

Exome Sequencing Reveals Cubilin Mutation as a Single-Gene Cause of Proteinuria

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ABSTRACT

In two siblings of consanguineous parents with intermittent nephrotic-range proteinuria, we identified a homozygous deleterious frameshift mutation in the gene *CUBN*, which encodes cubulin, using exome capture and massively parallel re-sequencing. The mutation segregated with affected members of this family and was absent from 92 healthy individuals, thereby identifying a recessive mutation in *CUBN* as the single-gene cause of proteinuria in this sibship. Cubulin mutations cause a hereditary form of megaloblastic anemia secondary to vitamin B₁₂ deficiency, and proteinuria occurs in 50% of cases since cubilin is coreceptor for both the intestinal vitamin B₁₂-intrinsic factor complex and the tubular reabsorption of protein in the proximal tubule. In summary, we report successful use of exome capture and massively parallel re-sequencing to identify a rare, single-gene cause of nephropathy.

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Protein-encoding exons constitute only 1% of the human genome but harbor 85% of mutations in single-gene disorders.¹ Among about 2800 Mendelian diseases, for which the causative gene has been identified, mutations affect the coding region or canonical splice sites and thereby the function of the encoded proteins.¹ However, PCR amplification of thousands of candidate exons is costly and impractical and has hampered discovery of single-gene disease causes. Recently, re-sequencing of entire coding regions of the human genome, the “exome,” with consecutive massively parallel (MP) re-sequencing has dramatically changed this situation.² This approach has been successfully applied to identify single-gene causes of rare diseases including Miller syn-

drome,² Kabuki syndrome,³ Schinzel-Giedion syndrome,⁴ Bartter syndrome,⁵ and nephronophthisis type 10.⁶

Nephrotic syndrome (NS) is a common kidney disease characterized by proteinuria, hypoalbuminemia, generalized edema, and hyperlipidemia. Identification of recessive single-gene causes of NS has provided important insights into the pathogenesis of this enigmatic disorder. Whereas *NPHS2* mutations explain 10 to 25% of childhood NS, and two thirds of all NS in the first year of life can be explained by mutations in four genes only (*NPHS1*, *NPHS2*, *WT1*, and *PLCE1*),⁷ most other recessive causes of NS are very rare (1 to 3% of cases).^{8–13} Whereas single-gene causes of NS occur in childhood and adolescence, in more than about 70% of cases the causative gene muta-

tion is still unknown.⁷ This forbids the use of cohort studies for gene identification and necessitates the ability to identify disease-causing genes in single families. We therefore combined whole genome homozygosity mapping with consecutive whole human exome capture (WHEC) and massively parallel re-sequencing to overcome this limitation.⁶ In this way we here identify a homozygous deleterious frameshift mutation within the *cubilin* gene (*CUBN*) as a novel unexpected cause of proteinuria in two siblings with intermittent nephrotic-range proteinuria. This approach will likely be very useful to rapidly solve cases of rare nephropathies.

RESULTS

Two siblings (A 2410-21 and A2410-22) from family A2410 were discovered to have proteinuria on routine examina-

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tion. The amount of proteinuria in both siblings had been fluctuating, sometimes reaching up to 2 g/d and then decreasing without any treatment (see Patients in the Concise Methods section). Given consanguinity of the parents of the two siblings with proteinuria, we hypothesized that its cause was a recessively inherited mutation. Therefore, we performed linkage analysis and homozygosity mapping in both affected siblings (A2410-21 and A2410-22) using Affymetrix 250K SNP StyI arrays. Nonparametric LOD score analysis¹⁴ was calculated for both siblings together, yielding seven segments of homozygosity by descent on chromosomes 3, 10, 14, 17, 21, and 22 with a total cumulative genomic length of 130 Mb (Figure 1). One segment of homozygosity on chromosome 10 coincided with *PLCE1*, a known locus for nephrotic syndrome. However, Sanger resequencing of all exons and adjacent exon-intron boundaries of *PLCE1* yielded no mutation in this gene.

We then performed whole human exome capture in one sibling (A2410-22) using NimbleGen 2.1M Human Exome Array with consecutive massively parallel re-sequencing on two lanes of an Illumina-GAIIX sequencing platform to identify the underlying disease-causing mutation. Sequencing generated 29.5 million single-end reads of 78 bases. Quality-filtered sequence reads were

aligned to the human reference genome assembly hg19 (NCBI build 37) using “CLC Genomics Workbench” software (CLC-bio, Aarhus, Denmark). About 20.1 million of these reads (68%) matched the targeted exons contributing to a median coverage of 11-fold (mean: 14-fold). No coverage was obtained for about 2.2% of all 180,000 targeted exons. CLC Genomics Workbench software was further used to call single nucleotide variants or small insertions/deletions. By filtering for homozygous-only variants, we identified 1968 variants from the reference sequence with at least fivefold sequence coverage. Of these, only 48 were not known single nucleotide polymorphisms, either reported in the database dbSNP130 or the 1000 genomes project. When examining the segments of homozygosity (132 Mb total), for which we had initially generated a positional candidate hypothesis by whole genome homozygosity mapping, only 11 nonsynonymous exonic variants remained. One of these changes was a novel 1-bp homozygous deletion (c.8355delA) in exon 53 of the gene *CUBN* (cubilin), resulting in a frameshift and a predicted premature truncation of the encoded protein (p.S2785fsX19) (Figure 2). We confirmed this mutation by exon-PCR and Sanger sequencing homozygously in both affected siblings and heterozy-

gously in both parents (Figure 3). We then searched for additional *CUBN* mutations in a large worldwide cohort of 540 families with NS in whom we had generated homozygosity mapping data by WGHM. Four affected individuals (A155-21, A849-21, A1605-21, and A2591-21) of these families showed a genomic segment of >2 Mb of homozygosity at the *CUBN* locus. However, direct Sanger sequencing of all exons of *CUBN* in these individuals did not yield any additional mutations (data not shown).

DISCUSSION

We here detected a novel 1-bp homozygous deletion of the *cubilin* (*CUBN*) gene as the cause of proteinuria in two siblings with NS using a combined strategy of homozygosity mapping and WHEC with massively parallel re-sequencing. The finding represented an unexpected cause of NS because intermittent gross proteinuria is rare in patients with *CUBN* mutations who are known to have Imerslund-Grasbeck syndrome (IGS; OMIM ID #261100), a form of congenital megaloblastic anemia due to vitamin B₁₂ deficiency caused by a defect in the vitamin B₁₂/intrinsic factor receptor (*CUBN*; OMIM ID #602997).

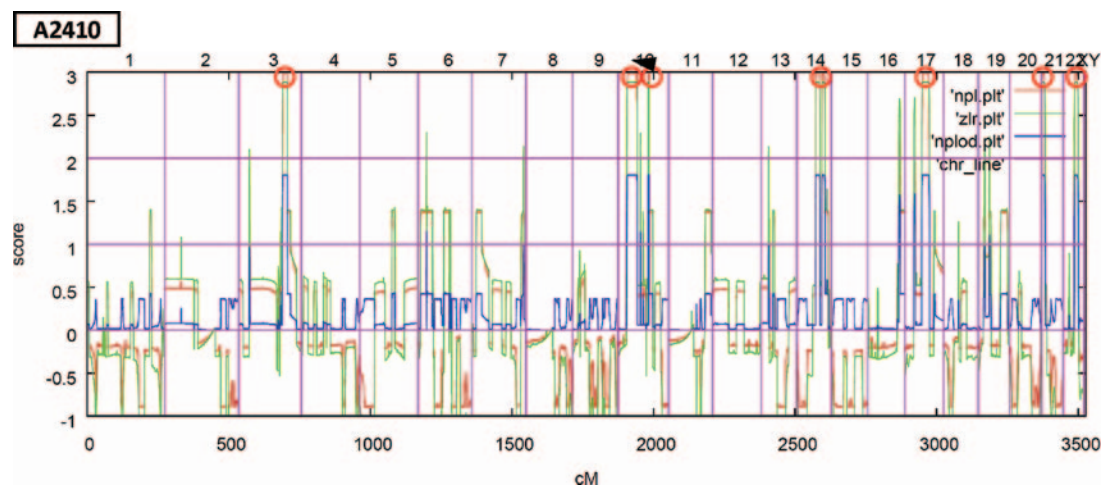


Figure 1. Nonparametric lod (NPL) scores across the human genome in two siblings with intermittent gross proteinuria of consanguinous family A2410 reveal four regions of homozygosity by descent. The x-axis shows Affymetrix 250K SNP StyI array SNP positions on human chromosomes concatenated from p-ter (left) to q-ter (right). Genetic distance is given in centimorgan (cM). Seven maximum NPL peaks (red circles) indicate candidate regions of homozygosity by descent. The arrow shows the region of homozygosity in chromosome 10, which includes *CUBN*.



Figure 2. The novel single-base homozygous deletion (c.8355delA) in exon 53 of the cubilin (*CUBN*) gene that causes nephrotic syndrome in family A2410. Note that the sequence shown depicts the plus strand of chromosome 10 and that *CUBN* is encoded on the reverse strand, therefore showing a deletion of a T (thymine) rather than A (adenine).

The renal effects of genetic ablation of cubilin in a mouse model has been reported previously.¹⁵ It was observed that proximal tubule cells did not localize the receptor protein “amionless” to the plasma membrane in the absence of cubilin, indicating a mutual dependency of cubilin and amionless to form a functional membrane receptor complex. Furthermore, cubilin-deficient mice exhibited markedly decreased uptake of albumin by proximal tubule cells, leading to proteinuria. It was shown that cubilin is essential for albumin reabsorption by proximal tubule cells.¹⁵ Recently, an association between a missense variant (I2984V) in the *CUBN* gene and both urinary albumin-to-creatinin ratio and microalbuminuria has been

identified in a genome-wide association study.¹⁶

In this study we detected by total human exome capture a novel homozygous deleterious *CUBN* mutation as the cause of proteinuria in a family in whom absence of megaloblastic anemia did not elicit any suspicion of IGS, most likely because of their young age. IGS represents the full clinical picture of *CUBN* mutations. This is an example of molecular genetics providing a diagnostic tool that is more sensitive and accurate than clinical diagnosis, a possibility that will be strongly enhanced as WHEC and massively parallel re-sequencing become more established.

Moreover, genetic diagnostics might inform therapy: Recently, another case

with IGS and *CUBN* mutation was reported. The patient was a 15-year-old girl of German descent who presented with megaloblastic anemia, selective proteinuria, and funicular myelosis. After intravenous vitamin B₁₂ therapy and consecutive intermittent intramuscular injection of vitamin B₁₂, the patient’s neurologic symptoms ceased completely after 2 months, hematologic parameters normalized within 5 months, and although proteinuria was persistent, renal function did not deteriorate.¹⁷ Likewise, the siblings described here might benefit from vitamin B₁₂ replacement therapy.

At the cellular level, IGS, a monogenic cause of megaloblastic anemia (MGA1: OMIM ID #261100), is due to defective intestinal B₁₂ malabsorption and/or re-

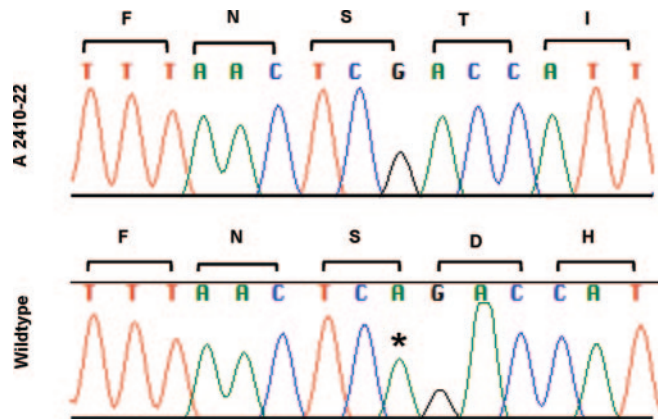


Figure 3. Novel homozygous mutation in *CUBN*. A novel homozygous of a single-bp deletion is shown that leads to a frameshift mutation in exon 53 of *CUBN* (c.8355delA; p.S2785fsX19) in patient A2410-22 who has intermittent nephrotic range proteinuria. Nucleotide sequence traces and deduced amino acid sequence are shown for mutated (top) and wild-type (bottom) sequences. Adenine deleted in A2410-22 is marked with an asterisk in the wild-type sequence. Mutation numbering is based on *CUBN* human reference sequence NM_001081.3, where +1 corresponds to the A of the ATG translation start codon.

nal tubular protein reabsorption. IGS occurs worldwide, but its prevalence is higher in several Middle Eastern countries and in Norway, and highest in Finland (0.8 in 100,000 individuals). By genetic mapping, Finnish-type IGS has been mapped to the *CUBN* gene locus, whereas Norwegian-type IGS has been mapped to the *AMN* gene locus.¹⁸ In families from the Mediterranean an Israeli Jewish family of Tunisian origin,¹⁸ a Tunisian family,¹⁹ and Turkish families²⁰ two different *AMN* mutations and three different *CUBN* mutations were detected. Outside these geographic regions, only scattered cases have been described, for example, South Africa,²¹ France,²² the United States,²³ and Taiwan.²⁴ The Scandinavian cases were typical examples of founder effects, whereas in the Mediterranean region, instead of a founder effect, high degrees of consanguinity exposed rare homozygous mutations in both genes.

We here describe a novel homozygous frameshift mutation in two siblings from family A2410. Sibling A2410-21 had intermittent nephrotic range proteinuria and sibling A2410-22 had intermittent nephrotic range proteinuria along with epilepsy as the only clinical presentations. Anemia was not a part of their clinical course. *CUBN* mutations represent a

recessive single-gene cause of proteinuria. Recessive single-gene disease causes convey full penetrance of a disease. They are thereby distinct from genetic variants that are found only to be associated with disease because associated variants usually explain only a low percentage of the phenotypic variance, as is the case for instance in the *MYH9* and *APOL1* variants that have been found in nephrotic syndrome.²⁵

In conclusion, the combination of homozygosity mapping in consanguineous siblings together with WHEC and massively parallel re-sequencing provides a tool for the evaluation of patients with undiagnosed genetic diseases. Our findings also indicate that IGS should be considered when diagnosing individuals with proteinuria to identify potentially treatable variants.

CONCISE METHODS

Patients

We obtained blood samples, pedigrees, and clinical information after receiving informed consent (<http://www.renalgenes.org>) from all patients. Approval for experiments on humans was obtained from the University of Michigan Institutional Review Board. One of the siblings of a consanguineous family from

Egypt (A2410-22) was found to have proteinuria during a routine urine analysis preceding an operation for hypospadias. A2410-22 had proteinuria of 790 mg/d. Serum creatinine was 0.4 mg/dl, serum protein 6.5 g/L, serum albumin 4.2 g/L with normal creatinine clearance, and normal C3 levels at 4 years of age. Then the other sibling of the family (A2410-21) was tested for proteinuria. Sibling A2410-21 had proteinuria of 380 mg/d. Serum creatinine was 0.4 mg/dl, total serum protein 6.8 g/L, and serum albumin 3.5 g/L. Creatinine clearance and complement 3 (C3) level were normal at 5 years of age. Both siblings were treated with ACE inhibitors with no improvement of their condition. The amount of proteinuria in both siblings had been fluctuating, sometimes reaching up to 2 g/d and then decreasing without any treatment. However, no edema developed and no immunosuppressive treatment was instituted. Renal biopsy was not performed. As for megaloblastic anemia, both siblings had normal hemoglobin levels and the red blood cell indices were also within normal range for their ages. A2410-22 has had complex partial seizures since he was 5-months old, requiring treatment with carbamazepine. He has been seizure free on carbamazepine for 1 year now.

Homozygosity Mapping

For genome-wide homozygosity mapping, the GeneChip Human Mapping 250K StyI Array from Affymetrix was used. Nonparametric LOD scores were calculated using a modified version of the program GENEHUNTER^{26,27} through stepwise use of a sliding window with sets of 110 SNPs. The program ALLEGRO was employed to identify regions of homozygosity as described,²⁸ using a disease allele frequency of 0.0001 and CEU marker allele frequencies.

Whole Human Exome Capture

Genomic DNA (10 μ g) from affected sibling A2410-22 was captured using the NimbleGen 2.1M Human Exome Array (Roche/NimbleGen) according to protocol. The NimbleGen 2.1M Human Exome Array contains oligonucleotide probes that target approximately 180,000 exons of 18,673 protein-coding genes from the consensus coding sequence (CCDS) set in addition to 551 micro-RNAs. To generate random start positions and to reduce fragment sizes to be appropriate for the Illumina

genome analyzer, high-throughput sequencing, captured and amplified DNA fragments in the range of 500 to 700 bp were subsequently modified using Bal31 exonuclease and DNase-I as published previously.²⁹

Massively Parallel Re-sequencing, Sequence Alignment, and Variant Calling

Library construction of the modified captured DNA fragments was performed using the “Genomic DNA Sample Prep Kit” according to the manufacturer’s instructions (Illumina, San Diego). The library was sequenced on two lanes of an Illumina genome analyzer (GAII) as 80 base single-end reads. Image analysis and base calling were generated by the Illumina pipeline 1.5.1 using default parameters. Subsequent sequence alignment to the human genome reference genome (hg19) and variant calling was performed using CLC Genomics Workbench software. Only variants with an allele frequency of more than 80% were called to identify homozygous variants.

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DISCLOSURES

None.

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