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ORIGINAL ARTICLE

Identification of novel pathogenic variants of *CUBN* in patients with isolated proteinuria

Huihui Yang[1] | **Lanfen He**[1] | **Hongjian Gong**[2] | **Chunhui Wan**[3] | **Juanjuan Ding**[1] | **Panli Liao**[1] | **Xiaowen Wang**[1]

[1]Department of Nephrology, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Center), Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China

[2]Clinical Research Center, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Center), Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China

[3]Precision Medical Center, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Center), Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China

Correspondence
Xiaowen Wang, Department of Nephrology, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Center), Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China.
Email: xiaowenwang331@163.com

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Abstract

Background: Although proteinuria is long recognized as an independent risk factor for progressive chronic kidney diseases, not all forms of proteinuria are detrimental to kidney function, one of which is isolated proteinuria caused by *cubilin* (*CUBN)*-specific mutations. *CUBN* encodes an endocytic receptor, initially found to be responsible for the Imerslund–Gräsbeck syndrome (IGS; OMIM #261100) characterized by a combined phenotype of megaloblastic anemia and proteinuria.
Methods: After analyzing their clinical and pathological characterizations, next-generation sequencing for renal disease genes or whole-exome sequencing (WES) was performed on four patients with non-progressive isolated proteinuria. *CUBN* biallelic pathogenic variants were identified and further analyzed by cDNA-PCR sequencing, immunohistochemistry, minigene assay, and multiple in silico prediction tools, including 3D protein modeling.
Results: Here, we present four patients with isolated proteinuria caused by *CUBN* C-terminal biallelic pathogenic variants, all of which showed no typical IGS symptoms, such as anemia and vitamin B12 deficiency. Their urine protein levels fluctuated between +~++ and estimated glomerular filtration rate (eGFR) were normal or slightly higher. Mild mesangial hypercellularity was found in three children's renal biopsies. A homozygous splice-site variant of *CUBN* (c.6821+3 (IVS44) A>G) was proven to result in the exon 44 skipping and premature translation termination by cDNA sequencing and immunohistochemistry. Compound heterozygous mutations were identified among the other three children, including another novel splice-site variant (c.10764+1 (IVS66) G>A) causing the retention of first 4 nucleotides in intron 66 by minigene assay, two unreported missense mutations (c.4907G>A (p.R1636Q); c. 9095 A>G (p.Y3032C)), and two reported missense mutations in China (c.8938G>A (p.D2980N); c. 9287T>C (p.L3096P)), locating behind the vitamin B12-binding domain, affecting CUB11, CUB16, CUB22, CUB23, and CUB27 domains, respectively.

Huihui Yang and Lanfen He have contributed equally to this work.

Conclusion: These results demonstrate that above *CUBN* mutations may cause non-progressive and isolated proteinuria, expanding the variant spectrum of *CUBN* and benefiting our understanding of proteinuria and renal function.

KEYWORDS

CUBN gene, isolated proteinuria, pathogenic variants

1 | INTRODUCTION

With advances in next-generation sequencing and cohort analysis of genotype and phenotype, it is proposed that not all forms of proteinuria are damaging, challenging a long-time-held opinion that persistent proteinuria was correlated with a decline in renal function and needed proteinuria-lowering treatments.

Mutations in *CUBN* were initially identified in a rare autosomal recessive IGS with megaloblastic anemia and a variable degree of proteinuria, due to inefficient uptake of vitamin B12 in the intestine and impaired reabsorption of selective protein in the renal proximal tubule, respectively (Aminoff et al., 1999; Amsellem et al., 2010). Mechanically, cubilin was one of the two receptors identified to be involved in protein reabsorption for glomerular ultrafiltrate, and the other is megalin. Both are large molecular weight proteins and can provide a broad interaction platform for multiple proteins required for reabsorption in structure. Notably, unlike megalin, cubilin is devoid of a transmembrane domain, whose membrane anchoring is dependent on amnionless (AMN), a 45-kD transmembrane protein (Larsen et al., 2018). Functional *CUBN* deficiency was proven to be associated with urine excretion of cubilin ligands such as albumin, transferrin, or apoA-I (Amsellem et al., 2010).

Compared with animal models with *CUBN* complete deletion, *CUBN* mutations in patients usually involve missense and truncated mutations, whose clinicopathologic features vary with the location of mutation site. Observations gleaned from human diseases with *CUBN* variants demonstrate that all IGS mutations can exclusively be found before or within the vitamin B12/intrinsic factor-binding region (CUB 5–8) (aa 66–1390), whereas proteinuria without vitamin B12 malabsorption was caused by mutations located after this region (aa 1487–3618) (Bedin et al., 2020; Böger et al., 2011; Kristiansen et al., 2000). Intravenous and intramuscular vitamin B12 injection can cure the patient's neurologic and hematologic symptoms for IGS patients with albuminuria. However, the proteinuria was persistent with normal renal function (Hauck et al., 2008; Ovunc et al., 2011). Therefore, the dysfunction of C-terminal CUB domains seems to be more tolerated than those related to vitamin B12 absorption.

In this study, we reported four children with chronically isolated proteinuria and normal renal function, in whom biallelic pathogenic variants of the *CUBN* gene were identified and characterized. These results indicated that some functional variations in *CUBN* with isolated proteinuria might not be damaging to renal function.

2 | MATERIALS AND METHODS

2.1 | Whole-exome sequencing and pathogenicity analysis

Genomic DNA was extracted from peripheral blood samples using the Blood Genome Column Medium Extraction Kit (Kangweishiji, China) according to the standard protocol. To detect the genetic causes of proteinuria, genetic variants were screened by WES or a panel including 348 kidney disease-related genes. Pathogenicity analysis of variants was performed according to the American College of Medical Genetics and Genomics (ACMG) practice guidelines. Bidirectional direct Sanger sequencing was performed to validate the screened variants. Prediction results of computational software (PolyPhen-2, MutationTaster, GenoCanyon, CADD_phred, and PROVEAN) were used to evaluate the pathogenic effects of the variants.

2.2 | RNA extraction and cDNA sequencing

Total RNA was isolated from the peripheral blood leukocytes of the patient and control individuals by TRIzol LS reagent, and RNA was reverse-transcribed using a SuperScript™ III First-Strand Synthesis System kit (Invitrogen, CA, USA) according to the manufacturer's protocol. To investigate the abnormal splicing of the variant of *CUBN*, we amplified *CUBN* cDNA using primers spanning exons 42 to 46 in the patient of case 1 and a normal control individual (NC). Then, the obtained PCR

products were analyzed by gel electrophoresis on a 2% agarose gel and further sequenced by the Wuhan QingKe Company.

2.3 | Plasmid construction and minigene assay

A pcDNA3.1 vector was used as the basic plasmid for the minigene. Three DNA fragments containing the CUBN exon 65, exon 66, and exon 67 and corresponding flanking sequences (~200 bp) were obtained by PCR of genomic DNA of human cell line HKE293T. Then, these DNA fragments were inserted into the basic vector by infusion methods, which was named pcDNA3.1-CUBN-wt. The variation of c.10764+1(IVS66) G>A was achieved by site-directed mutation DNA amplification, named pcDNA3.1-CUBN-mut.

HEK293T cells were transfected with 2 μg DNA of pcDNA3.1-CUBN-wt and pcDNA3.1-CUBN-mut, respectively. After 36 h of transfection, RNA extraction, cDNA sequencing, agarose gel electrophoresis, and Sanger sequencing were conducted to analyze splicing anomalies due to c.10764+1(IVS66) G>A.

2.4 | Histological analysis and microscopic observation

Renal biopsies were performed on three patients. Control samples of immunohistochemistry were taken from normal kidney tissue during tumor resection. The samples for light microscopy were fixed in neutral buffered formalin and processed according to the standard procedures of paraffin sections. Then, the sections were stained with HE, PAS, PASM, Masson, IF, and IHC, respectively. Digital images were obtained with a light microscope (Olympus). Rabbit monoclonal to cubilin C-terminal was purchased from Abcam (ab191073). The electron microscopic core laboratory of Renmin Hospital of Wuhan University performed electron microscopic samples.

2.5 | Molecular modeling and structural analysis

The 3D modeled structures of *CUBN* protein for the wild type and mutant types were prepared using homology modeling in SWISS-MODEL. Structural analysis and attribution of the residue interaction networks to the protein function were analyzed and visualized using the PyMOL software.

3 | RESULTS

3.1 | Clinical manifestations

The clinical and renal pathological features of our four patients are summarized in the table in Graphical Figure. All patients presented with isolated proteinuria (+~++) but without edema or decrease in serum albumin level. There were no abnormalities on physical examination, blood biochemical analysis, and developmental tests (Graphical Figure 1a). In a more detailed urine protein test, the urine β2-microglobulin was in the normal range for three patients and slightly increased in one patient, although the urine microalbumin and α1-microglobulin were always above the normal range. Notably, their serum creatinine level and eGFR showed no progressive pathological changes during the extended follow-up. Early treatment was fosinopril tablets (ACEi), later replaced with Chinese patent medicine for kidney protection, such as Bailing capsule (*Cordyceps sinensis*) and Huaiqihuang granules, based on our comprehensive considerations and the conclusions that patients with isolated proteinuria due to *CUBN* mutation did not respond to ACEi/ARB treatment (Bedin et al., 2020; Domingo-Gallego et al., 2022).

The 10-year-old boy of case 1 was found to have asymptomatic proteinuria at 3 months of age during the preoperative evaluation for transcatheter closure of atrial septal defect (ASD). The procedure of transcatheter closure was successful, but the amount of proteinuria fluctuated from 0.3–0.6 g/24 h (+~++). The light microscope histology with HE, PAS, PASM, and Masson staining was unremarkable except for mild mesangial proliferation in the 22 non-sclerotic glomeruli (Figure S1a). Immunofluorescence stains showed no IgG, IgA, IgM, C3, or C1q deposits. Staining of collagen type IV was also normal (Figure S1b). Meanwhile, no obvious foot process effacement or other ultrastructural lesions of podocytes were observed under electron microscopy (Figure S1c).

The remaining three boys in cases 2–4 were found to have proteinuria with chief complaints of frequent and urgent urination or bubble urine. At multiple follow-up examinations, their proteinuria persisted, but the renal function was normal. Meanwhile, no other clinical progression was found. Similar to the first patient, only slight mesangial hyperplasia in glomeruli was observed, and no deposits were discovered in immunofluorescence stains in patients 2 and 3 under light microscopy (Figure S2a). In addition, slight vacuolar degeneration was also observed in partial renal tubule epithelial cells in patient 2 (Figure S2a). In the electron microscopic results of patient 2, the foot process was partially fused,

and few electron-dense deposits were occasionally observed in the mesangial region (Figure S2b). Except for slight mesangial hyperplasia, no other abnormalities were observed in the electron microscopic results of patient 3.

3.2 | Identification of *CUBN* gene mutation

Considering that the first patient's parents were first cousins and there was no family history of kidney disease, we performed next-generation sequencing (panel including 348 kidney disease-related genes) to test whether the etiology of proteinuria was due to a recessively inherited mutation. A homozygous splice-site mutation of *CUBN* (c.6821+3 (IVS44) A>G) was detected in this patient, with his father and mother carrying the same heterozygous mutation, respectively. And no variants were identified in other candidate genes for kidney diseases. Direct sequencing of the cDNA-PCR product confirmed the skipping of exon 44, which introduced an early stop codon and generated cubilin-truncated protein ending at the CUB16 domain (Graphical Figure 1b and 1e). Immunohistochemistry stain further demonstrated that the signal of cubilin antibody against C-terminal was absent in the patient's renal proximal tubule compared to control (normal kidney tissue from tumor resection) (Graphical Figure 1c). Thus, we speculate that the isolated moderate proteinuria of this boy is caused by the homozygous mutation of cubilin.

To seek the cause of persistent proteinuria in the remaining three children, Trio-WES was performed, and biallelic pathogenic variants of *CUBN* were identified. Patient 2 carried compound heterozygous mutations c.4907 (exon 33) G>A (p.R1636Q) and c.10764+1 (IVS66) G>A, with the parents being carriers of each. The former was an unreported missense mutation locating at the CUB11 domain, described as 'probably damaging' with PolyPhen-2_HDIV and GenoCanyon, and 'tolerable' for CADD and PROVEAN. The other splice-site mutation was proven to cause retention of the first four bases of intron 66 (ATGA) in minigene assay, using pcDNA3.1(−)-hCUBN-exon 65-exon 66-exon 67 (the intron-associated flanking sequence of each exon is about 200 bp) expression plasmid to simulate the wild-type (WT) genome sequence and pcDNA3.1(−)-hCUBN-exon 65-exon 66(c.10764+1G>A)-exon 67 to mimic mutated (MT) genome sequence. The above results indicate that the cell selected c.10764+5_6 (IVS66) GT sequence as new splicing sites when the G at c.10764+1 was mutated to A. The intron 66 retention of first 4 nucleotides caused frameshift translation from aa3589 and early termination (Graphical Figure 1d).

Patient 3 was also proven to carry three compound heterozygous variation sites of *CUBN* gene. c.9287 (exon 59) T>C (p.L3096P) was inherited from his father, while c.9095 (exon 57) A>G and c.3371 (exon 24) A>G were inherited from his mother. Of note, the former (p.L3096P at the CUB23 domain) was the same as that of our patient 4's and a recently reported pathogenic site of *CUBN*, whose mutation caused a decrease in the number of hydrogen bonds within the protein using 3D structure model (Yang et al., 2022). One mutation site (c.9095 A>G; p.Y3032C at the CUB22 domain) from the mother was predicted to be harmful, while the other (c.3371 A>G; p.Y1124C at the gap between two Ca^{2+} binding sites) was predicted to be uncertain by above software (Graphical Figure 1e). Besides c.9287 (exon 59) T>C from the mother, c.8938 (exon 57) G>A (p.D2980N) was identified as the other compound heterozygous site from the father in patient 4, which was located at the CUB22 domain and predicted to be harmful (Graphical Figure 1e).

Since cubilin was essential in the homeostasis of vitamin B12 and anemic phenotype, we further detected the level of vitamin B12 and hemoglobin, both of which are within the normal reference range. The hemoglobin and the serum creatinine were also maintained normally in the follow-up duration for these four patients. Therefore, we infer that the corresponding correct amino acid of mutation site in our patients may be important for binding specific proteins in the glomerular filtration rather than vitamin B12.

3.3 | Protein structure modeling

To figure out the phenotype–genotype relationship, we located these *CUBN* variants in the corresponding *CUBN* protein domain and built 3D structure models of four missense mutations using SWISS-MODEL (Graphical Figure 1f). p.Arg1636Gln did not affect the hydrogen bond interaction in cubilin protein itself. However, since this site was located at the junction of two β-folded layers and was positively charged in the normal population, p.Arg1636Gln might affect charge interaction between cubilin and interactors, such as albumin, through negatively charged amino acid sites. Similarly, p.Tyr3032Cys did not affect the hydrogen bonding within cubilin. Because the phenyl ring also plays a major role in protein interaction, the loss of this functional group might explain the pathogenicity of this missense mutation site. p.Asp2980Asn and p. Leu3096Pro weakened the hydrogen bond interaction with Asn3020 and Phe3112, respectively, which destabilized cubilin protein structure.

4 | DISCUSSION

The measure of proteinuria has been helping us diagnose and assess kidney function, and persistent proteinuria is long known as an indicator of kidney damage. Pathologically, proteinuria caused by reduced reabsorption in the proximal tubule should be considered after ruling out the possibility of disturbance in the glomerular filtration barrier and other extrarenal factors. At the cellular level, interaction with receptors at tubular epithelial cells turns on the first step of protein reabsorption, which depends on the cooperation of cubilin, megalin, and AMN (Christensen et al., 2013). Therefore, dysfunction of cubilin appears to be detrimental to tubule reabsorption. However, a few reports have discovered that albuminuria due to reduced cubilin function could be an unexpectedly benign rather than a pathological condition, along with the first proposal by Bedin et al. that human C-terminal CUBN variants associated with chronic proteinuria and normal renal function in 2020 (Bedin et al., 2020; Domingo-Gallego et al., 2022; Jayasinghe et al., 2019; Ovunc et al., 2011). Meanwhile, the idea that some functional variations in *CUBN* might not be damaging but instead protective was also proposed, based on the results that diabetic mice and part diabetic individuals with lower expression of cubilin in the renal proximal tubule would reduce the reabsorption of glucose, probably due to both inhibition of glucose and protein uptake (Ahluwalia et al., 2019; Figueira et al., 2017; Simons, 2018). Bedin et al. found mild protective effects in GWAS in the general population; for some variants, associations were stronger when stratified for diabetes status (Bedin et al., 2020). Therefore, the cubilin variant sites associated with isolated proteinuria may be used as one of the therapeutic targets for diabetic patients in the future.

As a broad platform, cubilin is structurally characterized by 27 C-terminal CUB domains for efficient interaction with different ligands. The N-terminal consists of a stretch of 110 amino acids followed by 8 EGF-like repeats. The initial signal peptide would be cleaved by furin, and then, processed cubilin was transported to the plasma membrane through the post-Golgi vesicles (Kozyraki et al., 1998). The region where cubilin interacts with AMN for its membrane anchoring is also located at the N-terminal. Animal models showed that the expression and plasma membrane localization of cubilin and AMN are interdependent (Amsellem et al., 2010). The kidney biopsy from our patient 1 showed that the expression of AMN was unaffected with cubilin truncation losing CUB domains 17–27 (data not shown). After all, when AMN-binding site or N-terminal half of CUBN domains were affected, patients would show more than just isolated proteinuria. Another evidence was also recently presented by Gan et al. that the variants after the vitamin B12-binding domain of *CUBN* merely disrupted the membrane localization of AMN but did not affect its protein level, indicating the C-terminal domain of *CUBN* might be involved in the stability maintenance of AMN–cubilin binding (Gan et al., 2022). Besides minimal lesions of mild mesangial cell proliferation observed in patients 1 and 3, biopsies in patient 2 also showed slight fusion of the foot process and slight vacuolar degeneration in few renal tubule epithelial cells. The former may be related to the expression and function of cubilin in podocytes (Prabakaran et al., 2012; Yang et al., 2022). The latter might be associated with the injury of renal tubular epithelial cells by the overload of protein- and albumin-bound lipids (Ruggiero et al., 2014). Despite minor lesions on pathology examination in the first hospitalization, the eGFR in patient 2 remained in the normal or slightly higher range during long-term follow-up.

Collectively, we identified four patients with isolated proteinuria-associated *CUBN* biallelic mutations at the C-terminal. In addition to three reported mutations in China, one novel splice-site variant and two unreported missense mutations are also identified. We also provided experimental evidence for the effects of splice-site mutation c.6821+3 (IVS44) A>G and c.10764+1 (IVS66) G>A on *CUBN* mRNA processing and in silico evidence for the other pathogenic mutation sites, which involves in protein stability or ligand binding. Our results add to the growing list of *CUBN* abnormalities responsible for hereditary isolated proteinuria and illustrate the clinical heterogeneity of CUBN mutations based on the position of mutation. It is important for the pediatrician to consider inherited disorders of C-terminal *CUBN*-associated proteinuria through Trio-WES or cheaper kidney disease gene panel, when the patient presents early childhood onset and chronic proteinuria along with unaffected renal function, which helps us avoid unnecessary exposure to ACEi/ARB medications and biopsy.

AUTHOR CONTRIBUTIONS

YH drafted the manuscript and performed minigene assay. LH collected clinical data with the help of PL and JD. HG performed cDNA-PCR sequencing assay. CW collected and analyzed genetic data. XW interpreted the data and revised the manuscript. All authors have critically read and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL STATEMENT AND PATIENT CONSENT STATEMENT

Our study obtained all cases and control individuals from Wuhan Children's Hospital. Clinical information and peripheral blood samples were collected from the family after obtaining individual written informed consent. Written informed consent was obtained from a parent or legal guardian for participants under 18 years old. Genetic testing was performed in accordance with the Helsinki Declaration and approved by the ethics committee of Wuhan Children's Hospital (approval number: 2022R024-E01).

ORCID

Huihui Yang https://orcid.org/0000-0002-1836-1387

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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