

Genetic mutational testing of Chinese children with familial hematuria with biopsy-proven FSGS

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Received July 22, 2016; Accepted August 31, 2017

DOI: 10.3892/mmr.2017.8023

Abstract. Focal segmental glomerulosclerosis (FSGS) is a pathological lesion rather than a disease, with a diverse etiology. FSGS may result from genetic and non-genetic factors. FSGS is considered a podocyte disease due to the fact that in the majority of patients with proven-FSGS, the lesion results from defects in the podocyte structure or function. However, FSGS does not result exclusively from podocyte-associated genes, however also from other genes including collagen IV-associated genes. Patients who carry the collagen type IVA3 chain (COL4A3) or COL4A4 mutations usually exhibit Alport Syndrome (AS), thin basement membrane neuropathy or familial hematuria (FH). Previous studies revealed that long-time persistent microscopic hematuria may lead to FSGS. A case of a family is presented here where affected individuals exhibited FH with FSGS-proven, or chronic kidney disease. Renal biopsies were unhelpful and failed to demonstrate glomerular or basement membrane defects consistent with an inherited glomerulopathy, and therefore a possible underlying genetic cause for a unifying diagnosis was pursued. Genomic DNA of the siblings affected by FH with biopsy-proven FSGS was analyzed, and their father was screened for 18 gene mutations associated with FSGS [nephrin, podocin, CD2 associated protein, phospholipase C ϵ , actinin α 4, transient receptor potential cation channel subfamily C member 6, inverted formin, FH2 and WH2 domain containing, Wilms tumor 1, LIM homeobox transcription factor 1 β , laminin subunit β 2, laminin subunit β 3, galactoside α , integrin subunit β 4, scavenger receptor class B member 2, coenzyme Q2, decaprenyl diphosphate synthase subunit 2, mitochondrially encoded tRNA leucine 1

(UUA/G; TRNL1) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a like 1] using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technology. Then whole exome sequencing (WES) was performed in the two probands to ascertain whether there were other known or unknown gene mutations that segregated with the disease. Using mass array technology, a TRNL1 missense homozygous mutation (m. 3290T>C) was identified in the probands diagnosed with FH and manifested as FSGS on biopsy. In addition, a COL4A4 missense mutation c. 4195A>T (p. M1399L) in heterozygous pattern was identified using WES. None of these variants were detected in their father. In the present study, a mutation in TRNL1 (m. 3290T>C) was identified, which was the first reported variant associated with FSGS. The COL4A4 (c. 4195A>T) may co-segregate with FSGS. Screening for COL4A mutations in familial FSGS patients is suggested in the present study. Genetic investigations of families with similar clinical phenotypes should be a priority for nephrologists. The combination of mass array technology and WES may improve the detection rate of genetic mutation with a high level of accuracy.

Introduction

Focal segmental glomerulosclerosis (FSGS) is a description of a histological lesion, rather than a disease; characterized by focal and segmental glomerular sclerosis and podocyte foot-process effacement and its clinical manifestations include proteinuria and progressive renal failure. Current treatments for FSGS frequently fail to achieve remission (1,2). Therefore, unravelling the pathogenesis of FSGS is of primary concern for the development of targeted therapy.

The etiology of FSGS has been identified as diverse. FSGS may occur following immunologically-mediated injury, genetic factors, circulating permeability factor/s, and hemodynamic adaptations resulting in glomerular hypertrophy and direct podocyte injury also lead to FSGS (3). The most common clinical manifestation of FSGS is proteinuria, which may range from subnephrotic to nephrotic levels. However, a number of the patients with proven-FSGS present with hematuria (4).

The renal glomerular filtering apparatus consists of three major components: The fenestrated endothelial cell layer, the

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Key words: focal segmental glomerulosclerosis, familial hematuria, gene mutation, child

glomerular basement membrane (GBM) and the epithelial podocyte layer. Injury to any layer may result in red blood cells or protein escaping into the urine through a defect in the glomerular filtration barrier. It has been demonstrated that podocyte damage serves a central role in the pathogenesis of FSGS (5,6). A number of genes have also been demonstrated to be mutated in FSGS (7-24) (Table I), and most of the encoded proteins are localized in podocytes, whereas others are expressed in other tissues and cell types including in GBM (20-22,25-26). According to statistical analysis, 1/3-1/2 of children with isolated, persistent hematuria have a familial history (27-28). It has been demonstrated by previous studies that long-term persistent microscopic hematuria (MH) may lead to chronic kidney disease (CKD) (29-32). A total of ~14-50% of familial cases progress to end-stage renal disease (ESRD) on long-term follow-ups (33).

Alport's Syndrome (AS) and thin basement membrane neuropathy (TBMN), which occur most frequently in glomerular MH, result from defects in type IV collagen. The type IV collagen $\alpha 3\alpha 4\alpha 5$ chain is a major component of the GBM and a heterotrimer that is encoded by three genes: Collagen type IV $\alpha 3$ chain (COL4A3), COL4A4 and COL4A5 (34). During the last three decades, six genes (COL4A3, COL4A4, COL4A5, complement factor H related 5, myosin heavy chain 9 and fibronectin 1) have so far been identified in familial microscopic hematuria of glomerular origin (34). In addition to AS and TBMN, familial FSGS may also be a factor resulting in familial glomerular microscopic hematuria (GMH) (4).

It has been demonstrated by previous studies that long-term persistent MH may lead to renal injury regardless of TBMN, AS or other disease presenting with hematuria (29-32). Therefore, pediatric nephrologists need to be aware that children with familial hematuria and a family history of CKD have a high probability of developing proteinuria and progressing to renal failure in adult life. Especially at early stages when MH appears as an isolated warning sign, it is worth having a step-wise algorithm for deeper investigations of the etiology and pathogenesis of the disease.

Advances in DNA analysis technology may facilitate greater use of molecular diagnostics, which reduce the need to use invasive methods including renal biopsy (4). Importantly, molecular diagnostics may be performed at an early stage of disease, frequently providing a broader set of therapeutic options and an increased window of opportunity to ameliorate disease progression (35).

Recently the implementation of high-throughput sequencing technologies including mass array technology and whole exome sequencing (WES) make it possible to test multiple genes simultaneously in a single experiment faster and more efficiently (36). The performance of the next-generation sequencing may help to identify novel genes or novel unreported mutations and discover co-segregating genetic regions. However, the appropriate application and combination of sequencing methods with conventional gene-discovery strategies should be considered for each patient and research project (36). Only then may they be of use in making a diagnosis in a more precise way.

The present study reports on a family in which affected individuals exhibited familial hematuria and the siblings had biopsy-proven FSGS and normal GBM. Renal biopsies

demonstrated non-specific pathological alterations and failed to exhibit glomerular or basement membrane defects consistent with an inherited glomerulopathy, and therefore a possible underlying genetic cause for a unifying and definitive diagnosis was pursued. The present study hypothesized that FSGS in the siblings resulted from a defect in the 18 genes [*nephrin (NPHS1)*, *podocin (NPHS2)*, *CD2 associated protein (CD2AP)*, *phospholipase C ϵ (PLCE1)*, *actinin α 4 (ACTN4)*, *transient receptor potential cation channel subfamily C member 6, (TRPC6)*, *inverted formin, FH2 and WH2 domain containing (INF2)*, *Wilms tumor 1 (WT1)*, *LIM homeobox transcription factor 1 β (β LMX1B)*, *laminin subunit β (LAMB2)*, *LAMB3*, *galactosidase α* , *integrin subunit β 4*, *scavenger receptor class B member 2 (SCARB2)*, *coenzyme Q2 (COQ2)*, *decaprenyl diphosphate synthase subunit 2 (PDSS2)*, *mitochondrially encoded tRNA leucine 1 (UUA/G; TRNL1)* and *SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a like 1*]. Under this assumption, the siblings were identified as possessing a homozygous mutation for TRNL1 (m. 3290T>C), which may segregate with disease using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry technology. WES on the siblings was performed to identify the existence of other genes or mutations that co-segregate with familial hematuria or FSGS when mutated. The results demonstrated that the two sisters carried a single heterozygous mutation c. A4195T (p. M1399L) in the COL4A4 gene, which may serve a role in the pathology of FSGS and act as a modifier to TRNL1.

To the best of the authors' knowledge, this is the first report of a family with familial hematuria and proven-FSGS with a mutation in the TRNL1 gene, and with a mutation in the COL4A4 gene that co-segregated with disease. In addition, this may be the first study to use mass array technology and WES simultaneously in the identification of disease genes.

Materials and methods

Clinical data and DNA preparation. Clinical data and historical renal biopsies were reviewed where available. Following informed consent being obtained, DNA was obtained from the siblings (1~14 years old) and their father (33-34 years old; data not available from their mother) obtained from the Second Xiangya Hospital during the period March/2014-March/2015. The research was approved by the Ethics Commission of the Second Xiangya Hospital (Changsa, China). DNA was isolated from peripheral leukocytes using the DNA purification kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol.

Single nucleotide polymorphism (SNP) analysis using MALDI-TOF technology. The genes and SNPs were selected on the basis of currently available literature (22). The 15 genes were selected following searching databases including PubMed, the Online Mendelian Inheritance in Man (OMIM; www.ncbi.nlm.nih.gov/omim) and the Human Gene Mutation Database (nihlibrary.nih.gov/about-us/news/categories/3051). Database ClinVar and OMIM were searched for clinically relevant mutations or SNPs from the 18 genes. The database search identified 179 candidates. Certain candidates either lacked complete information or were not compatible for

Table I. Selected list of 18 genes associated with FSGS syndrome.

Author, year	Gene	Locus	Inheritance	Protein	Phenotype	(Refs.)
SD-associated and adaptor protein:						
Santin, 2009	NPHS1	19q13.1	AR	Nephrin	CNS/NS, FSGS	(7)
Tonna, 2008	NPHS2	1q25-q31	AR	Podocin	CNS, NS-childhood and adult onset, FSGS	(8)
Gigante, 2009	CD2AP	6p12.3	AD	CD2 associated protein	Early-onset NS, HIV nephropathy, FSGS	(9)
Hinkes, 2006	PLCE1	10q23.33	AR	Phospholipase C ϵ 1	Early-onset NS, DMS	(10)
Santin, 2009	TRPC6	11q22.1	AD	Transient receptor potential cation channel subfamily C member 6,	Adult onset NS, FSGS	(11)
Nuclear proteins:						
Hall, 2013	WT1	11p13	Sporadic, AD	Wilms' tumor 1	Adult onset NS, Denys-Drash and Frasier Syndromes, DMS, FSGS	(12)
Boyer, 2013	LMX1B	9q34.1	AR	LIM homeobox transcription factor 1, β	Nail-Patella Syndrome/NS only,	(13)
Boerkoel, 2002	SMARCAL1	2q34-q36	AR	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a-like 1	Schimke immuno-osseous dysplasia (syndromic immune complex nephritis)	(14)
Actin cytoskeleton and signaling						
Choi, 2008	ACTN4	19q13	AD	α -actinin 4	Adult onset NS, FSGS	(15)
Gbadegesin, 2011	INF2	14q32	AD	Inverted formin 2	Familial/sporadic NS; Charcot-Marie-Tooth, FSGS	(16)
Lowik, 2005	Mitochondrial MTTL1	mtDNA	Maternal inheritance	tRNA-LEU	MELAS syndrome; NS \pm deafness and diabetes	(17)
Diomed-Camassei, 2007	COQ2	4q21.23	AR	Coenzyme Q2 4-hydroxybenzoate polyprenyltransferase	Mitochondrial disease/isolated nephropathy	(18)
Lopez, 2006	PDSS2 GBM	6q21	AR	Prenyl diphosphate synthase subunit 2	Leigh syndrome, FSGS or collapsing glomerulopathy	(19)
Matejas, 2010	LAMB2	3p21	AR	Laminin β 2	Pierson syndrome: CNS with ocular abnormalities, isolated early-onset NS, DMS FSGS	(20)
Hatei, 2005	LAMB3	1q32	AR	Laminin β 3	Epidermolysis bullosa, junctional, non-herlitz type, somatic mosaic revertant, Junctional epidermolysis bullosa gravis of Herlitz	(21)

Table I. Continued.

Author, year	Gene	Locus	Inheritance	Protein	Phenotype	(Refs.)
Kambham, 2000	ITGB4	17q25	AR	β4-integrin	NEP syndrome-NS, epidermolysis bullosa and pulmonary disease	(22)
Berkovic, 2008	SCARB2 (lysosomal)	4q21.1	AR	Scavenger receptor class B member 2	Nephrotic syndrome, nephrotic syndrome with C1q deposits, progressive myoclonic epilepsy (Action myoclonus renal failure syndrome ± hearing loss)	(23)
Serebrinsky, 2015	GLA	Xq22.1	XLR	α-galactosidase A	Andeson-Fabry disease	(24)

AR, autosomal recessive; AD, autosomal dominant; XLR, X-linked recessive; CNS, central nervous system; FSGS, focal segmental glomerulosclerosis; NS, nephrotic syndrome; NEP, nephrotic syndrome; DMS, diffuse mesangial sclerosis; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; HIV, human immunodeficiency virus; GBM, glomerular basement membrane; NPHS1, nephrin; NPHS2, podocin; CD2AP, CD2 associated protein; PLCE1, phospholipase C ε; ACTN4, actinin α 4; TRPC6, transient receptor potential cation channel subfamily C member 6; INF2, inverted formin, FH2 and WH2 domain containing; WT1, Wilms tumor 1; LMX1B, LIM homeobox transcription factor 1β; LAMB, laminin subunit β; GLA, galactosidase a; ITGB4, integrin subunit β 4; SCARB2, scavenger receptor class B member 2; SD, slit diaphragm of podocytes; COQ2, coenzyme Q2; PDSS2, decaprenyl diphosphate synthase subunit 2; UUA/G, *TRNL1*, mitochondrially encoded tRNA leucine 1; SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a like 1; GLA, galactosidase.

MassArray technology. A total of 138 mutations were included in the assay.

Polymerase chain reaction (PCR). The assay was designed using MassARRAY® software (version 4.0; Sequenom, San Diego, CA, USA). The 138 mutations were assigned to six multiplex assays. PCR primers were designed using Mass ARRAY® Assay Design 4.0 Software (Sequenom) (Table II). PCR was first performed using the following protocol: 4 min at 95°C for activation of Faststart taq DNA polymerase (Roche Diagnostics, Basel, Switzerland, cat. no. 12032937001) and 30 sec at 95°C, 30 sec at 56°C, 1 min at 72°C for 45 cycles, followed by 5 min at 72°C. The PCR products were subjected to shrimp alkaline phosphatase (SAP) reaction for the degradation of residual dNTPs. The SAP reaction was performed as follows: 40 min at 37°C and 5 min at 85°C. Following this, extension reaction was performed by the following protocol: 30 sec at 94°C, 40 cycles for 5 sec at 94°C, from 5 sec at 52°C to 5 sec at 80°C for 5 cycles, and finally 3 min at 72°C. Then, the products were desalted using resin. The final products were analyzed by MALDI-TOF mass spectrometry (Mass ARRAY® Typer 4.0.5 Software, Sequenom) to identify the mass. SNP genotyping was performed on SEQUENOM®MassARRAY® platform using the iPLEX™ assay (Sequenom) (37).

WES analysis. WES was performed on the two siblings. A total of 6 μg sample DNA was prepared. First, the qualified DNA samples were randomly fragmented to generate 200-300 bp DNA fragments. The extracted DNA was amplified in a ligation-mediated (LM)-PCR, as described earlier. The NimbleGen human exome array (SeqCap EZ Human Exome Library; version 2.0; NimbleGen, Roche Diagnostics cat. no. 06465684001 or 06465692001) was used to capture the exons of the human genome. High-throughput sequencing was performed on a Hiseq2000 platform (Illumina), and the sequence of each library was generated as 90 bp paired-end reads. The raw image files were processed by Illumina base calling Software (Illumina Inc. San Diego, CA, USA, version 1.7; HCS1.5.15.1, RTA1.13.48, OLB 1.9.4). The obtained sequences were aligned to the reference genome [human genome build37 (hg19)] using Burrows-Wheeler Aligner (BWA; bio-bwa.sourceforge.net/; version: 0.5.9-r16). Single-nucleotide polymorphisms (SNPs) were detected by SOAPsnp (<http://soap.genomics.org.cn/soapsnp.html>; version 1.05) and small insertions/deletions (indels) were detected by SAMtools (version: 0.1.18; www.htslib.org/). Called SNP variants and indels were annotated and classified using ANNOVAR (www.ncbi.nlm.nih.gov/pmc/articles/PMC2938201/). Variants were filtered using data from dbSNP 142 and the 1000 Genomes Project.

Pathological diagnosis. The renal tissue was fixed in 10% neutral buffered formalin and stored at room temperature or 4°C. The fixed tissue was embedded in paraffin and 2-μm sections were cut. HE, PAS, PASM+Masson and Masson staining were performed at 37°C as described previously (22).

In silico analyses of the effect on protein structure and function. Selected bioinformatics tools were used to assess the effect of sequence variants on the structure and

Table II. Primer combinations for the 138 mutations used in the multiplex assays

WELL	SNP_ID	5' PCR primer	3' PCR primer	UEP_SEQ
W1	rs121912602	ACGTTGGATGGTCTTGAGGATCAGAACAC	ACGTTGGATGATGAGATGGCAACCCGAAAG	AACCACTCCGGCTTC
W1	rs121918233	ACGTTGGATGGGAAACAACTGGTTCAGC	ACGTTGGATGTTGCTTAGGAACCTGGCTTC	GCTGCCAAACCAATG
W1	rs28935493	ACGTTGGATGGTTATCATAGCTACAGCCC	ACGTTGGATGAGGAGACAACTTTGAAGTG	AAGCTGAGAGAGGT
W1	rs121912489	ACGTTGGATGCCAGGTACTTGATATTC	ACGTTGGATGTGCTCCGTACCACTTCAGAT	AGCCTGGTATCTCCTA
W1	rs121912462	ACGTTGGATGAGATGAGTCTCATCTCCC	ACGTTGGATGTCCAAACCAAGGCTCACACACA	ccTCCCTCTCTGCAGAC
W1	rs121918231	ACGTTGGATGAGTTGAAATGTCTCCAGCGG	ACGTTGGATGACATGAAGCTAAGTATCTG	ccCCAGCGGCTATTGGA
W1	rs80356680	ACGTTGGATGACCTGCTTGTGGAGGAC	ACGTTGGATGTACTGGGTGACGTAGTCTC	GGAGGACCCGGTTTCTC
W1	rs121434393	ACGTTGGATGTCTGCTTCAACTCACCTTC	ACGTTGGATGAAGATATGACTGCAGGCC	TCACTTCACACTCTCCT
W1	rs121912490	ACGTTGGATGATCTGGCAATGTGAGTGGAG	ACGTTGGATGTGGGTACGGTAGAAGAAAG	TGTGAGTGGAGGTGTGTG
W1	rs28941777	ACGTTGGATGCTTCTCTGTCCATTAGGTG	ACGTTGGATGTATGAGTCTGTGTGGGTTC	CCATTTAGGTGTGAAACCA
W1	rs119473037	ACGTTGGATGCTGTGGAGGAAGCAGTCAT	ACGTTGGATGGCAATCACCACTATCTTGCG	tgacGCAGTCATGCTGCGG
W1	rs28935485	ACGTTGGATGCTCTCTCTTGTGTAAT	ACGTTGGATGCAGAGGGCCATCTGAGTTA	ggTggGGCCCTCAGCTGGAAT
W1	rs118203956	ACGTTGGATGATGAAACAAAGGCACTGGAG	ACGTTGGATGAATCTGGTCACAGCAAAACAC	cccggGAGAGCTTCTCCTCCT
W1	rs121909119	ACGTTGGATGGAGATATCGGGCTTGAAAC	ACGTTGGATGTGTGACTCACACAGTTGACG	AAGATTTCATCTTTGTAGCCC
W1	rs267606710	ACGTTGGATGGTTTTAGGAAAGAACTGG	ACGTTGGATGCCCTCTAGATTACTTCTCATTG	AGGAAAGAACTGGAAAAACTG
W1	rs28939695	ACGTTGGATGTCTCTCCCGCCAGATCC	ACGTTGGATGAAACACACACAGCCTCACCC	gactGCCCAGAAACTGTGGAAT
W1	rs267606955	ACGTTGGATGCAATTGTCAAGTTGGTGTAG	ACGTTGGATGTTGATGTCTAAGCATGCCC	gggggaTGACTCGGAGCCAGATC
W1	rs28935490	ACGTTGGATGCCCTGATGATGGCAATTACG	ACGTTGGATGACATCAGCCCTCAAGCCAAA	ccctATGGCAATTACGTCCTTAT
W1	rs104894841	ACGTTGGATGCAGGAATCATCATGTCAGC	ACGTTGGATGATCTGTTTTCACAGCCCCA	ATCATCAATGTCAGCAAAATTC
W1	rs121912466	ACGTTGGATGGATAAAGAGGGCACCCGTA	ACGTTGGATGACCTCTGACCACTCCGAAC	cccCCCTCCTGGAACTCCACCATG
W1	rs121912461	ACGTTGGATGTGCTGTCTCCACTCTGGC	ACGTTGGATGACTCCATGTCCGATGATCTG	atcccCTCTGGCCCTGCGGTACCC
W1	rs121907904	ACGTTGGATGTGCTGTGCACTGTAAAGTGG	ACGTTGGATGCTGAGACCAGTGAGAAACG	GACAGCTTAAATAATCTCTTATTG
W1	rs199474665	ACGTTGGATGGCCCGGTAATCGCATAAAAC	ACGTTGGATGGTTGGCCATGGGTATGTTGT	caaaaTTTACAGTCAGAGGTTCAA
W1	rs118203955	ACGTTGGATGAAGACCACTGACTCCATGAC	ACGTTGGATGATCCACAAATCTCTTCCAAAG	cccaTCAGCTCCTGTAGTCTTACAT
W1	rs74315344	ACGTTGGATGCCTTTGCCCTCTTGTCTCC	ACGTTGGATGAGCTCTGAGGATGGAGAGGA	aaacGCCCTCTTGTTCCTCCTGTGC
W1	rs121912603	ACGTTGGATGGCTTCTTTATCCTCTTTGGG	ACGTTGGATGAACATCTGGAAGACAGACC	TGGGATTTCTTCATTAATATCAAACT
W1	rs121912482	ACGTTGGATGGGATTCACAGCAACTCAAAG	ACGTTGGATGCCCATAGTTCCATGGACAAG	aagaAACTCAAAAGTCAAAAAATTCAA
W1	rs121912488	ACGTTGGATGTGTCTCCCAACGTGTGTAG	ACGTTGGATGCAGACCTGTTGAAGATCACC	ggtagGTAGACGAGTCAGGTTACCC
W1	rs121912485	ACGTTGGATGCAATGCTCTGTTGTCTGG	ACGTTGGATGACTGTCTGCAAGAAGATG	ggTGGGAGCCCTGGCTGCAATGGCCT
W1	rs80356681	ACGTTGGATGACCTCCAGCGCCTACTAT	ACGTTGGATGTCAGCATGGCCGTGACAGAA	cccaaCAGCGCCTACTATGCTGTGTCC
W1	rs121912605	ACGTTGGATGGTTGAAGCCATTGATCGCAG	ACGTTGGATGTCGTTGCTGAGGCAATGAAC	gtccACTCTGACCTGCGCAATCATATAT
W1	rs74315347	ACGTTGGATGAAGGAGGCCCAAGAAATCAAGC	ACGTTGGATGAGCAATGGCATCTATCTCC	ctcagCCAAACTTTTCTGCTAGATC
W1	rs267606954	ACGTTGGATGTACAGAATCTCTGACTAAC	ACGTTGGATGGATGATGTAGAAGAAAGACGC	catAATCTCCTGACTAACAAAGTGGATC
W1	rs121918232	ACGTTGGATGTCTCCATTTCAAAGGAGAG	ACGTTGGATGTGGCACTGGGTGTCTCTCTG	tggaacAACGTTTAAATATACCTGTAGTAA

Table II. Continued.

WELL	SNP_ID	5' PCR primer	3' PCR primer	UEP_SEQ
W1	rs74315343	ACGTTGGATGGGTTGTACAAGAGTATGAAAAG	ACGTTGGATGGAGTGTCTTTTACCAGGGC	cCAAGAGTATGAAAGAGTA ATTATAITTC
W2	rs119473038	ACGTTGGATGAAAGGGTGATCCTGTGTGC	ACGTTGGATGAGAAAGTTGGCTTGACTGCG	ACACCAGCCATGTCC
W2	rs119473035	ACGTTGGATGCAGAGCTGAGAAATTATGG	ACGTTGGATGTTGGGATGGGCTTGCTTG	TTGGCAGAAACAGCAT
W2	rs121434390	ACGTTGGATGTTAAACGTTGAGTGAGTGGC	ACGTTGGATGGGAACGCTTTTGGATGCAG	aATCTTCGCACCACT
W2	rs119473033	ACGTTGGATGAGCTCAITTCCTCCCAACAG	ACGTTGGATGAATTGCTCTCAGAAAGCCCG	ACAGGCCCTGATTCAA
W2	rs104894835	ACGTTGGATGTGACATGAAGCGCTCCCA	ACGTTGGATGCCTCGTTTCTCGGACATC	ctaGTCTTGCCAATCCA
W2	rs74315346	ACGTTGGATGATCCAGAGTTGGAGACG	ACGTTGGATGCTCCTCCTCTCTTTAGGTC	TCAACCTTGTTGGTAGGTA
W2	rs121912601	ACGTTGGATGTTCAAGGCATTACCTTTCAGC	ACGTTGGATGGTCTTTTGGAGCAAGAAGTG	TTACCTTTCAGCATCATTC
W2	rs121907909	ACGTTGGATGCTTCTCTGTCCATTAGGTG	ACGTTGGATGATGATGCTCTGGTGTGGGTC	CCAGTGTA AAACCTTGTCAG
W2	rs2071225	ACGTTGGATGGATGATGTTCTGGGTTCTC	ACGTTGGATGTTAAAGCCCAAGTTACCCG	gaatCTGCAITGTCAAGGT
W2	rs121907908	ACGTTGGATGAGAGGATGGGCGTTGTGTG	ACGTTGGATGAGCCACACTGAGCCTTTTTC	gggGTTGTGTGGTTATCGC
W2	rs61747728	ACGTTGGATGAACCACTATGAAGCGTCTCC	ACGTTGGATGCTAAGTACCTTTGCATCTTG	taatCGTCTCCTAGCACATC
W2	rs2717-192515	ACGTTGGATGTTCTGGGCTCACTATCTCAC	ACGTTGGATGGCAGAAAGCAITGTGTACTCC	gAAGGGCCACATATAAAGAG
W2	rs104894837	ACGTTGGATGATGTCGTAGTATCCAAAAC	ACGTTGGATGAGCAAAAGACTGAAGCTAGG	CGTAGTATCCAAAACCTCCAG
W2	rs2717-192520	ACGTTGGATGCTGTGTCAGCAACATCAACA	ACGTTGGATGGCTGACATGTGATTCCTG	CTGGTTAAAGATGTCCAGTC
W2	rs121912492	ACGTTGGATGTGCAAAACACAACACACGTTGG	ACGTTGGATGGCAGGTCAAGATAGAAATCC	ccaccACACACGTGGCCTCAAC
W2	rs28935197	ACGTTGGATGGAATCATCAATGTCAGCAAAA	ACGTTGGATGGAAGTAAACAGAAAGAGTC	gaACTGTGCGGATTTCTGTATAA
W2	rs121909118	ACGTTGGATGCTTACATCCTAACAGGTCAG	ACGTTGGATGTCTGCAGGAACCTTTATACCG	TTTCAGTGACTATGAGAGTGTA
W2	rs28935492	ACGTTGGATGCTCTTATTACCTGTCTAAGC	ACGTTGGATGATTGCCATCAATCAGGACCC	catcTACCTGTCTAAGCTGGTAC
W2	rs267607207	ACGTTGGATGATCGACAGAGGCACTGATG	ACGTTGGATGAGAGAGCTTGCCAAAGTGCC	gggggaTCAGA AGGAGGACTTCAA
W2	rs28942089	ACGTTGGATGCCAGCAATGAGAAGTGAACC	ACGTTGGATGTTTACAGACCAGCTCAAAAAGAC	ctAAGTGAACTTACAAACCTGTAT
W2	rs267606919	ACGTTGGATGGACGCAGGAGGAGGTGTCT	ACGTTGGATGGGTACCTCTGAGTGAGGGAA	actCAGGAGGAGGTGTCTTATTC
W2	rs199474663	ACGTTGGATGTTGTAAAGATGGCAGAGCCC	ACGTTGGATGAGAGGAATTGAACCTCTGAC	tfggtAGAGCCCCGGTAATCGCATA
W2	rs267607183	ACGTTGGATGGCAGGTGCTTACCGATAAAC	ACGTTGGATGCAACGCCGTCATCTTGGG	ccctTGCTTACCAGATAAACTCGTTC
W2	rs121909486	ACGTTGGATGAGACACTGGCAGCTGAGAC	ACGTTGGATGGTGGGCTCTTACCTTTTG	gcgGGTCCAGGTCTGGTTTCAGAA
W2	rs121912487	ACGTTGGATGCTCTGTAGGTCCAACTTAAC	ACGTTGGATGGAGAAAGTAAACCACTGACC	ccctcATTCCAGCAACTCAAA GTCAA
W2	rs104894834	ACGTTGGATGCCCAAAAGAGATT CAGAAGGC	ACGTTGGATGACATAATTAGCTAGC TGGCG	aaagtCAGACTTCAGGCAGACCCCT CAG
W2	rs121912467	ACGTTGGATGTTACCCGTGTAG CCGTAGG	ACGTTGGATGTTGGGCCCATGAAGA AAGTG	ggggccGGTTCCTCAATAAAGCAGC ATCC
W2	rs2717-192517	ACGTTGGATGCCCTCTGTCCATTCAITCTT	ACGTTGGATGGGAGACATGGTAACAAAGTCA	ccTTAAACCTGTTTAAATTTCTTCTCAG
W2	rs121907911	ACGTTGGATGAGTTCACTGGCACAGCCGGA	ACGTTGGATGTTAGGAACATCTTGGCCTG	gacgGGCACAGCCGGAGCCTGTGCGTA
W2	rs121907911	ACGTTGGATGAGTTCACTGGCACAGCCGGA	ACGTTGGATGTTAGGAACATCTTGGCCTG	gacgGGCACAGCCGGAGCCTGTGCGTA
W2	rs121434394	ACGTTGGATGGGAAATTAAAGCAGGACATCTC	ACGTTGGATGAAGTTCTGTAGTCTTCTG	ctcccTTAAAGCAGGACATCTCAAGTCTC
W2	rs28935488	ACGTTGGATGTTCTCCTCTCTTGTGTTG	ACGTTGGATGAGGGCCATCTGAGTTACTTG	gcATTATTTCATCTCTTTTCTCAGTTAG
W2	rs121912465	ACGTTGGATGAGCAAAACCGTGCAGAAAGG	ACGTTGGATGAGTAGGCGCAGTCTTATCC	ccgtTGCAAGAAAGGCCCCCAGTGAAGAGC
W2	rs80356682	ACGTTGGATGCCGGATCCTAGATGCAAGA	ACGTTGGATGACCTCCTCTGCTGTGACTG	ctcgGCAAAGAGTAAGATTGAGCAGATC

Table II. Continued.

WELL	SNP_ID	5' PCR primer	3' PCR primer	UEP_SEQ
W3	rs104894836	ACGTTGGATGTGATGCAGGAATCTGGCTCT	ACGTTGGATGTGCACTGGGAGCGCTTCAT	CTGGCTCTTCTCTGGC
W3	rs104894849	ACGTTGGATGTATACAGGCCACTCTTTTACC	ACGTTGGATGGGGCTGTAGCTATGATAAAC	AGCAACTGCGATGGT
W3	rs267607208	ACGTTGGATGTATGTCTGTGAGGCTCAGG	ACGTTGGATGGGAGGACTTCAACAGCAAAC	GGGTATGGGCAGAGA
W3	rs28935196	ACGTTGGATGTGGGAGTTTGGATACTACG	ACGTTGGATGCTGTACAGTAACAACCATC	CCAGACCTTTGCTGAC
W3	rs104894847	ACGTTGGATGAGAACTACATCTGGGCTGCG	ACGTTGGATGTGCTCTAGCCCCAGGATGT	CTGGCTTCGCTTCTCTG
W3	rs121907905	ACGTTGGATGTCTTCCCCAAGGTGAGAAAC	ACGTTGGATGTGCTCTTTGAGCTGGTCTG	AGTGTGACTTCAAGGAC
W3	rs104894833	ACGTTGGATGAGACCATGAGCTCTGCCATC	ACGTTGGATGTGGGAGGTACCTAAAGTTTC	TCTCCATGAAGAGCTTCT
W3	rs104894838	ACGTTGGATGTCTCATACAGGTTATAAGC	ACGTTGGATGAAGGCCACATATAAAGAGG	AAGCATTTGTACTCCTG
W3	rs121909489	ACGTTGGATGTTCCTGATGCGAGTCAACGA	ACGTTGGATGAAGTAGCAGCTGGTGGTGA	TGGCACGAGGAGTGTTC
W3	rs121909491	ACGTTGGATGTGGAAGGCTCTTCGCTGCTG	ACGTTGGATGATGATCAGAGCAAGGGCAGCG	GGGTGGTGGAGATGGTC
W3	rs137853042	ACGTTGGATGACTGGCTCTCTCATATTCG	ACGTTGGATGCACCTTCATCTCGGAAGTTC	aTCATATTCGTTCTCTGACTC
W3	rs80338755	ACGTTGGATGTCTCCATGACCACGATGCTC	ACGTTGGATGTCTTGCCAGATGTTCAAGG	ctaaCTCCGCCCTGGGTGTTG
W3	rs199474668	ACGTTGGATGTGAACTCTGACTGTAAAG	ACGTTGGATGTTATATACCCACACCCACC	tgTTATGCGATTACCCGGGCT
W3	rs267606918	ACGTTGGATGGCGCTTACCAGTGCATGTG	ACGTTGGATGCCACATCTGACAACAAGAC	AGTGCATTTGGACAATGGG
W3	rs119473034	ACGTTGGATGCTTGCCTCTTACAGAGGAGC	ACGTTGGATGATAACTTCTCAGCTCTGCGG	AGGA AAAAGATTGAAAGAGAAT
W3	rs121912491	ACGTTGGATGCAGACCTGTTGAAGATCAC	ACGTTGGATGTGTCTCTCCCAACGTGTGTAG	cccTGAAAGATCACCAACCTAC
W3	rs2717-192513	ACGTTGGATGCAGTCTCTGTAATGAACAAG	ACGTTGGATGAGACTTTCAGGCAGACCCTCA	ccctCATAAATTAGCTAGCTGGC
W3	rs121434392	ACGTTGGATGAGACAATGACAGTTAAGCCG	ACGTTGGATGAGAAAACAGAAAGCATGACTCG	TAGGCATTAATCTAGATCTGG
W3	rs199474660	ACGTTGGATGACAGTCAGAGGTTCAATTCC	ACGTTGGATGTGGGTACAAATGAGGAGTAGG	AGAGGTTCAATCTCTCTCTTAA
W3	rs267606879	ACGTTGGATGTACCGATAAACTCGTCCCG	ACGTTGGATGTGATCAACGCCGTCATCTTG	ggaaaTCGTTCCGCAGCTGGGTG
W3	rs2717-192524	ACGTTGGATGTATTACCTGTCTAAGCTGG	ACGTTGGATGTCAAGCCAAAGCTCTCCTTC	ccctTCCTGATTGATGGCAATTAC
W3	rs121918230	ACGTTGGATGCTGTGATCCATCTGTTTGCC	ACGTTGGATGAATTTCTTTACCTGATGGGC	CTGGAGTTATGTGGACACTAATAT
W3	rs104894842	ACGTTGGATGGGGCCACTTATCACTAGTTG	ACGTTGGATGCAGGCTAAGCCTGAGAGAG	gaAGGGAGACAACCTTTGAAGTGTG
W3	rs121909488	ACGTTGGATGAGGTGGTACACGCACCTCCAG	ACGTTGGATGTGTGCATCCGAGGCTCTTC	cccgtTGGGGGCGATCTTCTCCATG
W3	rs121912486	ACGTTGGATGGAGAAGTAACCACTGACC	ACGTTGGATGCTCTGTAGGTCCAACCTAAC	gttgTGACTTTGAGTTGCTGGAATC
W3	rs104894844	ACGTTGGATGCTTTCATCACACAGCTCCTCC	ACGTTGGATGATGTGACTTCTTAAACCTTG	gggtggAAAAGGAAGCTAGGGTTCTAT
W3	rs119473036	ACGTTGGATGAGTACTTAAAGACTTTGCC	ACGTTGGATGTGACCTTCTTAGCAAGTTGG	ggacAGACTTTTGCCAAGTTTCTTACA
W3	rs267606953	ACGTTGGATGGAATGACATCCTGTGCAGTG	ACGTTGGATGCCAAGTGCATGATGTTTCTC	gacgaGGGGTGTCTTTGATGACTGTTC
W3	rs74315345	ACGTTGGATGATCTGACGCCCTTAGTTAC	ACGTTGGATGTGGTGGCGCTGTTGGAGAG	tatCAGATAGTGGTGTGTAATCCGTAC
W4	rs121909490	ACGTTGGATGAAGCGACCCCGACCATCCT	ACGTTGGATGACCTCGAAGGAGGCCTTGA	CTCACCAACGACGACG
W4	rs104894848	ACGTTGGATGGCTTCATGTGCAACCTTGAC	ACGTTGGATGACATGGA AAAAGCAAAGGG	CCAGATTCTCGCATCA
W4	rs199474659	ACGTTGGATGTTGTTAAGATGGCAGAGCCC	ACGTTGGATGAGAGGAATTGAACTCTGAC	LAGAGCCCGGTAATCG
W4	rs121912468	ACGTTGGATGTGGAGGAGACGACATTTGAAG	ACGTTGGATGACAGCACTCTTCTCTGTATTC	GACATTGAAGGCCAGA
W4	rs267607071	ACGTTGGATGTCTCCGACAGTTGGAACCTGC	ACGTTGGATGACATCCGATCGATGGCTC	tcTGGCACAGGTCTCTCC
W4	rs142059681	ACGTTGGATGCGATAACCAACAACGCC	ACGTTGGATGACCTGAAATGCCTCTGAAGAC	CACACAACGCCCATCCTC

Table II. Continued.

WELL	SNP_ID	5' PCR primer	3' PCR primer	UEP_SEQ
W4	rs121907907	ACGTTGGATGACACAGTGTGACTTCAAGGAC	ACGTTGGATGGAAGTGAACCTACAAACCTG	AGACCAGCTCAAAAAGACA
W4	rs121912484	ACGTTGGATGCCACGCTCCTCCAGCCAG	ACGTTGGATGATGCGGACCTCCGGAGCAG	cccaCAGCCCAGGCCCTGA
W4	rs121912463	ACGTTGGATGACAGCTTGGGCCTGTCCAAAC	ACGTTGGATGTCTCATCTCCCTTCTTGTCTC	tAACcAGGCTCACACACAC
W4	rs104894851	ACGTTGGATGTATCTGTTTTCACAGCCCCA	ACGTTGGATGCAGGAATCATCAATGTCAGC	ATACAGAAATCCGACAGTA
W4	rs74315348	ACGTTGGATGCTGAAGCGCAAAAGACAAGCC	ACGTTGGATGACGAGCAGGCTTCTCTAAAG	gaAAAGACAAGCCAAAGTG
W4	rs121908415	ACGTTGGATGCCCTTGTGTTCACTTGCAG	ACGTTGGATGAAAGGCATGTTAGAAAGCTGG	ggaaGGCCCCGGCCCCGACGAG
W4	rs121912604	ACGTTGGATGTTCTGTGGGACAAGAACTGC	ACGTTGGATGATCCATGCTGTCCAGATCTC	caccAGAACTGCCCATGTAT
W4	rs121434391	ACGTTGGATGAGAAGCAAAGCATCCCCAAC	ACGTTGGATGGAATGCCCTACAGTTGGCAG	TCTGTAAATTTCCAGATGCTCA
W4	rs28935487	ACGTTGGATGTTGAACAAGGAGGGCTCAAG	ACGTTGGATGCCAGGAGAGAATGTTGATG	GAGGGCTCAAGTTTTACCATA
W4	rs104894852	ACGTTGGATGCTTCAAGGTTAAGAAGTCAC	ACGTTGGATGCTGCATTGTATTTCTAGCTG	ctTTAAGAAAGTCACATAAAATCCC
W4	rs104894827	ACGTTGGATGTAGCCTGGGCTGTAGCTATG	ACGTTGGATGTTACAGGCCACTCCTTTACC	ggttGGCTGTAGCTATGATAAAC
W5	rs2717-192514	ACGTTGGATGGAGGAACCCAGAACTACATC	ACGTTGGATGAGGGAATGCCAGGAAACGA	CTGCGGCTTTCGCT
W5	rs267606880	ACGTTGGATGCGGAGTAGTTGACCACAGAG	ACGTTGGATGACGGAGGCCAACCTGGAGA	ACAGAGGGCATCTGG
W5	rs2717-192511	ACGTTGGATGCTAGCTTCTTTTACAGGG	ACGTTGGATGAGTTGCTTCCCTGGTAAAG	ACAGGGAGGAGCTGTG
W5	rs2717-192516	ACGTTGGATGGCAGAAGCATTTGTACTCC	ACGTTGGATGCAGTTCTATTGATTTCTGGG	CCCTTTCAAAAAGGTGAG
W5	rs121909487	ACGTTGGATGTTGAAGGCTCTTCGCTGCTG	ACGTTGGATGAGTCAGAGCAAGGGCAGCG	GAGGATGGTCCGGGGTTC
W5	rs28935495	ACGTTGGATGAGGGCCATCTGAGTTACTTG	ACGTTGGATGGTTTCTCTCTCTTGTGTTG	ataCAGCTGAGGCCAAAG
W5	rs2717-192521	ACGTTGGATGATAGGAACAAGCCTACCCG	ACGTTGGATGGTTGATGTTGCTGGACCAGG	GGGTCTTGAAACAAGGAGG
W5	rs104894845	ACGTTGGATGAGCAAAGGACTGAAGCTAGG	ACGTTGGATGTCGTAGTATCCAAACTCCC	ATGTTGGAAATAAAACCTGC
W5	rs28941778	ACGTTGGATGTATGAGTCTCTGTGTGGTCTC	ACGTTGGATGCTTCTCTGTCCATTAGGTG	gtTGTGGGTCTTCAGGTGGT
W5	rs121908417	ACGTTGGATGCTCCTGAAAGGCATGGTAG	ACGTTGGATGCTTGTGTTCACTTGCAGAC	gggcGGCATGGTAGAAGCTGG
W5	rs121907910	ACGTTGGATGGAAGTGAACCTACAAACCTG	ACGTTGGATGACCCAGTGTGACTTCAAGGAC	cccTTTTGGAGCTGGTCTGAAC
W5	rs121434395	ACGTTGGATGAAGTTCTGCTAGGTCTTCTG	ACGTTGGATGAGCAGGACATCTCAAGTCTC	TGAGATTTTCTTCAAGGAGTT
W5	rs104894843	ACGTTGGATGGTTTATCATAGCTACAGCCC	ACGTTGGATGAGGGAGACAACCTTTGAAGTG	caaaCTAAGCCTGAGAGAGGTC
W5	rs2717-367202	ACGTTGGATGGCTGACATTGATGATTCCTG	ACGTTGGATGCTGGTCCAGCAACATCAACA	gTTCTGGAAAAAGTATAAAGAGT
W5	rs199474658	ACGTTGGATGGCCCGGTAATCGCATAAAAC	ACGTTGGATGGTTGGCCATGGGTATGTTGT	aGGTAATCGCATAAAAACTTAAAAAC
W5	rs104894828	ACGTTGGATGTGAAGGAGAGCTTTTGGCTTG	ACGTTGGATGCAAGTAACCTCAGATGGCCCT	gatgTGGCTTGAGGGCTGATGTGT
W6	rs121912483	ACGTTGGATGACTGGCACCCGAACATCCTG	ACGTTGGATGACCTGGCGAGTGTACCAGTA	CATCCTGCCAGCTCT
W6	rs2717-192510	ACGTTGGATGTTACAGGCCACTCCTTACC	ACGTTGGATGGGGCTGTAGCTATGATAAAC	CCAGGGAAGCAACTG
W6	rs121907902	ACGTTGGATGTATGAGTCTCTGGTGGGTCTC	ACGTTGGATGCTTCTCTGTCCATTAGGTG	TGGGTCTTCAGGTGG
W6	rs121909492	ACGTTGGATGTTCTGAAACCAGACCTGGAC	ACGTTGGATGACCTGTTCCTCTCTCTCTGA	GCTGCCAGTGTCTCTC
W6	rs151195362	ACGTTGGATGTCAGCCAAAGCTCTCCTTC	ACGTTGGATGTAATTACCTGTCTAAGCTGG	CAGGACCCCTTGGGCA
W6	rs2717-192522	ACGTTGGATGCGCAGCCAGATGTAGTTCT	ACGTTGGATGTTAAAAAGCCAGGTACCCG	acGTAGTTCTGGGTTCCCTC
W6	rs2717-192519	ACGTTGGATGGCTGACATTGATGATTCCTG	ACGTTGGATGCTGGTCCAGCAACATCAACA	TTGATGATTCCTGGAAAAAG
W6	rs199474661	ACGTTGGATGAGAGGAATTGAACCTCTGAC	ACGTTGGATGACAGGGTTTGTAAAGATGGC	GTAAAGTTTTAAAGTTTATGCGA

function of the receptor. A total of two indirect *in silico* predictors, PolyPhen2 (Polymorphism Phenotyping version 2; genetics.bwh.harvard.edu/pph2/index.shtml) and SIFT (sift.jcvi.org), were used to evaluate the possibly damaging effects of single amino-acid substitutions on the expression of the proteins of these genes. To identify potential pathogenic mutations, additional analysis focused on the variants that are listed in OMIM as being associated with FSGS (even if frequent) and also any other indels and nonsense variants. For the missense variants, the high risk variants were determined by a minor allele frequency (MAF) of 1% or unknown (using 1,000 Genomes population data; www.1000genomes.org/node/506).

Results

Quality control. Mass array technology assigns a quality code for each genotyping call. Codes A, B, C, D and I stand for conservative, moderate, aggressive, low possibility and bad spectrum, respectively. The lower the order of the code from A to Z, the higher the quality of the genotyping calls. Code A indicates the highest quality and code D and I indicate no genotyping call (reported as NA). The overall quality of the assay is summarized in Table III. The total number of genotyping calls are 5,658, which is a product of 138 (the number of variations) and 41 (the number of samples). The percentage of code A calls is 90.84% and the sum of code A, code B and code C calls is 98.09%. Overall, the assay achieved a good quality. The assay quality was also investigated to see the distribution of no call genotypes (quality code D and I) among the mutations identified. Two variations (rs121912601, rs2717-192514) did not get genotypes from >10% samples and genotyping of 4 variations (rs121918230, rs121907911, rs28935487, rs267607183) failed in >20% samples. As the Mass array genotyping assay is multiplexed, these mutations/SNPs are likely to be susceptible to assay condition variations. The performance of the assay may be improved by redesigning the PCR primers and extension primers for these variations.

The two affected sisters were selected for exome sequencing. For each participant, 4,4017,835 bases were created and covered on the target. The sequence data were generated with a x177 average coverage for each subject. An average coverage of the target region was 98.96 and 99.27% of the target region had at least x4 coverage. For each participant, 21,134 single-nucleotide variants were identified, of which 9,986 were missense mutations and 135 were nonsense (premature termination) mutations.

Clinical characteristics. A total of two female siblings presented with MH at 9 and 6 years-old, respectively. The oldest sibling was referred to the Second Xiangya Hospital for persistent MH (8 months) with macroscopic hematuria initially. The physical examination revealed no abnormalities and the older sibling did not suffer from hypertension, sensorineural deafness, or eye involvement. Laboratory tests revealed only MH, which was demonstrated to be glomerular hematuria by the urinary sediment test (erythrocytes 100,000/high power field; 70% of glomeruli; urinary protein 0 mg/dl), with normal renal function. Values obtained in the hematological, biochemical and serological tests were: Serum creatinine,

Table III. Analysis of overall quality of the assay.

Class	Count	Percentage, %
Conservative	5,140	90.84
Moderate	363	6.42
Aggressive	47	0.83
Low possibility	100	1.77
Bad spectrum	8	0.14
Total	5,658	100

26.8 μ mol/l; hemoglobin, 111 g/l; total protein, 61.5 g/l; uric acid, 83.6 μ mol/l; cholesterol, 3.5 μ mol/l; complement component, 31.16 g/l and blood urea nitrogen 4.58 μ mol/l.

A renal biopsy was performed in hospital and this demonstrated severe glomerular alterations consistent with FSGS (Fig. 1). Due to continuous MH and positive family history with renal disease, a renal biopsy was performed at the Second Xiangya Hospital. On light microscopy, characteristic lesions of focal glomerulosclerosis were present in 2 of 28 glomeruli (Fig. 1A). Sclerotic glomeruli were present in 1 of 28 glomeruli and small arteries exhibited loss of smooth muscle fibers. (Fig. 1B). There was mild mesangial matrix proliferation. Vacuolation of the tubular epithelial cells, loss of the brush border of lumen surface and inflammatory cell infiltration was observed (Fig. 1C). Mitochondria in podocytes demonstrated normal morphology (original magnification, x10,000; data not shown). On immunofluorescence, focal segmental coarse granular deposits of immunoglobulin G(+) and proliferation of endothelial cells were observed. Electron microscopy exhibited diffuse podocytic foot process effacement (Fig. 1D). The endothelium was swollen and hypertrophied, however the GBM exhibited a normal structure and thickness. Paramesangial deposits were noted. Massive tubules with swollen tubular epithelial cells, edema in the interstitium and inflammatory cell infiltration were noted (data not shown).

The patient was born following a full-term normal pregnancy as the first child of unrelated Chinese parents. The family history was remarkable in that multiple family members were affected by isolated MH or other renal disease in her father's pedigree (Fig. 2). The family history revealed that the parents were Chinese in origin and non-consanguineous. Her sister was also identified as being affected by isolated MH, histologically characterized as FSGS. Her father was diagnosed with CKD by qualified doctors 8 years ago, in another hospital. Her paternal aunts and paternal cousins have also been identified as exhibiting hematuria. Urinalyses and blood chemistries identified isolated MH. None of the affected individuals had ESRD, sensorineural hearing loss, or eye complications including lenticonus. Their mother was well and was not known to have any kidney disease.

Variants of TRNL1 in the family. Using Massarray technology, the same mutation in TRNL1 (m. 3290T>C) was identified in the two sisters, which was not demonstrated by polyphen-2 and SIFT. However, it was predicted to be a pathogenic alteration based on OMIM and Pubmed. This mutation mtT3290C was

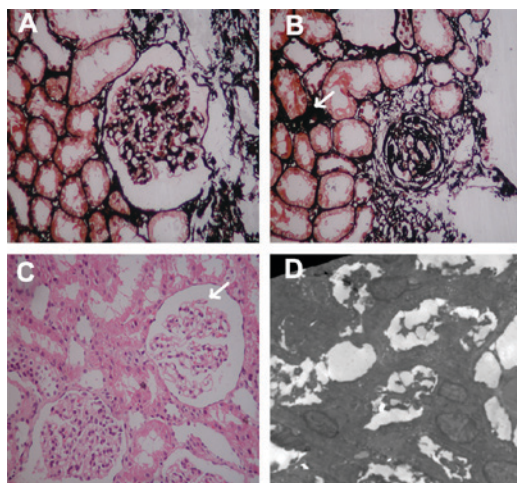


Figure 1. Light and electron microscopic images of the renal biopsy taken from the proband. (A) Light microscopy, PASM staining. A glomerulus is exhibited with segmental sclerosis, hyalinosis and adhesion to Bowman's capsule, consistent with an FSGS lesion. (B) Light microscopy, PASM staining of the renal tubules and glomeruli. Exhibited is globally sclerotic glomeruli with small arteries demonstrating loss of smooth muscle fibres (arrow). (C) Light microscopy, periodic acid-Schiff staining of the renal biopsy taken from the proband. Exhibited is a glomerulus with segmental sclerosis, hyalinosis (arrow), consistent with an FSGS lesion, vacuolation of the tubular epithelial cells, effacement of the brush border of lumen surface and inflammatory cell infiltration. (D) Electron microscopy of kidney section, (original magnification, x5,000). Glomerular segment with extensive podocyte foot process effacement. The endothelium was swollen and hypertrophied. The glomerular basement membrane has normal structure and thickness. Electron-dense deposits were observed in the paramesangial regions. Massive tubules with swelling tubular epithelial cells, edema in the interstitium and inflammatory cell infiltration (not shown). FSGS, focal segmental glomerulosclerosis; PASM, Periodic Schiff-Methenamine Silver.

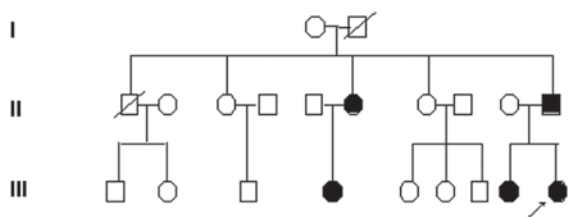


Figure 2. Pedigree of the presenting family. Multiple family members demonstrated asymptomatic isolated hematuria. Octagons represent females and squares represent males. Shaded, affected; shaded with arrow, proband. Diagonal lines represent family members who died because of other disease.

first detected by Opdal *et al* (38) in 1 of the sudden infant death syndrome (SIDS) cases and it suggested that mutations in mitochondrial DNA (mtDNA) may serve a role in certain cases of SIDS. It was speculated that mtT3290C may segregate with FSGS (38). The results are exhibited in Fig. 3. No significant sequence mutations were observed in the other 17 genes analyzed.

Variants of COL4A3 in two sisters with familial FSGS. WES was performed on the proband and her sister, and this identified a heterozygous candidate COL4A4 missense mutation c. 4195A>T(p. M1399L) which was not identified by polyphen-2 and SIFT. Examination of the mutation using 1,000 Genomes provided evidence that the identified sequence variant is a rare polymorphism with a MAF of 0.0022. In addition, the mutation

that was identified was in the NC1 (trimeric noncollagenous) domain. It was previously demonstrated that mutations of COL4A3/COL4A4/COL4A5 in the NC1 domain disrupt heterotrimer formation in podocytes and subsequently inhibit secretion into the GBM domain (39). These results indicate that the substitution is pathogenic and may lead to FSGS.

Discussion

In the present study, a Chinese family presenting with GMH, or CKD were investigated. Renal biopsies from the proband and her sister demonstrated FSGS and normal GBM. Her father reached CKD 8 years ago. A genetic analysis was performed on 15 genes associated with FSGS in the proband and her father, and a homozygous m. 3290T>C missense mutation in TRNL1 was identified in the two siblings. Next generation sequencing of the siblings was performed to reveal a second mutation, a heterozygous c. 4195A>T missense variant in COL4A4. To the best of the authors knowledge, this is the first report of the two aforementioned mutations that may co-segregate with disease in familial hematuria, histologically characterized by FSGS.

It has been demonstrated that podocyte damage is sufficient to cause FSGS, which results from a number of podocyte-specific gene mutations (7-24). In the present study, Massarray sequencing of 18 podocyte-specific genes in a family including two sisters and their father was performed. In the family, no mutations were identified in the genes most frequently reported including NPHS1, NPHS2, CD2AP, PLCE1, ACTN4, TRPC6, INF2 and WT1.

mtDNA including COQ2 and PDSS2 (18-19), has also been identified to be associated with focal glomerulosclerosis. Human mtDNA which encode subunits of enzyme complexes involved in oxidative energy metabolism may result in various diseases and syndromes and the most severely affected organs are the brain, heart, skeletal muscle, sensory organs and the kidney, in mtDNA associated diseases (40). In recent years, the involvement of the kidney has been concerned in mitochondrial cytopathies by nephrologists. tRNA^{Leu} (UUR) gene also called mitochondrial tRNA^{leucine} 1, is a hotspot in mitochondrial disease and has a high incidence of mutations (41). The tRNA^{Leu} (UUR) mutation is associated with the mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome. Renal tubular dysfunction and FSGS have been associated with MELAS (42,43). It has been reported that the mtDNA mutation may also cause isolated renal disease in patients who were not diagnosed as MELAS (44-46). It was reported by Lowik, *et al* (17) that 3243A-G may be identified in a steroid-resistant nephrotic syndrome with histological signs of FSGS. They concluded that mtDNA abnormalities lead to a steroid-resistant nephrotic syndrome with histological signs of FSGS. In the present study, an mtT3290C mutation in TRNL1 in two siblings was identified. It was first reported by Opdal *et al* (38) that the mtT3290C mutation may serve a role in various patients with the SIDS cases (40).

The T3290C mutation is located in the TΨC loop of the TRNL1 gene, disrupting the three-dimensional shape of this tRNA. It has been proposed that a common pathogenic mechanism associated with mutations in this particular mtDNA gene may be a decreased steady-state level of tRNA^{Leu} (UUR) and

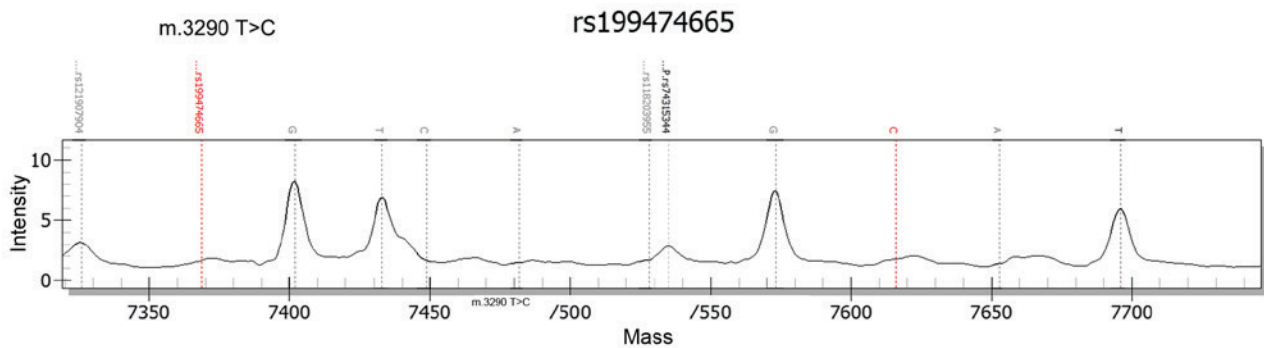


Figure 3. Sequence analysis of tRNA leucine 1 in the two affected siblings. Two patients are homozygous for a missense mutation (m.3290T>C).

a partial impairment of mitochondrial protein synthesis (47). Opdal *et al* (40) proposed that any mtDNA mutation may affect oxidative energy metabolism and thereby induce adenosine triphosphate depletion (40), and m. T3290C mutation may affect the complex structural podocyte composition by affecting metabolic and energy requirements. Hence, there are several reasons to suggest that m. 3290T>C in TRNL1 gene may be involved in FSGS. In addition, homozygous mutation was identified in the two sisters and their father, whereas mtDNA exhibited maternal inheritance. However, blood from the mother was not available and it is unclear whether these mutations are somatic or inherited. To the best of the authors knowledge, the present study is the first to document the development of FSGS and isolated hematuria with the mitochondrial T3290C transition.

However, the affected cases were in the father's pedigree including paternal aunts and paternal cousins, and it were not consistent with typical maternal inheritance.

This suggests that mutations in other genes may also be involved in the development of FSGS. The list of genes implicated in the development of FSGS is updated continuously. The introduction of more comprehensive screening technologies including WES allows simultaneous screens for mutations in other potentially relevant genes and contributes to the detection of novel genes or mutations (48,49), instead of testing for one gene at a time, or screening for certain known mutations only.

As the sisters exhibited normal GBMs, the type IV collagen-associated genes were not initially screened. However, using WES technology a single heterozygous mutation c. A4195T (p. M1399L) in the COL4A4 gene, which encodes the $\alpha 4$ chain of type IV collagen, the most important structural component of the GBM, was identified.

Though the variant was not demonstrated by SIFT and polyphen-2, the COL4A4 mutation that was identified in the siblings is most likely disease-causing. Firstly, certain studies suggest that COL4A3 and COL4A4 mutations may cause a wide spectrum of disease phenotypes from AS to FSGS (39,50,51). Malone *et al* (51) was the first to document COL4A3 and COL4A4 mutations associated with primary FSGS. The authors identified seven variants in COL4A3 and COL4A4 in a cohort of 70 families with a pathological diagnosis of familial FSGS of unknown cause. Notably, each of these variants were heterozygous and no mutations in known FSGS-associated genes were identified (51). The authors

hypothesized that mutations in mature GBM collagen (IV) may have a direct role in the pathogenesis of FSGS and that the phenotypes induced by mutations in mature GBM collagen (IV) genes may phenocopy primary FSGS. Secondly, the mutation that was identified in these variants is exhibited at very low frequencies of 0.0022 by 1,000 Genomes. In addition, the mutation is located in the NC1 domain where the variant may disrupt heterotrimer formation in the podocyte and subsequent secretion into the GBM domain (39). Molecular and bioinformatics analyses suggested that the mutations in the conserved glycine-rich regions or in the NC1 carboxy terminus of the involved proteins are deleterious (52). It is now recognized that the mature type IV collagen network, $\alpha 3\alpha 4\alpha 5$, originates solely in the podocytes (53). Kruegel *et al* (54) proposed that podocyte receptors may recognize the mutated COL4 leading to upregulation of podocyte profibrotic factors, including transforming growth factor- β , connective tissue growth factor and matrix metalloproteinases-2, -9 and -10. These data add support to the hypothesis that these variants may cause disease.

The COL4A4 mutations follow an autosomal dominant or recessive inheritance pattern. The patients with heterozygous mutations in the COL4A3/COL4A4 are more common in the carrier state of atherosclerotic renal artery stenosis and TBMN than autosomal dominant AS, and familial hematuria and GBM morphology are typical clinical features of these diseases (54,55). The patients with COL4A4 mutations documented in the present study had significant hematuria at diagnosis. Biopsies in the families in the present study demonstrated the typical signs of FSGS on light microscopy and foot process effacement on EM. However, in the present study there were no consistent GBM ultrastructural alterations in the siblings with COL4A4 variant and there was no decrease in collagen (IV) staining in the GBM. In addition, these phenotypes also lack extra-renal manifestations including deafness or ocular symptoms, which are characteristic of AS. In the two patients, there was not enough supportive evidence that was consistent with AS or TBMN and the sisters were diagnosed as familial hematuria rather than AS or TBMN.

Whether or not the reported heterozygous variant (c4195A>T) alone in the present study is sufficient to cause FSGS, or is only partially penetrant, the study by Malone *et al* (51) demonstrated that the variants in COL4A3/COL4A4 c may be associated with FSGS, however the possibility of the presence of other modifier genes and/or

other acquired factors cannot be excluded (51). These genes or factors may determine the phenotypic heterogeneity that leads to variability in disease progression and results in an unpredictably benign course or long-term progression of hematuria to proteinuria, and ESRD (56). Podocyte foot process effacement was a constant result in the present report, and it suggests that the observed phenotype may be due to podocyte abnormalities. So it is possible that the variable phenotypes demonstrated in the present study may be due to variants in COL4A4 acting as disease modifiers for FSGS and this is consistent with the view of Bullich *et al* (57).

FSGS-associated genes frequently follow an autosomal dominant or recessive inheritance pattern, therefore a mutation in mtDNA may have been overlooked. In addition, the WES cannot be performed to analyze mtDNA mutations. The results of the present study demonstrated that Massarray technology and WES technology were complementary, each with its own advantage. The combination of Massarray technology and WES may improve the detection rate of genetic mutation with an increased level of accuracy.

At present, monogenic FSGS subtypes have been reported by genetic studies primarily focusing on familial FSGS. However, a rare study on the potential role of combinations of mutations in different genes was reported in FSGS (12,13). The present study, to the best of the authors knowledge, is the first report to document two relevant genes co-segregated with FSGS.

It has been proposed that hematuria is the forgotten CKD factor (32). In a number of families carrying these mutations, certain members continue to exhibit chronic and isolated MH for the rest of their lives, whereas others develop proteinuria later on in life, usually with hypertension and a variable gradual progression to CRF leading to ESRD (58,59).

Therefore, the term familial hematuria (FM) would be appropriate to use instead of misnomer benign familial hematuria and the pediatric nephrologist must give a correct prediction of prognosis to the children with hematuria and to avoid misdiagnosis. Genetic testing benefits include early diagnosis, highly-targeted therapy and an ESRD onset delay. Genetic investigations may be more definitive and diagnostic than renal biopsies.

For the initial treatment of FSGS, the Kidney Disease Improving Global Outcomes 2012 guideline (60) recommends that corticosteroid and immunosuppressive therapy be considered only in idiopathic FSGS associated with clinical features of nephrotic syndrome (17). There is no evidence to suggest corticosteroids or immunosuppressive therapy in the treatment of the mutation induced FSGS.

The treatment for the two sisters consisted of Chinese traditional medicines including huaqihuang and shenyansiwei capsules. Regular follow-up surveys were carried out in the clinic. The older sister has had enalapril administered up to this point as proteinuria was detected five months following diagnosis with FH. Currently, the proteinuria is in remission and hematuria is reducing gradually. Blood pressure was relatively well regulated and renal function was normal therefore steroid, and immunosuppressive therapy was not instituted.

The results of the present study demonstrate that it may not be possible to take a detailed three generational family history in every pediatric out-patient clinic, however it is always

worth asking if there is a family history of kidney problems, especially if these have occurred in relatively young people. Screening for COL4A mutations in FSGS, particularly when presenting with FH, is recommended.

Whether the variants in COL4A4 were inherited from their father and TRNL1 were inherited from the healthy mother, has not been resolved. Next, the blood of the proband's parent and other affected family members should be obtained to screen for COL4A4 and TRNL1 genes. Then, the pathogenic mechanism of two variants should be verified by animal or *in vitro* experiments.

In the present study, the sisters with mtDNA mutation did not manifest features including hearing loss, diabetes mellitus, neuromuscular symptoms or cardiomyopathy. The family members should be followed closely to identify the development of associated conditions including diabetes mellitus and cardiomyopathy.

Heterozygous carriers of COL4A3 or COL4A4 mutations, irrespective of gender, may be asymptomatic, may have hematuria (carriers of recessive disease) or may progress to ESRD (58,59). Therefore, the family members require long-term follow-up.

In the present study, a missense mutation in the COL4A4 and TRNL1 genes were identified, which may be responsible for MH with FSGS in this family. Screening for COL4A mutations in familial FSGS patients is recommended. Genetic investigations of families with similar clinical phenotypes should be a priority for nephrologists. The combination of Massarray technology and WES may improve the detection rate of genetic mutation with a high level of accuracy.

Acknowledgements

The present study was supported partially by the National 'Twelfth Five-Year' Science and Technology Support Project (2012BAI03B02), Ministry of Science and Technology of China, and by Xiangya Excellent Physician Award (ZWY, 2013), Central South University, China.

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