution of a highly conserved arginine by tryptophan (Fig. 4). By sequencing of the respective exons in all available members of each family, we found full segregation of the mutations. None of the mutations was detected among 200 control chromosomes. In 12 unrelated individuals with CNS and variable associated manifestations, who had earlier been negative for mutational screening in the nephrin, podocin and *WT1* genes, no *LAMB2* mutations were detected, indicating that *LAMB2* defects are specifically associated with Pierson syndrome.

Immunohistology and western blotting

To study the consequences of the observed *LAMB2* mutations on protein expression, we examined kidney, skeletal muscle and ocular tissues from different patients by immunofluorescence and western blotting using laminin β 2 antibodies directed against the N-terminal region. In individuals with the homozygous nonsense mutation (family 2), laminin β 2 immunoreactivity of the GBM was virtually absent (Fig. 5A–D), and lack of laminin β 2 could also be demonstrated by western

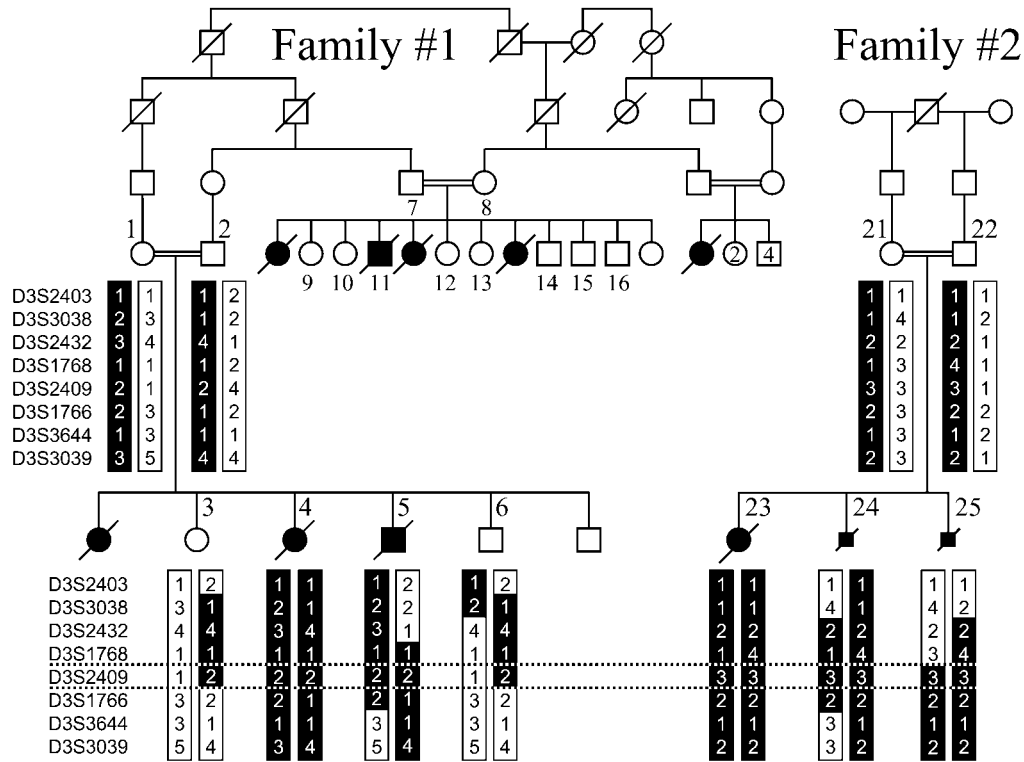


Figure 2. Pedigree of the large family originating from Lebanon with multiple offspring affected by Pierson syndrome (family 1) and the results of haplotype analysis of eight polymorphic microsatellite markers on 3p in one branch of this family and a consanguineous Turkish couple with three affected children (family 2). Numbered family members (1–16, 21–25) were available for linkage studies. The shared disease-associated haplotypes are represented by a black background. The homozygous interval in family 1 and recombination in patient 25 define the centromeric and telomeric boundaries, respectively (indicated by dotted lines).

Table 1. Two point lod scores calculated for eight 3p microsatellite markers

Marker/ θ	0.00	0.05	0.10	0.20	0.30	Z_{max}	θ_{max}	Position on the chromosome (cM)
D3S2403	–∞	–1.098	–0.359	0.098	0.152	0.330	0.065	37.20
D3S3038	–∞	–0.221	0.447	0.604	0.361	0.163	0.630	44.81
D3S2432	–∞	1.541	1.601	1.169	0.629	0.077	1.622	57.92
D3S1768	–∞	0.423	0.704	0.665	0.421	0.134	0.739	61.52
D3S2409	3.495	3.063	2.632	1.790	1.019	0.000	3.495	70.60
D3S1766	0.662	2.654	2.449	1.721	0.929	0.043	2.659	78.64
D3S3644	–∞	0.603	0.957	0.814	0.446	0.130	0.704	91.18
D3S3039	–∞	–1.301	–0.442	0.078	0.126	0.259	0.134	103.72

The position on the chromosome was determined according to the Marshfield data (<http://research.marshfieldclinic.org/genetics>).

blotting of muscle proteins (Fig. 6). Similar immunofluorescence results were obtained in kidney tissues of affected individuals from family 3 and family 5 (data not shown). In frozen kidney tissue of one patient with the homozygous missense mutation R246W (family 4), significant reduction but not a complete lack of laminin β 2 could be demonstrated by western blotting (Fig. 6). By densitometry, a 7-fold reduction of laminin β 2 relative to laminin β 1 expression was calculated. Accordingly, immunostaining for laminin β 2 was absent in eye sections of one aborted fetus homozygous for the mutation R1562X (family 2; data not shown). Eye sections of normal controls revealed that specific laminin

β 2 immunoreactivity is present not only in the retina and lens capsule as already known (data not shown), but also most prominently in the ciliary and iris muscles (Fig. 5E–H).

DISCUSSION

We demonstrate here that *LAMB2* mutations can be consistently found in patients with Pierson syndrome, a newly delineated entity characterized by CNS and distinct ocular anomalies. All but one mutant *LAMB2* allele predict a C-terminal truncation of the protein owing to premature

Table 2. Compilation of clinical data on the affected families

Family no.	Ethnic origin	Parental consanguinity	No. of affected children	Renal manifestations	Ocular manifestations	Others	Age of death	Mutation	Exon	Reference
1	Lebanese	Yes	8	CNS with DMS, early-onset RF	Non-reactive miosis, 'buphthalmos' ^a	ASD and Ebstein anomaly in one affected child	1-44 days	3015delG homozygous	21	(5)
2	Turkish	Yes	3	CNS with DMS, early-onset RF	Microcoria, lenticonus, corneal and retinal anomalies	—	Prenatal-32 days	R1562X homozygous	28	(5)
3	German	No ^b	1	CNS with DMS, early-onset RF	Non-reactive miosis, 'Rieger anomaly', spherophakia	Severe muscular hypotonia and psychomotor delay, blindness	1.8 years ^c	5259insA homozygous	30	—
4	Portuguese	Yes	2	NS starting at 1-5 months, DMS	Miosis, nystagmus, cataracts	Muscular hypotonia, hemiparesis, abnormal movements	8 months	R246W homozygous	7	(8)
5	Polish	No	1	CNS with DMS, early-onset RF	Unilateral microphthalmia, contralateral 'glaucoma' with pinhole fixed pupil, cataract	—	75 days	4504delA Q1507X compound heterozygous	27	(12)

CNS, congenital nephrotic syndrome; DMS, diffuse mesangial sclerosis; RF, renal failure; ASD, atrial septal defect.

^aOcular anomalies not documented in all affected individuals.

^bBoth parents originate from a German isolate in Romania.

^cSurvived under chronic peritoneal dialysis from the age of 6 weeks and died after kidney transplantation.

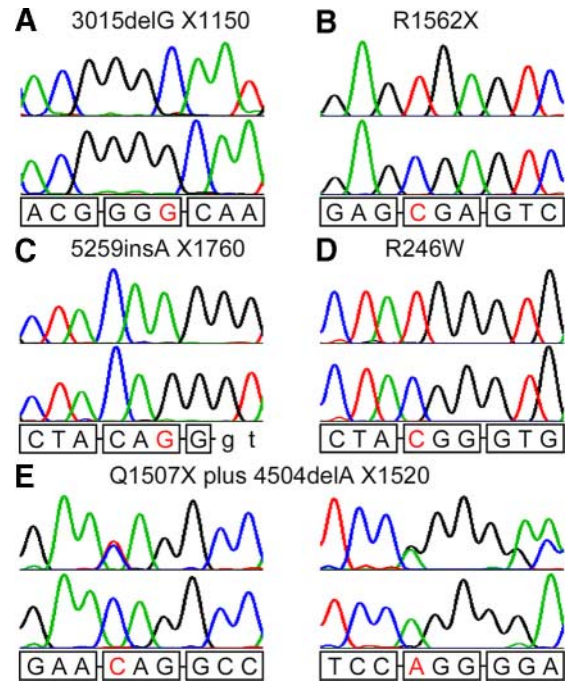


Figure 3. Results of sequence analysis showing mutant sequences from affected individuals of each family (upper lane) compared with the respective sequence of a normal control (lower lane). Normal sequences are indicated at the bottom of the respective chromatograms with boxes indicating triplet codons and letters in red highlighting the sequence deviations.

translational stop codons. Even with the least extensive truncation of the protein by only 39 amino acids (family 5), no significant laminin β 2 immunoreactivity of the GBM could be demonstrated. We assume that these truncating mutations disturb the assembly of the mutated protein into laminin heterotrimers, as it was shown that a peptide sequence of approximately 50 amino acid residues forming the C-terminal alpha-helical domain of the laminin chains represents the critical site for the initiation of laminin chain assembly (13). In the kidney tissue of a patient with the homozygous missense mutation R246W, significant reduction of laminin β 2 could be demonstrated by western blotting (Fig. 6). The mutation is located within a highly conserved sequence motif of domain VI that is crucial for laminin polymerization to form BMs (14). One might suggest that laminin β 2 deficiency as evidenced by western blotting results from increased degradation of the protein either due to decreased stability within the BM network or related to primary misfolding of the protein chain. Preservation of some residual function, on the other hand, might be an explanation for a somewhat milder phenotype with prolonged survival in this family (Table 2). Taken together, these findings clearly indicate that Pierson syndrome is caused by the absence or severe quantitative reduction in laminin β 2 expression rather than a dysfunctional protein.

Laminin β 2 is one component of the laminins, a family of heterotrimeric extracellular glycoproteins consisting of variable assemblies of α -, β - and γ -chains and representing major BM constituents that play important roles in cell adhesion, proliferation, differentiation and migration (15,16).

laminin beta 2 [Homo sapiens] gi 1335202	221	VLDPAIPI PDPYSSRIQNLLKITNL	R	VNLTRLHHTLGDNLL	260
laminin, beta 2 [Mus musculus] gi 2497599	224	VLDPAIPI PDPYSSRIQNLLKITNL	R	VNLTRLHHTLGDNLL	263
laminin beta 2 [Rattus norvegicus] gi 6981142	224	VLDPAIPI PDPYSSRIQNLLKITNL	R	VNLTRLHHTLGDNLL	263
laminin beta 2-like chain [Gallus gallus] gi 45383784	221	VLDPAIPI RDPYSPAIQDLVRVTNL	R	VNLTKLHHTLGDNLL	260
laminin related (lam-1) [C. elegans] gi 17541466	213	VLSPHIVTENPYADEIESTLLKITNL	R	FNFTKLHHTLGDLL	252
laminin B1 chain [D. melanogaster] gi 4388541	228	VLEPPNINVTDPYAEHVQNQLKMTNL	R	IQMTKLHKLGDNLL	267
laminin, beta 1 [Danio rerio] gi 27545301	206	VLDPAFRIEDPYSFRIQNMLKITNL	R	VKFTKLHHTLGDNLL	245
laminin beta 1 [Homo sapiens] gi 186876	209	ALDPAFKIEDPYSFRIQNLLKITNL	R	IKFVKLHHTLGDNLL	248
laminin beta 3 [Homo sapiens] gi 1151215	193	LMDLASAI PASQSKKIQELGDI TNL	R	VNFTKLAPV PQRGS	232
laminin beta 4 [Homo sapiens] gi 46276866	202	VLDPSFEIENPYSPIYQDLVITL TNL	R	INFTKLHHTLGDALL	241
laminin, beta 4 [Danio rerio] gi 27545303	204	ALDPSFDIHDYPDSTIQGLITL TNL	R	VNFTRLLL TLGDILL	243
beta-netrin [Homo sapiens] gi 11139301	200	ALSPPHDTENPYSAKVQEQLKITNL	R	VQLLKRQSCPQRND	239
beta-netrin [Mus musculus] gi 11120055	200	ALSPPYDIENPYSAKVQEQLKITNL	R	VRL LKRQSCPQIN	239
netrin-1 [Gallus gallus] gi 529419	225	LDGRPTAHDFDNSPVLQDWVTATDI	K	VTF SRLHTFGDENE	264
netrin-2 like protein [Homo sapiens] gi 2052395	196	QDSSPPGLDLDSSPVLQDWVTATDV	R	VV LTRPSTAGDPRD	235
netrin-2 [Gallus gallus] gi 529421	195	LDGRPSAQDFDSSPVLQDWVTATDI	R	VVFSRPHLFRRLGG	234
netrin-A precursor [D. melanogaster] gi 2497607	217	LEGRPSSRDLDSSPVLQDWVTATDI	R	VVFHRLQRDPDQAL	256
netrin-B precursor [D. melanogaster] gi 2497608	218	LEGRPSANDLDSSLVLQDWVTATDI	R	VVFHRLLELPPQLK	257

Figure 4. Alignment of human laminin $\beta 2$ and laminin $\beta 2$ orthologs from various species, as well as the human β -laminin paralogs and members of the laminin-like netrins shows high conservation of the arginine residue (white frames) at position 246 of laminin $\beta 2$ and the surrounding sequence motif. Conserved amino acids are shown with black background and similar amino acids with a gray background. Alignments created according to outputs of BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) and the Conserved Domain Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd>; CD: smart00136.10, LamNT).

This report describes only the third human phenotype resulting from mutations in members of the laminin family, after Herlitz-type epidermolysis bullosa including two variants (MIM 245660, 226650 and 226700) that can be caused by mutations in the genes encoding one of the laminin-5 components (*LAMA3*, *LAMB3* and *LAMC2*) and congenital muscular dystrophy due to merosin deficiency (MIM 607855, *LAMA2*). Laminin $\beta 2$ (formerly s-laminin) was initially defined by an antiserum to an extract of lens capsule, which stained synaptic BM of the neuromuscular junction (17), but it was subsequently found to be also abundant in some non-muscle BMs, like perineurial, glomerular and arterial BMs (18). In laminin $\beta 2$ -deficient mice, a lethal phenotype of massive early-onset proteinuria was observed, suggesting a critical role in proper maturation or function of the renal filtration apparatus (11). Laminin-11, the major laminin of the mature GBM and composed of $\alpha 5$ -, $\beta 2$ - and $\gamma 1$ -chains (19), was shown to promote podocyte process development in cell culture (20). In addition to the renal alterations, the knockout mouse model exhibited abnormalities of the neuromuscular junctions, proposing laminin $\beta 2$ to be a muscle-derived regulator of nerve-terminal differentiation (21). Specific expression patterns of laminin $\beta 2$ were described in the human ciliary body (22) and corneal BL (23). Regarding the role of laminin $\beta 2$ in ocular development, however, most research focused on the significance for retinal differentiation. In laminin $\beta 2$ -deficient mice, shortened inner and outer segments of photoreceptors and disrupted synaptic connections between photoreceptors and second order cells were demonstrated (24). Moreover, laminin $\beta 2$ has also been found at distinct sites of the developing central nervous system in rats (25). In contrast

to the renal phenotype of laminin $\beta 2$ deficiency, the functional consequences of developmental deficiencies in neural, retinal and other tissues remained unclear. Knockout mice were reported to display no apparent neurologic phenotype at birth, but it was suggested that the observed presynaptic defects might contribute to the weakness and eventual death of the mutants occurring between days 15 and 30 (21). In addition, abnormal electroretinograms consistent with synaptic disruption in the outer retina were documented (24).

The human phenotype corresponds well to the known expression patterns of laminin $\beta 2$ and the manifestations in laminin $\beta 2$ deficient knockout mice. The findings presented here thus provide the missing link between the data derived from experimental studies and the significance of the protein in human development. However, aside from the functions inferred from the animal model, our investigations add some new aspects on the potential physiological role of laminin $\beta 2$ in humans. Specifically, they point out the importance of laminin $\beta 2$ for the proper development of structures of the anterior eye segment, particularly the intraocular muscles and lens, demonstrating that a distinct pattern of ocular maldevelopment is associated with human laminin $\beta 2$ deficiency. The renal histomorphological phenotype (diffuse mesangial sclerosis) in human laminin $\beta 2$ deficiency differs from the histological findings reported in knockout mice where only minimal changes were observed. This discrepancy, however, may reflect interspecies differences or the natural evolution of renal damage, as we reported earlier that the changes are milder in fetuses than in newborns (5). Additionally, although most patients known to the authors died in the newborn period without being recognized to have an obvious neurological

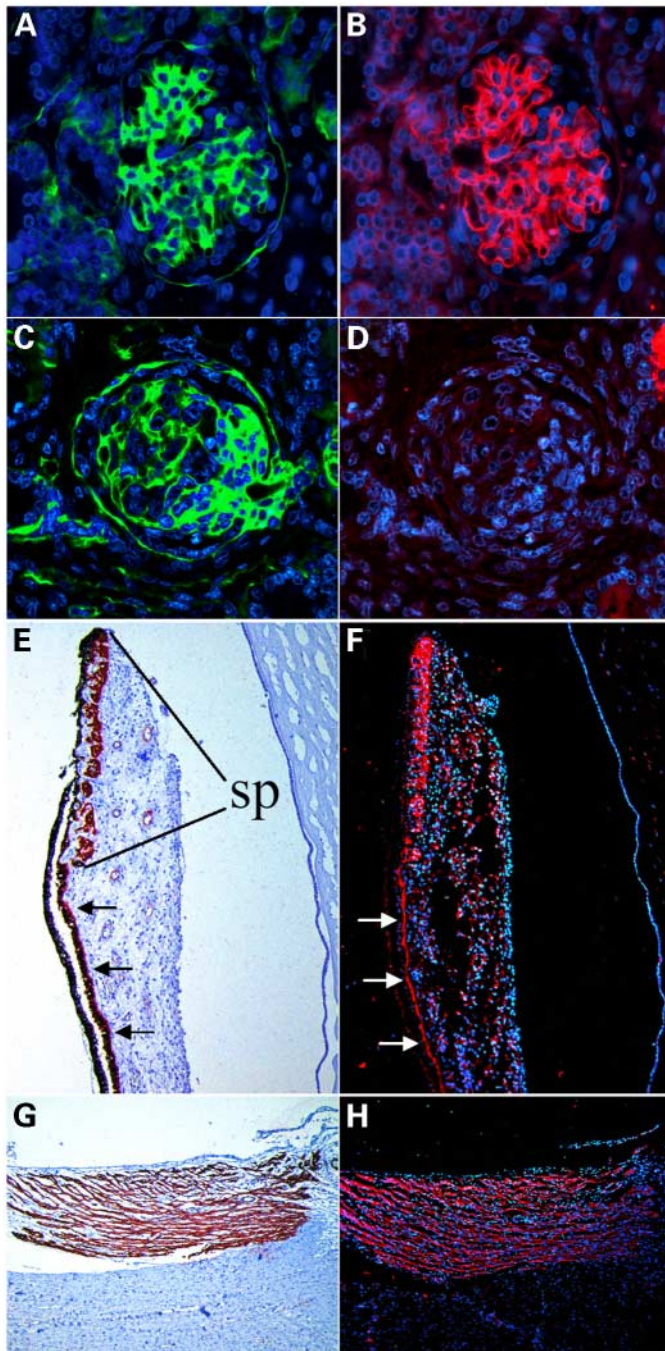


Figure 5. Immunohistology: Fixed, paraffin-embedded tissue sections analyzed by simultaneous indirect immunofluorescence using antibodies against collagen IV as a control protein for glomerular BL (green; A and C) and against laminin $\beta 2$ (red; B, D, F, H). Nuclear stain with DAPI (blue) is displayed as background. (A and B) Normal neonatal kidney; (C and D) kidney tissue from a newborn patient with the mutation R1562X showing a structurally altered glomerulus with positive staining for collagen IV (C) but lack of laminin $\beta 2$ immunoreactivity (D). (E–H) Corresponding eye sections of a newborn control analyzed by immunoperoxidase using antibodies against smooth muscle actin (E and G) and by immunofluorescence using laminin $\beta 2$ antibodies (F and H). Specific expression of laminin $\beta 2$ surrounding muscle cells of sphincter pupillae (sp) (F) and ciliary muscle (H); bandlike laminin $\beta 2$ expression at the basal site of the anterior layer of the posterior iris epithelium that forms the dilatator pupillae (arrows).

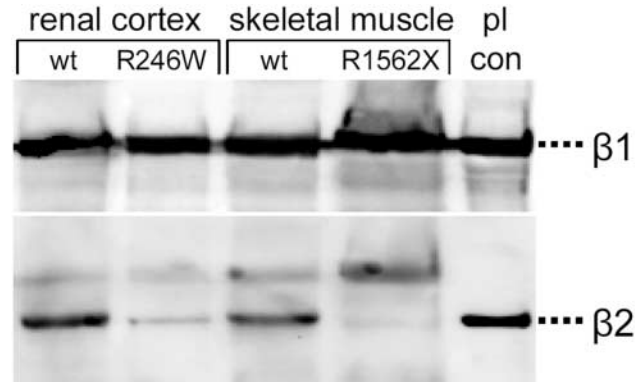


Figure 6. Western blotting of proteins from renal cortex of an affected infant with the mutation R246W and from skeletal muscle of an affected fetus carrying the mutation R1562X compared with tissues of age-matched controls (wt). Placental laminins (pl con) served as a positive control. Consecutive staining of the blot for laminin $\beta 2$ (bottom) and laminin $\beta 1$ (top) showing complete lack of laminin $\beta 2$ immunoreactivity in the mutant R1562X and severe reduction in the mutant R246W.

phenotype, the clinical findings in the two previously reported sibs (8) and in particular one patient who is first reported here and who survived until the age of 1.8 years under chronic dialysis (family 3, Table 2; see Materials and Methods: Study population) now provide preliminary evidence that laminin $\beta 2$ deficiency is associated with significant neurological and developmental deficits. These may reflect the functional importance of the protein at the neuromuscular junction and in the central nervous system. These issues will have to be addressed by future research and should be considered before the decision is made to rescue affected children from renal insufficiency by kidney replacement therapy.

Previously identified molecular causes of CNS emphasized the pathogenetic significance of impaired podocyte–podocyte interactions at the slit diaphragm which represents a highly specific renal structure. In contrast, we report on CNS associated with defects of a protein which has more pleiotropic effects. Our current understanding suggests that human laminin $\beta 2$ deficiency is consistently and specifically associated with the particular oculorenal syndrome described here. Our findings, however, place components of the molecular interface between GBM and podocyte foot processes in the focus of future research for candidates for isolated and, in particular, syndromic NS. Remarkably, the significance of laminins in the extracellular matrix as triggers for process formation is not the only mechanism shared by podocytes and neuronal cells (26). Therefore, defects in other genes involved in podocyte morphogenesis should particularly be searched for in disorders presenting with CNS and neurological features.

MATERIALS AND METHODS

Study population

Patients were identified as having Pierson syndrome in the presence of CNS and microcoria, the clinical hallmarks of this disorder. Most affected individuals died in the newborn

period from renal insufficiency. Survival until the age of 8 months in both affected siblings from a family reported before (8) is considered to reflect a somewhat milder expression. Only one patient survived infancy owing to chronic dialysis. By the time of her death at 1.8 years she exhibited severe neurological deficits previously not described in Pierson syndrome: marked muscular hypotonia, psychomotor retardation and apparent blindness (Table 2; detailed clinical report in preparation). In addition to the patients with definite Pierson syndrome, 12 unrelated individuals with CNS and variable associated manifestations, in whom no mutations had been detected in the nephrin, podocin and *WT1* genes, were included for *LAMB2* mutational screening.

The study was approved by the Institutional Review Boards. After obtaining informed consent, blood samples were drawn from all available members of the two families included in the linkage studies. DNAs from deceased patients and aborted fetuses were obtained from stored biopsy specimens, Guthrie cards and abortion material. Stored frozen tissue samples from the affected siblings of the family described in a previous publication (8) served for molecular genetic and western blot analyses. Tissues used in this study were derived from stored autopsy or biopsy specimens and were obtained after informed parental consent had been given.

Linkage analysis

A genome scan using microsatellite markers was initiated in one of the families (family 1), a large inbred sibship of Lebanese origin (Fig. 1), and terminated upon finding evidence of linkage after typing of 124 markers covering approximately one-third of the genome (R. Fenski and A. Reis, unpublished data). The locus on chromosome 3p was confirmed and refined in the second consanguineous family (family 2) using a panel of microsatellite markers from chromosome 3p from the Weber panel (Research Genetics). Two-point lod score calculations were performed by the LINKAGE program package (27) with the help of the computer programs LINKRUN and MKS, using an autosomal recessive fully penetrant model.

Mutational analysis

We developed primers flanking all 32 exons of *LAMB2* from the genomic sequence. Oligonucleotide sequences and PCR conditions are available on request. We carried out bidirectional direct sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 3730 capillary sequencer (Applied Biosystems) and evaluated sequences using the DNASTar software package.

Antibodies

We obtained antibodies against collagen IV and smooth muscle actin commercially (DakoCytomation, clones 1A4 and CIV 22, respectively). The polyclonal antibody against human laminin β 1 (H-300) was purchased from Santa Cruz Biotechnology. The polyclonal anti-laminin β 2 antibody 409 was a gift from Lydia Sorokin (Experimental Pathology, Lund University, Lund, Sweden). It is directed against recombinant laminin

β 2 domain VI corresponding to amino acids 30–250 (S. Agrawal, M. Durbeej, M. Sixt, H. Korner, I. Nelissen, G. Opdenakker and L.M. Sorokin, submitted for publication).

Western blot analysis

We carried out western blot analysis on deep frozen muscle and kidney tissues according to standard procedures. The tissues had been stored at -70°C until examination. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies, as previously reported (28). Placental laminins containing both laminin β 1 and laminin β 2 were used as a positive control. They were derived from placenta extract by immunoaffinity chromatography, as described previously (29). The antibodies 409 and anti-laminin β 1 were diluted 1:500 and 1:1000, respectively. Immunoreactivities were detected by ECL reaction in a Lumi-Imager F1 (Roche, Mannheim, Germany) according to the manufacturer's instructions. Densitometry was performed using the software LumiAnalyst, version 3.1 (Roche, Mannheim, Germany).

Immunofluorescence and immunohistochemistry analysis

Tissue samples were fixed with 10% formalin and embedded in paraffin. Sections of 4 μm were used for immunolocalization studies for detecting endogenous laminin β 2 and collagen IV. All primary antibodies were applied at 4°C in a moist chamber overnight. Secondary antibodies were labeled either with Cy-2 or Cy-5 (Dianova, Hamburg, Germany). Nuclear counterstain was performed with DAPI; 70% glycerine was used as mounting medium. For double-labeling immunofluorescence using anti-collagen IV and anti-laminin β 2 antibodies, tissue sections were pretreated with hyaluronidase (2 mg/ml in PBS, pH 5 for 60 min at 37°C) and pronase (2 mg/ml in TB, PBS 7.3; Sigma, Taufkirchen, Germany, for 60 min at 37°C) and incubated with a mixture of both antibodies with anti-laminin β 2 antibody used at a $10\times$ higher concentration. The anti-laminin β 2 antibodies were detected using Cy-2-labeled goat anti-rabbit antibody (Dianova). For the detection of the anti-collagen IV antibodies, sections were first incubated with biotin-labeled goat-anti-mouse antibodies (Dianova) and then with peroxidase-labeled streptavidin. Subsequently, the tyramide amplification system (PerkinElmer, Boston, MA, USA) was used. Finally, the signals were detected using Cy-5-labeled streptavidin (Dianova). Nuclear staining was again performed using DAPI.

To show the distribution of smooth muscle in sagittal eye sections from unaffected individuals, we subjected sections to immunohistochemistry with a monoclonal anti-human smooth muscle actin antibody (DakoCytomation) as described previously (30) using alkaline phosphatase as detection enzyme and 3-hydroxy-2-naphthylacid 2,4-dimethylanilide as substrate. Nuclei were counterstained with hematoxylin.

The images were visualized with an Olympus AX70 fluorescence microscope and digitally captured using AnalySIS Docu software (SoftImaging, Stuttgart, Germany).

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REFERENCES

- Jalanko, H., Kääriäinen, H. and Norio, R. (2002) Nephrotic disorders. In Rimoin, D.L., Connor, J.M., Pyeritz, R.E. and Korf, B.R. (eds), *Principles and Practice of Medical Genetics*, 4th edn. Churchill Livingstone, London, Vol. 2, pp. 1708–1719.
- Somlo, S. and Mundel, P. (2000) Getting a foothold in nephrotic syndrome. *Nat. Genet.*, **24**, 333–335.
- Koziell, A., Grech, V., Hussain, S., Lee, G., Lenkkeri, U., Tryggvason, K. and Scambler, P. (2002) Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. *Hum. Mol. Genet.*, **11**, 379–388.
- Patek, C.E., Fleming, S., Miles, C.G., Bellamy, C.O., Ladomery, M., Spraggon, L., Mullins, J., Hastie, N.D. and Hooper, M.L. (2003) Murine Denys–Drash syndrome: evidence of podocyte de-differentiation and systemic mediation of glomerulosclerosis. *Hum. Mol. Genet.*, **12**, 2379–2394.
- Zenker, M., Tralau, T., Lennert, T., Pitz, S., Mark, K., Madlon, H., Doetsch, J., Reis, A., Müntefering, H. and Neumann, L.M. (2004) Congenital nephrosis, mesangial sclerosis and distinct eye abnormalities with microcoria: an autosomal recessive syndrome. *Am. J. Med. Genet.*, in press.
- Pierson, M., Cordier, J., Hervouet, F. and Rauber, G. (1963) An unusual congenital and familial congenital malformative combination involving the eye and kidney. *J. Genet. Hum.*, **12**, 184–213.
- Braga, S., Monn, E., Zimmermann, A. and Oetliker, O. (1989) Congenital nephrotic syndrome with congenital buphthalmos: a new genetic entity? *Prog. Clin. Biol. Res.*, **305**, 205–209.
- Glastre, C., Cochat, P., Bouvier, R., Colon, S., Cottin, X., Giffon, D., Wright, C., Dijoud, F. and David, L. (1990) Familial infantile nephrotic syndrome with ocular abnormalities. *Pediatr. Nephrol.*, **4**, 340–342.
- Nielsen, K.F. and Steffensen, G.K. (1990) Congenital nephrotic syndrome associated with Lowe's syndrome. *Child Nephrol. Urol.*, **10**, 92–95.
- Schneller, M., Braga, S.E., Moser, H., Zimmermann, A. and Oetliker, O. (1983) Congenital nephrotic syndrome: clinico-pathological heterogeneity and prenatal diagnosis. *Clin. Nephrol.*, **19**, 243–249.
- Noakes, P.G., Miner, J.H., Gautam, M., Cunningham, J.M., Sanes, J.R. and Merlie, J.P. (1995) The renal glomerulus of mice lacking s-laminin/laminin beta 2: nephrosis despite molecular compensation by laminin beta 1. *Nat. Genet.*, **10**, 400–406.
- Swietlinski, J., Maruniak-Chudek, I., Niemir, Z.I., Wozniak, A., Wilinska, M. and Zacharzewska, J. (2004) A case of atypical congenital nephrotic syndrome. *Pediatr. Nephrol.*, **19**, 349–352.
- Nomizu, M., Utani, A., Beck, K., Otaka, A., Roller, P.P. and Yamada, Y. (1996) Mechanism of laminin chain assembly into a triple-stranded coiled-coil structure. *Biochemistry*, **35**, 2885–2893.
- Colognato, H., Winkelmann, D.A. and Yurchenco, P.D. (1999) Laminin polymerization induces a receptor-cytoskeleton network. *J. Cell Biol.*, **145**, 619–631.
- Tunggal, P., Smyth, N., Paulsson, M. and Ott, M.C. (2000) Laminins: structure and genetic regulation. *Microsc. Res. Technol.*, **51**, 214–227.
- Yurchenco, P.D., Amenta, P.S. and Patton, B.L. (2004) Basement membrane assembly, stability and activities observed through a developmental lens. *Matrix Biol.*, **22**, 521–538.
- Sanes, J.R. and Hall, Z.W. (1979) Antibodies that bind specifically to synaptic sites on muscle fiber basal lamina. *J. Cell Biol.*, **83**, 357–370.
- Hunter, D.D., Shah, V., Merlie, J.P. and Sanes, J.R. (1989) A laminin-like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. *Nature*, **338**, 229–234.
- Miner, J.H. and Patton, B.L. (1999) Laminin-11. *Int. J. Biochem. Cell Biol.*, **31**, 811–816.
- Kobayashi, N., Mominoki, K., Wakisaka, H., Shimazaki, Y. and Matsuda, S. (2001) Morphogenetic activity of extracellular matrices on cultured podocytes. Laminin accelerates podocyte process formation in vitro. *Ital. J. Anat. Embryol.*, **106**, 423–430.
- Noakes, P.G., Gautam, M., Mudd, J., Sanes, J.R. and Merlie, J.P. (1995) Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin beta 2. *Nature*, **374**, 258–262.
- Wang, T.H., Lindsey, J.D. and Weinreb, R.N. (1994) Laminin subtype distribution in the human ciliary body. *Invest. Ophthalmol. Vis. Sci.*, **35**, 3776–3782.
- Ljubimov, A.V., Burgesson, R.E., Butkowski, R.J., Michael, A.F., Sun, T.T. and Kenney, M.C. (1995) Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. *Lab. Invest.*, **72**, 461–473.
- Libby, R.T., Lavalley, C.R., Balkema, G.W., Brunken, W.J. and Hunter, D.D. (1999) Disruption of laminin beta2 chain production causes alterations in morphology and function in the CNS. *J. Neurosci.*, **19**, 9399–9411.
- Hunter, D.D., Llinas, R., Ard, M., Merlie, J.P. and Sanes, J.R. (1992) Expression of s-laminin and laminin in the developing rat central nervous system. *J. Comp. Neurol.*, **323**, 238–251.
- Kobayashi, N. (2002) Mechanism of the process formation; podocytes vs. neurons. *Microsc. Res. Technol.*, **57**, 217–223.
- Lathrop, G.M. and Lalouel, J.M. (1984) Easy calculations of lod scores and genetic risks on small computers. *Am. J. Hum. Genet.*, **36**, 460–465.
- Sorokin, L.M., Conzelmann, S., Ekblom, P., Battaglia, C., Aumailley, M. and Timpl, R. (1992) Monoclonal antibodies against laminin A chain fragment E3 and their effects on binding to cells and proteoglycan and on kidney development. *Exp. Cell Res.*, **201**, 137–144.
- Sixt, M., Hallmann, R., Wendler, O., Scharffetter-Kochanek, K. and Sorokin, L.M. (2001) Cell adhesion and migration properties of beta 2-integrin negative polymorphonuclear granulocytes on defined extracellular matrix molecules. Relevance for leukocyte extravasation. *J. Biol. Chem.*, **276**, 18878–18887.
- Aigner, T., Neureiter, D., Volker, U., Belke, J. and Kirchner, T. (1998) Epithelial-mesenchymal transdifferentiation and extracellular matrix gene expression in pleomorphic adenomas of the parotid salivary gland. *J. Pathol.*, **186**, 178–185.