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Mutations in the *INF2* gene account for a significant proportion of familial but not sporadic focal and segmental glomerulosclerosis

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Abstract

Mutations in the inverted formin 2 gene (*INF2*) have recently been identified as the most common cause of autosomal dominant focal and segmental glomerulosclerosis (FSGS). In order to quantify the contribution of various genes contributing to FSGS, we sequenced *INF2* where all mutations have previously been described (exons 2 to 5) in a total of 215 probands and 281 sporadic individuals with FSGS, along with other known genes accounting for autosomal dominant FSGS (*ACTN4*, *TRPC6* and *CD2AP*) in 213 probands. Variants were classified as disease-causing if they altered the amino acid sequence, were not found in control samples, and in families segregated with disease. Mutations in *INF2* were found in a total of 20 of the 215 families (including those previously reported) in our cohort of autosomal dominant familial nephrotic syndrome or FSGS, thereby explaining disease in 9 percent. *INF2* mutations were found in 2 of 281 individuals with sporadic FSGS. In contrast, *ACTN4* and *TRPC6*-related disease accounted for 3 and 2 percent of our familial cohort, respectively. *INF2*-related disease showed variable penetrance, with onset of disease ranging widely from childhood to adulthood and commonly leading to ESRD in the third and fourth decade of life. Thus, mutations in *INF2* are more common, although still minor, monogenic cause of familial FSGS when compared to other known autosomal dominant genes associated with FSGS.

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DISCLOSURES

[+]Moumita Barua, MD[1]: None

[+]Elizabeth J. Brown, MD[1,2]: E.J.B is named as an inventor of a genetic test for *INF2* mutations

Victoria T. Charoonratana, BSc[1,2]: None

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Hua Sun, MD[1]: None

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Keywords

INF2; FSGS; nephrotic syndrome; mutations; familial FSGS; sporadic cases

INTRODUCTION

Glomerular disease, characterized by focal and segmental glomerulosclerosis histology (FSGS), is a challenging disease to treat due to its frequent relapsing unremitting course and high rate of progression to end-stage renal disease (ESRD). For unclear reasons, FSGS has a rising incidence, now becoming a common cause of glomerular disease in both children and adults worldwide.[1–4] Although only a minority of those affected by FSGS have a family history of this lesion, the study of hereditary forms has helped inform our understanding of the molecular pathogenesis of FSGS. Mutations in *ACTN4*, *TRPC6* and *CD2AP* are all rare causes of FSGS, although never quantified in the literature.[5–8] In contrast, several recent studies suggest that mutations in the *INF2* gene account for a significant proportion of hereditary cases.[9–11] INF2 belongs to the formin family, a group of heterogeneous actin binding proteins that regulate a variety of cytoskeleton-dependent cellular processes.[12–16] Moreover, *INF2* has been implicated in individuals with Charcot-Marie-Tooth disease who manifest FSGS as part of this syndrome.[17]

We previously reported *INF2* as a cause of autosomal dominant FSGS in 11 of 93 families screened. Initial screening of the entire gene revealed disease-causing mutations only in exons 2 to 4. In this study, we expand on our initial report by mutational analysis of the DNA sequence encoding the diaphanous inhibitory domain (DID) of *INF2* in a total of 215 probands from autosomal dominant FSGS families and also in 281 individuals with apparent sporadic disease. Known autosomal dominant FSGS genes including *ACTN4*, *TRPC6* and *CD2AP* were also screened in 213 probands for comparison.

RESULTS

INF2 exons 2, 3, 4 and 5 were sequenced by Sanger method in the DNA belonging to 215 probands from autosomal dominant FSGS families and 281 sporadic cases in order to evaluate variation in the DID domain of the gene. Given the absence of mutations detected outside of this domain based on our own experience and other published reports, we restricted mutational screening to these exons.[9–11, 17] Thirteen missense variants in 20 families that segregated with disease were identified, thereby explaining disease in 9% of our cohort with hereditary proteinuric renal disease (Tables 1 and 2). Individuals whose clinical status was defined as indeterminate given their young age were also found to have the mutations in some instances. Eleven of these families were reported previously. These tables summarize results from our entire cohort with *INF2*-related disease.[11] We also now include sporadic cases of FSGS and identify mutations in 2 individuals (Tables 1 and 3). Scores from PolyPhen-2 software analysis (http://genetics.bwh.harvard.edu/pph2) to predict the functional effects of missense *INF2* variants ranged from 0.789 to 1.000, predicting that *INF2* variants were possibly or probably damaging. No *INF2* variants were present in any of the 10800 control chromosomes assayed or referenced in dbSNP, 1000 Genomes Project or

in the National Heart, Lung and Blood Institute's Exome Sequencing Project. All mutations affected highly conserved residues (Figure 1).

Some of the mutations identified in our cohort have been described before, whereas others are novel (Tables 2 and 3). Certain codons appear to be hot spots for mutations.[9, 10, 17–19] For example, five families have a mutation affecting arginine at the 218th position. Three of these families share the p.R218Q mutation while two share the p.R218W mutation. None of these families are known to be related and this mutation has been described by other groups as well. Mutations in the arginine at the 214th position are also frequently seen in our cohort and others; two of our families have an arginine to histidine mutation and one family has an arginine to cysteine mutation. Finally, two families share the p.S186P mutation but are not known to be closely related.

Though we believe that there are certain "hotspots" for mutations, an alternative explanation is that these mutations could have been passed from a common ancestor. However, in family FG-JN, with the recurrent mutation p.R218Q, the mutation appears to have arisen *de novo* in an apparent founder individual within the pedigree. We do not have this individual's DNA sample but several of his unaffected siblings share the haplotype on which the mutation is found in later generations (data not shown). These unaffected siblings do not harbor the mutation thus suggesting that their affected sibling is the founder.

Our clinical data suggests that affected families are predominantly Caucasian, though this may reflect bias in our cohort. Affected individuals who have had a kidney biopsy generally show focal segmental glomerulosclerosis, usually of the not otherwise specified variant but collapsing in one instance, on histology. Affected individuals generally develop disease during adolescence or early adulthood, which frequently leads to end-stage renal disease in the third or fourth decades of life (Table 4). Ages of onset for renal disease and ESRD ranged from 8 to 72 years and 13 to 67 years, respectively. Most of the families were moderately sized, with 2 to 19 affected individuals per family.

Disease penetrance is variable. Within families, there are rare individuals who harbor the variant but remain clinically unaffected into their sixth and seventh decades of life. Urine screening has revealed microalbumin ratios >250mg/g creatinine in some of these asymptomatic individuals. There are rare individuals who carry a mutation but have no proteinuria. There was at least one adult without any proteinuria.

Of note, one affected female individual from family FS-V with the p.L81P variant developed motor weakness but the cause was unclear. She was diagnosed with FSGS at the age of 15 and developed ESRD at the age of 30. In light of the recent publication linking Charcot-Marie-Tooth (CMT) to *INF2* mutations, she is undergoing further evaluation for this disorder.[17] The *INF2* mutations described in individuals with CMT disease with associated FSGS do not overlap with individuals with familial FSGS (Figure 2). The underlying mechanism causing individuals with *INF2* mutations to develop kidney disease and/or neurological manifestations as part of CMT disease still remain unknown.

Of the 281 sporadic individuals screened, 2 *INF2* mutations were identified. One was a mutation in exon 2 leading to a serine to proline change at the 129th position, which was not

found in control chromosomes. The other mutation was a heterozygous in-frame 9 base pair deletion (c.305_314delTCAGCTGCG, p.del102_104VSC) in exon 2. This individual was diagnosed with primary FSGS on biopsy but also has a long standing history of sarcoidosis involving the skin. Four unaffected individuals from this family, including both parents, were also sequenced and not found to have this variant thereby indicating it to be *de novo*.

Examination of the burden of coding variants in the entire *INF2* gene documented in the 1000 Genomes Project and the Exome Sequencing project is telling in that the ratio of non-synonymous (NS) to synonymous (S) variants in exons 6 to 22 is higher than the ratio for exons 2 to 5 in both datasets. In the 1000 Genomes project, this ratio is 36/20 for exons 6 to 22 and 1/9 for exons 2 to 5 (p=0.002 by right-tailed Fisher Exact test). Similarly, in the Exomes Sequencing Project, the ratios are 57/38 versus 4/9, in exons 6 to 22 and exons 2 to 5, respectively (p=0.045 by right-tailed Fisher Exact test). This suggests that coding variants in exons 2 to 5 of *INF2* are less tolerated than coding variants in exons 6 to 22, resulting in a higher rate of purifying selection in the first coding exons.

Sequencing by Sanger and next generation method of known autosomal dominant FSGS genes—*ACTN4*, *TRPC6*, and *CD2AP*—was also performed in 213 probands. Only the first 10 exons of the *ACTN4* gene were sequenced by Sanger method given that all disease-causing mutations have been localized to this part of the gene.[5, 6] Novel missense variants in *ACTN4* and *TRPC6* that segregated were identified in 7 and 5 or 3% and 2% of families, respectively (Supplementary Tables 1 and 2). No novel variants were found in *CD2AP*. These variants were not present in any of the 10800 control chromosomes assayed or referenced in dbSNP, 1000 Genomes Project or in the National Heart, Lung and Blood Institute's Exome Sequencing Project.

DISCUSSION

The most recent gene to be identified as causing a form of autosomal-dominant FSGS is inverted formin 2 (*INF2*).[11] Mutations in this gene have been demonstrated to be a major cause of autosomal dominant FSGS, previously reported to account for up to 16% to 17% of familial cases.[9, 10] We report a lower proportion of *INF2*-related disease, 9%, in a larger familial cohort than has been previously tested. We also show it to be a more common, albeit still minor, monogenic cause of FSGS when compared to other known gene culprits including *ACTN4*, *TRPC6*, and *CD2AP*. Conversely, similar to other reports, our results indicate that mutations in *INF2* are rarely found in sporadic cases of FSGS, with only 2 identified cases in our cohort of 281 sporadic individuals.

Identified variants in *INF2*, *ACTN4*, *TRPC6*, and *CD2AP* were ultimately determined to be disease-causing if they a) segregated with disease b) were not present in control chromosomes in dbSNP, 1000 Genomes Project and the Exome sequencing project and 3) predicted to be damaging by *in silico* analysis. For each mutation, every affected individual tested for whom we had a DNA sample harbored the variant. For *INF2*, there were occasional family members who carried the family variant and had asymptomatic proteinuria or indeterminate microalbuminuria (25–250 mg/g creatinine). Many of these

individuals are children and may not have manifested clinical disease yet, however, there are rare adults with disease-associated mutations who do not have clinical kidney disease.

We include in this report a list of all *INF2*, *ACTN4*, and *TRPC6* mutations found in screening our expanded cohort of autosomal dominant families with FSGS, including those published earlier as part of the initial discovery of these genes.[5–7, 11] Most of the mutations are missense with only one structural variant in a sporadic case—a finding that is not surprising given the significantly higher frequency of missense variants in the human genome. We now exclude two missense variants previously reported as disease-causing, *INF2* p.A13T and *TRPC6* p.N143S as both have now been discovered in controls in the Exome sequencing project (Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [date (January, 2011) accessed]).

The recessive gene, *NPHS2* or *podocin*, has also been previously sequenced in the sporadic cohort given that it has been found in sporadic cases of FSGS, though usually in children.[19] Novel missense variants in *NPHS2* were identified in 6 or 3% of sporadic individuals.[20] One deletion was detected in *NPHS2*.[20]

The new *INF2* families we have identified since our first report of *INF2* mutations have a similar phenotype. Our current data continue to support the trend that individuals with *INF2*-related disease typically present in the second to fourth decade of life with onset of renal failure in the third decade. However, there continues to be wide inter-family and intra-family variability for ages at which disease onset and ESRD occurs. There were several cases of affected children manifesting proteinuria and ESRD in adolescence, suggesting that *INF2* should be considered in the pediatric population as well.

Previous work on genes identified as causing familial forms of nephrotic syndrome and/or FSGS such as *ACTN4*, *TRPC6*, and *CD2AP* highlight the importance of the podocyte slit diaphragm and actin cytoskeleton in the pathogenesis of disease.[5, 21–25] INF2 is also highly expressed in the podocyte and belongs to the formin family, a group of heterogeneous actin binding proteins that regulate a variety of cytoskeleton-dependent cellular processes.[12–16] INF2, in particular, is known to accelerate both actin polymerization and depolymerization. Our work on the initial *INF2* discovery families showed that all mutations were contained within the diaphanous inhibitory domain (DID), suggesting an important role for this domain in its signaling pathway (Figure 2).[11] Furthermore, the burden of coding variants in the entire *INF2* gene documented in the 1000 Genomes Project and the Exome Sequencing project is weighted to outside of the DID, suggesting that coding variants in exons 2 to 5 of *INF2* are less tolerated than coding variants in exons 6 to 22. The INF2 protein exists in 3 isoforms, 2 of which are long forms and 1 of which is a short form lacking the C-terminal portion downstream from the DID. Thus, we hypothesize that there is something inherently more biologically relevant about the short splice variant (NM_032714) of INF2 compared to the long splice variants (NM_022489 and NM_001031714). Given that only *INF2* exons 2 to 5 were sequenced in this study, it is possible that mutations outside of the DID have been missed. However, previous studies sequencing the entire *INF2* gene have localized

mutations to this domain only, suggesting that mutations outside of the DID domain are relatively infrequent if they exist at all.[9–11, 17–19]

INF2's depolymerization activity is regulated via an autoinhibitory interaction of its DID with its diaphanous autoregulatory domain (DAD).[26] Subsequent work has shown that INF2-DID interacts with the DAD of the mammalian diaphanous-related formins (mDias), which are a family of Rho effectors that also regulate actin dynamics[27]. INF2 bearing the p.E184K, p.S186P and p.R218Q mutations decreased the binding affinity of INF2 DID with the mDia DAD.[28] We hypothesize that mutations within the DID domain of INF2 may interrupt the binding conformation of INF2 to the DAD domains of itself and the mDias leading to altered cytoskeletal rearrangements. To date, all FSGS-associated *INF2* mutations alter highly conserved residues within the DID domain.

Familial studies have yielded significant insight into our current understanding of FSGS. Despite the fact that mutations in *INF2* do not contribute significantly to sporadic disease, they are a relatively common cause of familial FSGS. The lessons learned from the biological study of *INF2* and disease-causing *INF2* mutations are likely to be relevant to the broader patient population with sporadic disease. Though the primary insult leading to disease may be different, the downstream effects leading to altered actin dynamics and glomerulosclerosis may reveal common targets for therapy.

Thus, mutations in *INF2* explain a greater, albeit still minor, proportion of hereditary FSGS compared to *ACTN4*, *TRPC6* and *CD2AP* while not contributing significantly to sporadic disease. *INF2*-related disease has variable penetrance, presenting in the second to fourth decades of life, though childhood onset has also been documented. *INF2* mutations cluster in exons 2 to 4, representing the DID domain, and highlights the importance of this domain and its ability to regulate actin assembly and disassembly in the podocyte.

METHODS

Patients

A total of 912 individuals belonging to 215 families and 281 sporadic cases of FSGS were included in this study. Familial cases were defined as 2 or more affected individuals. All families had an inheritance pattern consistent with autosomal dominance. Affected status was defined as having either a reported history of nephrotic syndrome or biopsy-proven FSGS, having documented proteinuria with urine microalbumin >250mg/g creatinine in a family with at least one case of documented FSGS or nephrotic syndrome. We obtained blood, sputum, or isolated DNA and clinical information after receiving informed consent from participants in accordance with the Institutional Review Board at the Beth Israel Deaconess Medical Center and Brigham and Women's Hospital. Clinical information was obtained from telephone interviews, questionnaires and physician reports.

Mutation Analysis

Genomic DNA was extracted from blood or sputum samples using standard procedures. For *INF2*, mutational screening was restricted to exons 2, 3, 4 and 5. As positive controls, we included families in which *INF2* mutations had been previously identified. The first 10 of 21

exons in *ACTN4* were sequenced. All 13 exons in *TRPC6* and 18 exons in *CD2AP* were screened. Sanger sequencing was performed using Big Dye terminator cycle sequencing kit and analyzed with an ABI Prism 3130 XL DNA analyzer. Primer sequences are available on request. Sequence chromatograms were analyzed using the Sequencher software (Gene Codes, Ann Arbor, MI). *INF2* was sequenced in all probands and sporadic cases. A minority of the probands had next generation sequencing performed. A shotgun sequencing library for each sample was constructed and captured using either the Nimblegen 2.1M Human Exome kit or the Nimblegen SeqCap EZ Exome v2. Enriched libraries were then sequenced on a GAII. The potential pathogenicity of variants were assessed *in silico* using PolyPhen-2 software analysis (http://genetics/bwh.harvard.edu/pph2). Variants were compared against dbSNP 134 (ftp://ftp.ncbi.nih.gov/snp/organisms/human_9606/VCF/v4.0/00-All.vcf.gz), 1,000 Genomes Project (ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/release/20110521/ALL.wgs.phase1_intergrated_calls.20101123.snps_indels_svs.sites.vcf.gz) and the Exome Sequencing Project (Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [(January 2012) accessed]). Where a novel variant was identified in a family, all available affected and unaffected family members were sequenced to investigate if the variant segregated with disease. If any affected individual of a family did not harbor the variant of interest, it was excluded as disease-causing.

Structural Model

Three-dimensional models of the human *INF2* (amino acids 1 through 234) were designed using the Phyre[2] threading program based on primary sequence conservation and known protein structures.[29] The model was manipulated using the program PyMOL (DeLano, W.L. The PyMOL molecular graphics system 2002).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Summary of INF2 substitutions, with red representing affected residues reported in families with FSGS and green representing residues reported to be altered in individuals with CMT disease. Disease-segregating alterations shown aligned with wild-type INF2 protein sequence from humans, gorilla, bovine, horse, mouse and chicken. All of the disease alterations occur in evolutionarily conserved residues.[11]

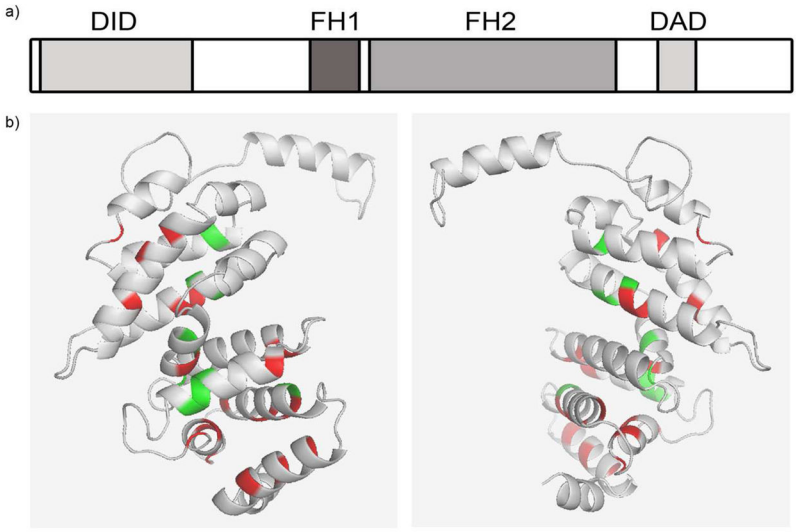

Figure 2.

(a) Schematic showing INF2 protein domain structure. All *INF2* mutations identified thus far occur in exons 2 to 4, which localizes to the diaphanous inhibitory domain (DID) of the protein, albeit only exons 2 to 5 were sequenced in the majority of individuals in our cohort. FH1/2 = formin homology domains 1 and 2, DAD = diaphanous activating domain (b) Three dimensional model of the first 234 amino acids of INF2 as modeled in Phyre[2.29] Affected residues identified in families with FSGS are indicated in red. All known pathogenic mutations cluster in the DID, represented by grey ribbons. Green represents residues mutated in individuals with CMT disease. Figure was generated using PyMOL (DeLano, W.L. The PyMOL molecular graphics system 2002).

Table 1

Percentage of families and sporadics affected by *INF2*-related kidney disease in the current cohort compared to other groups reported in the literature.[9,10]

	Barua *ET AL*	**Boyer *ET AL*[9]**	**Gbadegesin *ET AL*[10]**
Total number of families with *INF2* mutations	20	9	8
Total number of families tested	215	54	49
Percentage of families with *INF2* mutations	9	17	16
Number of sporadics with *INF2* mutations	2	1	0
Total number of sporadics tested	281	84	31
Percentage of sporadics with *INF2* mutations	0.7	1	0

Table 2

List of *INF2* heterozygous missense mutations by family and *in silico* protein function prediction according to Polyphen-2 software. Mutations that were found in other families, either in our cohort or published in the literature, are indicated.

Family ID	Exon Number	Nucleotide Change	Amino Acid Change	Polyphen-2 Prediction	Polyphen-2 Score	Found in other cohorts?	Previously Reported?
FG-BR	4	c.556T>C	p.S186P	Probably damaging	0.988	Y	Y[11]
FG-DM	4	c.593T>G	p.L198R	Probably damaging	0.995	Y	Y[9]
FG-EA	4	c.652C>T	p.R218W	Probably damaging	1.000	Y	Y[11]
FG-EF	4	c.641G>A	p.R214H	Probably damaging	1.000	Y	Y[9,10]
FG-EP	2	c.125T>C	p.L42P	Probably damaging	0.995	N	Y[11]
FG-ER	4	c.556T>C	p.S186P	Possibly damaging	0.789	Y	Y[11]
FG-FG	4	c.641G>A	p.R214H	Probably damaging	1.000	Y	Y[9-11]
FG-GY	4	c.550G>A	p.E184K	Probably damaging	0.999	Y	Y[10]
FG-HT	3	c.472C>G	p.H158D	Probably damaging	1.000	N	N
FG-JN	4	c.653G>A	p.R218Q	Probably damaging	1.000	Y	Y[9-11]
FG-JY	4	c.640C>T	p.R214C	Probably damaging	1.000	Y	N[9,10]
FG-KM	2	c.217G>A	p.G73S	Probably damaging	0.999	N	N
FG-KQ	4	c.653G>A	p.R218Q	Probably damaging	1.000	Y	N[9-11]
FG-LL	4	c.542T>G	p.V181G	Probably damaging	0.989	N	N
FG-LP	4	c.529C>T	p.R177C	Probably damaging	1.000	N*	N[9,10]
FG-LW	4	c.653G>A	p.R218Q	Probably damaging	1.000	Y	N[9-11]
FG-LY	3	c.451T>C	p.C151R	Probably damaging	1.000	N	N
FG-ME	4	c.652C>T	p.R218W	Probably damaging	1.000	Y	N[9-11]
FS-B	4	c.658G>A	p.E220K	Probably damaging	0.999	N	Y[9,18]
FS-V	2	c.242T>C	p.L81P	Probably damaging	0.995	N	N[11]

An asterisk (*) indicates that this residue has been mutated in other families with FSGS but to a different amino acid. Families that were reported in our original INF2 discovery paper published in 2009 are also shown.[11] Alterations in nucleotide and amino acid sequence are reported using the following NCBI RefSeq accession numbers: INF2 – NM_022489 and NP_071934.

Table 3

List of *INF2* heterozygous variants found in sporadic individuals with FSGS and *in silico* protein function prediction according to Polyphen-2 software. Alterations in nucleotide and amino acid sequence are reported using the following NCBI RefSeq accession numbers: INF2 – NM_022489 and NP_071934.

Affected Individual	Exon Number	Nucleotide Change	Amino Acid Change	Polyphen-2 Prediction	Polyphen-2 Score	Found in other
FG-FU 13	2	c.305_314delTCAGCTGCG	p.del102_104VSC	N/A	N/A	No
CPMC 105	2	c.385T>C	p.S129P	Probably damaging	0.895	No

Table 4

Clinical characteristics for the 20 families affected with *INF2*-related disease.

Family ID	Self-reported Ethnicity/Country	Ages at disease onset	Number affected	Ages at ESRD	Number with ESRD	Histology	Number with Biopsy
FG-BR	European ancestry/Canada	12–67	19	32–67	6	FSGS	7
FG-DM	European ancestry/Ireland and USA	13–18	2	21–29	2	FSGS	2
FG-EA	African American/USA	27–33	3	30–40	2	FSGS	1
FG-EF	European ancestry/USA	19–35	5	49	1	FSGS	3
FG-EP	European ancestry/USA	11–13	3	13–14	3	FSGS	3
FG-ER	European ancestry/Canada	13–60	9	20–50	6	Unclear	3
FG-FG	European ancestry/USA	12–72	7	17–60	3	FSGS	3
FG-GY	African American/USA	17–30	8	17–30	7	FSGS	2
FG-HT	European ancestry/USA	13–29	9	21–22	9	FSGS	4
FG-JN	European ancestry/USA	22–45	10	30–45	4	FSGS	At least 1
FG-JY	European ancestry/Australia	21-?	2	35	1	FSGS	3
FG-KM	European ancestry/Poland	15–24	4	25–44	2	FSGS	2
FG-KQ	Asian/USA	?	2	26-?	2	FSGS	1
FG-LL	European ancestry	19	2	19	1	FSGS	At least 1
FG-LP	European ancestry/USA	17–27	4	25–28	3	FSGS	1
FG-LW	European ancestry	22–30	4	27–30	3	FSGS	1
FG-LY	European ancestry/France	18	4	33	3	FSGS	1
FG-ME	European ancestry	15–16	3	-	0	FSGS	1
FS-B	European ancestry/USA	13–21	5	23–30	4	FSGS	3
FS-V	European ancestry/Sweden and Denmark	15	6	30	2	FSGS	1