Mutations in *INF2* Are a Major Cause of Autosomal Dominant Focal Segmental Glomerulosclerosis

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ABSTRACT

The recent identification of mutations in the *INF2* gene, which encodes a member of the formin family of actin-regulating proteins, in cases of familial FSGS supports the importance of an intact actin cytoskeleton in podocyte function. To determine better the prevalence of *INF2* mutations in autosomal dominant FSGS, we screened 54 families (78 patients) and detected mutations in 17% of them. All mutations were missense variants localized to the N-terminal diaphanous inhibitory domain of the protein, a region that interacts with the C-terminal diaphanous autoregulatory domain, thereby competing for actin monomer binding and inhibiting depolymerization. Six of the seven distinct altered residues localized to an INF2 region that corresponded to a subdomain of the mDia1 diaphanous inhibitory domain reported to co-immunoprecipitate with IQ motif–containing GTPase-activating protein 1 (IQGAP1). In addition, we evaluated 84 sporadic cases but detected a mutation in only one patient. In conclusion, mutations in *INF2* are a major cause of autosomal dominant FSGS. Because IQGAP1 interacts with crucial podocyte proteins such as nephrin and PLCε1, the identification of mutations that may alter the putative INF2–IQGAP1 interaction provides additional insight into the pathophysiologic mechanisms linking formin proteins to podocyte dysfunction and FSGS.

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FSGS is a histologic pattern of injury leading to end-stage kidney disease (ESKD) in approximately 40% of cases of isolated proteinuria/nephrotic syndrome.[1] The recognition of familial forms of FSGS has grown, and several human genes highly expressed in podocytes have been identified to cause this entity[2]; these findings highlighted the crucial role of the podocyte in the glomerular filtration barrier function. Although an autosomal recessive inheritance underlies most cases of inherited FSGS occurring in childhood, a few genes have been shown to be involved in the rare autosomal dominant (AD) juvenile and adult forms of the disease. Indeed, mutations in *ACTN4*, encoding

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α-actinin 4, and *TRPC6*, encoding a member of the transient receptor potential superfamily of ion channels, have been identified in patients presenting late-onset proteinuria with a slow progression to ESKD and may account for approximately 4 and 6% of familial FSGS, respectively.[3–7] Whereas the discovery of *TRPC6* unexpectedly suggested the implication of calcium signaling in the pathogenesis of FSGS, the identification of *ACTN4* emphasized the importance of an intact podocyte actin cytoskeleton in kidney function.

Recently, Brown *et al.*[8] found heterozygous mutations in the formin *INF2* gene segregating with FSGS in 11 (12%) of 93 families with age at diagnosis and ESKD varying from 11 to 72 years and 13 to 67 years, respectively. Formins are widely expressed proteins governing several dynamic events that require remodeling of the actin cytoskeleton such as cell polarity, cell and tissue morphogenesis, and cytokinesis.[9] To date, 15 mammalian formin genes have been identified, among which are the best studied diaphanous-related formins (DRF)—mDia1, mDia2, and mDia3—but also the inverted formins (INF). In the C-terminal half, DRF proteins contain the formin homology domains FH1/FH2 and the diaphanous autoregulatory domain (DAD) region, whereas the diaphanous inhibitory domain (DID) is localized at the N-terminal half. The FH2 domain directly mediates actin assembly, and the FH1 domain accelerates, through profilin-actin complexes, filament elongation. Interaction between DID and DAD blocks the ability of the FH2 to interact with actin, and this inhibition is relieved by the Rho GTPases.[10] In contrast to other formins, INF2-mediated polymerization is not inhibited by the DID–DAD interaction. In addition, INF2 has the unique ability to accelerate depolymerization, an activity requiring the C-terminus; the C-terminus can sequester actin monomers in 1:1 complexes and is also required for rapid filament severing. DID binding to the DAD compete for actin monomer binding, thereby inhibiting depolymerization.[11,12]

Although the study by Brown *et al.* suggested that *INF2* may play a significant role in FSGS, this finding has not yet been confirmed by others. In addition, the role of *INF2* mutations among cases with a sporadic appearance has never been evaluated. We therefore aimed to determine the prevalence of *INF2* mutations in a cohort of 54 families (78 patients) with a glomerular proteinuric disorder of apparent AD inheritance. These patients presented proteinuria at a median age of 20.5 years (range 2 to 52 years), and FSGS was documented in at least one affected member in 80% of families (Table 1).

Seven missense *INF2* mutations were found in nine families (28 patients), which translates to a detection rate of 16.7% (Table 2). Median age at onset of proteinuria among mutated patients was 27 years (compared with 15.5 years among familial cases without mutations; $P = 0.026$); proteinuria was isolated and in the nephrotic range (at least transiently) for most cases. Full-blown nephrotic syndrome was noted in four patients. Median age at ESKD was 36 years (range 20 to 70 years). No significant statistical difference was observed regarding the percentage of patients reaching ESKD ($P = 0.085$) and the age at onset of ESKD ($P = 0.629$) between familial cases with or without mutations. FSGS was documented in at least one affected member of each family with *INF2* mutation. Mutations segregated with disease in each respective family, although the screening of seven additional at-risk asymptomatic relatives (from three families) revealed a mutation in two cases, confirming the incomplete penetrance of the disease (Supplemental Figure S1). Indeed, in family F carrying the p.R214C, one affected member presented proteinuria at 5 years of age whereas a relative was still free of renal manifestations at 26 years of age. In family H, carrying the p.R218Q, proteinuria was detected at 10 and 24 years of age in two sisters, with ESKD at 30 years of age for one of them, whereas their mother had only isolated microalbuminuria (100 mg/d) at 66 years of age. Significant intrafamilial phenotypic variability was also noted with a wide range of age at presentation and ESKD. For example, in family A, carrying the p.L76P mutation, one member required dialysis at the age of 28 years whereas another presented moderate proteinuria without ESKD at the age of 79 years.

Given the occurrence of incomplete penetrance in AD FSGS cases and the reports of neomutation in *ACTN4*-, *TRPC6*-, and *INF2*-related FSGS,[3,6,8] we undertook the search for *INF2* mutations in 84 sporadic cases. The clinical presentation of sporadic and familial cases was similar. Only one already identified mutation (p.R218Q) was detected in a single patient, who presented proteinuria and FSGS lesions at 15 years of age. However, a complete renal evaluation and DNA samples were not available for his seemingly healthy parents, precluding the distinction between a *de novo* mutation and a familial case with low penetrance and variable expressivity.

Among the seven mutations identified in familial cases, four were novel mutations. We detected the p.R218Q and p.E220K in families from France, and these substitutions were also previously identified in families from the United States (European ancestry).[8] In addition, we found the p.R177H and the p.R214C mutations each in two seemingly nonrelated families (from France). We studied a possible founder effect by haplotype analysis using six markers flanking the locus. No common ancestral disease-associated haplotype was found, suggesting no founder effect (data not shown). All these four single-base mutations involve a CpG dinucleotide, consistent with a chemical model of mutation *via* methylation-mediated deamination.[13] Because this represents by far the most common single-base substitution involved in human disease,[14] we can speculate that these mutations are recurrent. Finally, the p.L198R, which was present in a family from North Africa in our series and in a family from Ireland/United States (European ancestry) in the Brown *et al.* study, does not affect a CpG doublet; however, on the basis of the ethnicity of the involved families, an underlying founder effect seems unlikely.

Table 1. Patient characteristics ($n = 162$)

Characteristic	All Cohort, *n* (%)[a,b]	Familial, *n* (%)[a,b]			Sporadic Cases, *n* (%)[a,b]
		Familial Cases	With Mutations	Without Mutations	
No. of cases	162	78	28	50	84
No. of families	138	54	9	45	84
Ethnic origin					
n	136 families	52 families	9 families	43 families	84
North Africa	5 (3.7)	3 (5.8)	1 (11.1)	2 (4.7)	2 (2.4)
Middle-East	2 (1.5)	1 (1.9)	1 (11.1)	0 (0.0)	1 (1.2)
Europe	115 (84.5)	46 (88.5)	7 (77.8)	39 (90.7)	69 (82.1)
Other	14 (10.3)	2 (3.8)	0 (0.0)	2 (4.7)	12 (14.3)
Renal histologic lesions					
n	124 families	45 families	9 families	36 families	79
FSGS	90 (72.6)	36 (80.0)	9 (100)	27 (75.0)	54 (68.4)
MGC	32 (25.8)	7 (15.6)	0 (0)	7 (19.4)	25 (31.6)
terminal kidney	2 (1.6)	2 (4.4)	0 (0)	2 (5.6)	0 (0)
Age at onset of proteinuria (years)					
n	130	50	18[c]	32	80
median (range)	19.0 (0.5 to 55.0)	20.5 (2.0 to 52.0)	27.0 (5.0 to 44.0)	15.5 (2.0 to 52.0)	19.5 (0.5 to 55.0)
mean ± SD	20.6 ± 12.1	21.6 ± 13.6	26.0 ± 10.6	19.1 ± 14.6	20.2 ± 11.0
ESKD					
n	155	71	26	45	84
no. of patients reaching ESKD	80 (49.4)	39 (54.9)	18 (69.2)	21 (46.7)	41 (48.8)
Age at onset of ESKD (years)					
n	75	34	16	18	41
median (range)	35.0 (7.0 to 73.0)	33.0 (7.0 to 73.0)	36.0 (20.0 to 70.0)	31.5 (7.0 to 73.0)	35.0 (13.0 to 62.0)
mean ± SD	36.4 ± 12.9	35.6 ± 14.4	36.1 ± 11.2	35.1 ± 17.1	37.0 ± 11.7

MGC, minimal glomerular changes.
[a]The number of available data are shown as *n*.
[b]The denominator used to calculate percentages is the number of available data.
[c]Among the nine families with *INF2* mutations, genetic testing was performed for seven asymptomatic relatives (from three families) and revealed an *INF2* mutation in two cases; one was free of renal manifestations at 26 years of age, whereas the other presented microalbuminuria with normal renal function and BP at 66 years of age. Only symptomatic patients are included in the cohort.

Table 2. Spectrum of *INF2* mutations and associated phenotypes among familial and sporadic cases with glomerular proteinuric disease

Family	Familial/ Sporadic	Origin	Nucleotide Alterations(s)	Predicted Effect on Protein	Exon	Age at Diagnosis[b]	*n*[c]	Age at ESKD[b]	*n*[d]	Histology
A	Familial	Europe	c.227 T>C	p.L76P	2	27 to 44	7	28 to 70	6	FSGS
B	Familial	Europe	c.530 G>A	p.R177H	4	24	4	ND	1	FSGS
C	Familial	Europe	c.530 G>A	p.R177H	4	19 to 27	2	27 to 29	2	FSGS
D	Familial	Oman	c.577 T>C	p.Y193H	4	24	1	No ESKD at 25 years	0	FSGS
E	Familial	North Africa	c.593 T>G	p.L198R[a]	4	28 to 36	3	29 to 36	3	FSGS
F	Familial	Europe	c.640 C>T	p.R214C	4	5 to 44	5	20 to 42	3	FSGS
G	Familial	Europe	c.640 C>T	p.R214C	4	16 to 37	2	41	2	FSGS
H	Familial	Europe	c.653 G>A	p.R218Q[a]	4	10 to 24	2	30	1	FSGS
I	Familial	Europe	c.658 G>A	p.E220K[a]	4	ND	2	No ESKD at 42 years	0	FSGS
J	Sporadic[e]	Europe	c.653 G>A	p.R218Q[a]	4	15	1	No ESKD at 28 years	0	FSGS

All the mutations were found in the heterozygous state. Mutation numbering is based on the cDNA reference sequence (GenBank accession no. NM_022489.3). ND, not determined.
[a]These mutations were reported in Brown *et al.*[8]
[b]Ages given as a range over the family members.
[c]Number of affected individuals with available age at onset of proteinuria.
[d]Number of affected individuals with available age at ESKD.
[e]Segregation of the mutation in the family cannot be tested.

All variants were localized in exons 2 and 4. They caused nonconservative changes in highly conserved amino acids of the N-terminal half of the protein (Figures 1 and 2A). None were present in any of the 350 chromosomes assayed. In an attempt to make functional predictions of the mutations found in our study, we used a human INF2 *in silico*

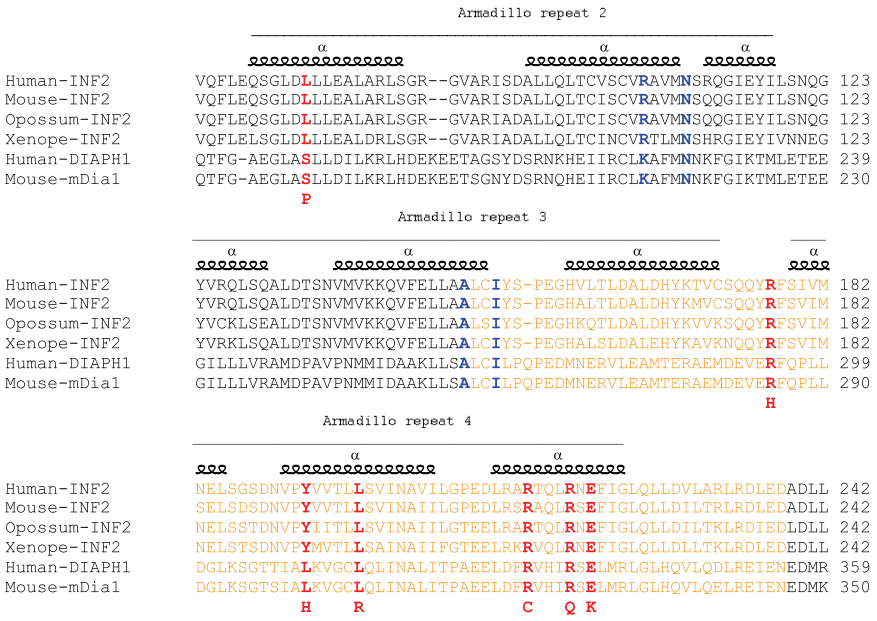

Figure 1. Protein sequence alignment of human, mouse, opossum, and *Xenopus* INF2; human DIAPH1 (the *human* homolog of *mDia1*); and mouse mDia1 N-terminal region. Amino acids 66 through 242 are shown for the human, mouse, opossum, and *Xenopus* INF2 corresponding to amino acids 181 through 359 and 172 through 350 of human DIAPH1 and mouse mDia1, respectively. Altered residues are shown in red. Residues important for the DID–DAD interaction are shown in blue (R106, N110, A149, and I152, corresponding to K213, N217, A256, and I259 in mDia1). The corresponding INF2 region of a mDia1 DID subdomain reported to interact with IQGAP1 (INF2 amino acids 149 through 238, corresponding to mDia1 amino acids 256 through 346) is represented in yellow. All substitutions occur in highly conserved amino acids. The secondary structure of the protein is shown above the sequence. The DID is composed of four armadillo repeats, each of which is formed by three adjacent α-helices. Six of the mutations identified in this series are localized in the fourth DID armadillo repeat.

model based on the available mDia1 DID crystal structure. The DID domain is composed of four armadillo repeats, a structural motif of three helices arrayed in a superhelical coil.[9–11] The core of the DAD segment is an amphipathic helix, binding to the concave surface formed by the central helices of the armadillo repeats. Six of the seven altered residues identified are localized in the corresponding INF2 region of a mDia1 DID subdomain reported to co-immunoprecipitate with IQ motif–containing GTPase-activating protein (IQGAP1; Figure 2B, a through e).[15] Within this subdomain, the residues are localized in three adjacent helices forming the fourth armadillo repeat (Figure 2Be). Residues R177 and E220 are localized at the protein surface (Figure 2Bb) and are interconnected. Residues L198, R214, and R218 are buried in the DID core (Figure 2Ba) and directed toward the long helix connecting the fourth armadillo repeat to the distant DID C-terminal part, whereas residue Y193 is localized at the interface between the third and fourth armadillo domains. Their mutation may induce changes in the amino acid side chain polarity (L198R), acidity/basicity (Y193H, R214C, R218Q, and E220K), or length (R177H and R214C). Finally, the L76P variant, part of the second armadillo repeat, seems to lie close to important residues for the DID–DAD interaction (Figure 2Bf).[8,10]

This study confirms that *INF2* mutations explain a high proportion of AD FSGS cases. Indeed, we detected mutations in this gene in almost one fifth of families with this type of disease, which is significantly higher than what has been reported for *ACTN4* and *TRPC6*.[3–7] Interestingly, all of the 13 human *INF2* mutations reported to date are restricted to two exons, encoding the DID. Indeed, 10 are localized in exon 4 and three are in exon 2. Although alteration of INF2 subcellular localization and disruption of actin behavior have been hypothesized, the precise mechanisms explaining how mutations in the INF2 DID region may lead to a proteinuric phenotype remain unclear. It is therefore of great interest that six of the seven altered residues found in our study are localized in the corresponding INF2 region of a mDia1 DID subdomain interacting with IQGAP1. Indeed, IQGAP1 has been identified as a Dia1-binding protein that is necessary for its subcellular location[15]; it interacts with Dia1 through a region within the DID after the RhoA-mediated release of Dia1 autoinhibition. IQGAP1 is also involved in actin cytoskeleton dynamics[16] and has been shown to interact with the podocyte proteins nephrin[17] and PLCε1.[18] Mutations in the genes encoding nephrin and PLCε1 have been identified in patients with nephrotic syndrome, some of whom presented FSGS.[18,19] On the basis of these observations, we may speculate that these six mutations, localized in the fourth armadillo domain, might alter the DID structure and a potential INF2–IQGAP1 interaction, leading to a disorganized podocyte architecture and the development of a glomerular proteinuric disorder. In addition, changes in the amino acid side-chain polarity, acidity/basicity, or length potentially induced by these mutations are likely to alter the superhelical structure and the function of the protein. Finally, the change in the amino acid side-chain orientation induced by the L76P substitution, localized in the second armadillo domain, may interrupt crucial interactions in this region of the protein.

Although the finding that all human *INF2* mutations identified so far affect the DID has a pathophysiologic relevance, it is striking that no mutation in the C-terminal part of the protein, encoding the DAD, has been identified. We may hypothesize that the collapse of the endoplasmic reticulum observed in cells expressing INF2 containing DAD mutations may be lethal in humans.[12] Animal

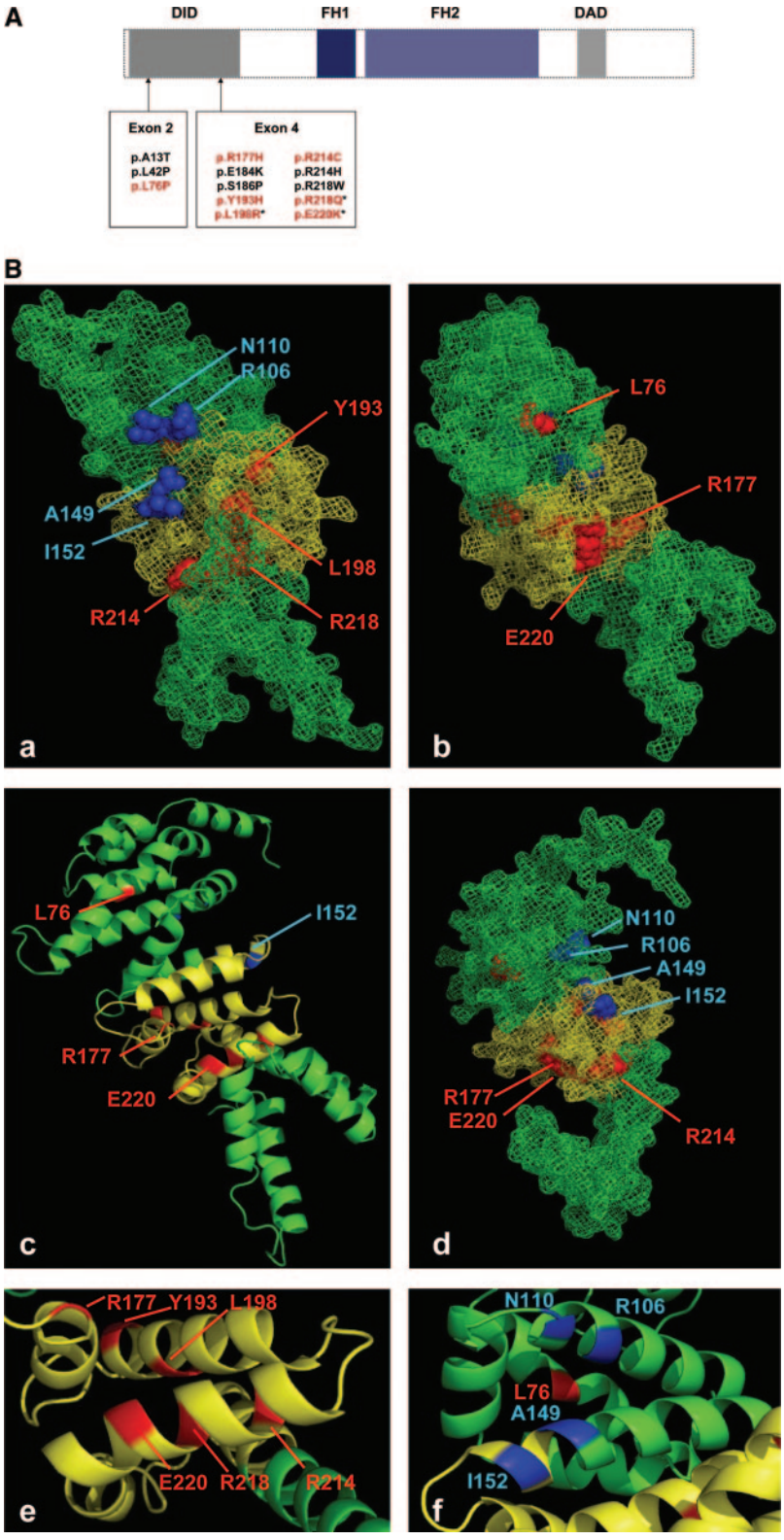

Figure 2. All mutated residues are located in the DID. (A) INF2 protein domain structure (two-dimensional representation) and location of *INF2* mutations. Mutations identified in this study are shown in red. Mutations identified by Brown *et al.*[8] are shown in black. *Mutations also found by Brown *et al.*[8] (B) Three-dimensional model of the N-terminal portion of human INF2, based on the structure of mDia1. Amino acids 1 through 330 of INF2 (corresponding to amino acids 46 through 440 of mDia1) are shown. Positions of the altered residues identified in our study are represented in red. Residues important for the DID–DAD interaction are shown in blue. The corresponding INF2 region of a mDia1 DID subdomain reported to interact with IQGAP1 (INF2 amino acids 149 through 238, corresponding mDia1 amino acids 256 through 346) is represented in yellow. (a) Global front view of INF2. Y193, L198, R214, and R218 are buried into the DID core. (b) Global rear view of INF2. R177 and E220 are localized at the protein surface. (c) Global cartoon view of the DID displaying the four armadillo repeats. (d) Profile (right) view of INF2. Residues important for the interaction with DAD (R106, N110, A149, and I152, corresponding to K213, N217, A256, and I259 in mDia1) are shown in the upper right of the figure; the core of the DAD segment binds to the concave surface formed by the central helices of the DID armadillo repeats. The altered residues R177, R214, and E220 are localized in the DID subdomain co-immunoprecipitating with IQGAP1. The concave surface observed in the lower left of the figure may represent an IQGAP1-binding pocket. (e) Close-up view of the fourth armadillo repeat. Six of the seven mutations identified in this study are localized in the three α-helices forming the fourth DID armadillo repeat. (f) Close-up view of the putative DID–DAD interaction domain. The altered residue L76 is part of this interacting domain.

or cellular models should be developed with the aim to characterize better the phenotype associated with mutations that alter the INF2 C-terminus.

In conclusion, *INF2* is a major gene of AD FSGS. Screening for *INF2* mutations, at least in exons 2 to 4, needs to be strongly considered in patients with an AD familial history of FSGS, even before *ACTN4* and *TRPC6*. Conversely, this gene seems less involved in sporadic cases of FSGS. All of the mu-

tations identified to date are localized in the DID region of the protein, suggesting its critical role for INF2 function. Whereas patients with disease-associated *INF2* mutations may have a defect in actin-mediated podocyte structural maintenance and repair, the identification of mutations in a mDia1 DID subdomain co-immunoprecipitating with IQGAP1 provides additional insights into the pathophysiologic mechanisms linking formin proteins to podocyte dysfunction and FSGS.

CONCISE METHODS

Patients

A total of 162 patients belonging to 138 families were included in this study. These patients were divided into familial cases (n = 78), defined as families with two (or more) affected cases compatible with AD inheritance, and sporadic cases (n = 84). Mutations in the *ACTN4* and *TRPC6* genes were excluded for all familial cases. Most (84%) families were from Europe. Patients presented proteinuria between the age of 0.5 and 55.0 years, with a median age of 19.0 years. Microscopic hematuria and early-onset hypertension were rarely noted. Among patients with available data, 49% reached ESKD at a median age of 35 years (range 7 to 73 years). A renal biopsy was performed in 124 families; FSGS, minimal glomerular changes, and terminal kidney were noted in at least one affected member in 72, 26, and 2% of these families, respectively. Patient characteristics are summarized in Table 1. No significant difference was noted between patients with familial and sporadic cases in ethnicity, histology, and clinical presentation. Informed consent was obtained for all participating families, and the study was approved by the Comité de Protection des Personnes "Ile de France II."

Genotyping and Mutation Analysis

Genomic DNA was extracted from peripheral blood by standard methods. The complete coding sequence and exon-intron boundaries of *INF2* gene were amplified by PCR (23 exons, GenBank accession no. NM_022489.3) for all familial cases. Given the absence of mutation detection outside exons 2 and 4 among familial cases, mutational screening was restricted to exons 2, 3, and 4 among sporadic cases. Sequencing was performed using a Big Dye terminator cycle sequencing kit and analyzed with an ABI Prism 3130 XL DNA analyzer (Applied Biosystems, Foster City, CA). PCR and sequencing primers are listed in Supplemental Table 1. Sequence chromatograms were analyzed using the Sequencher software (Gene Codes, Ann Arbor, MI). The absence of the mutations among 350 control alleles was confirmed. Mutational data were described using the nomenclature of the Human Genome Variation Society (www.hgvs.org/mutnomen). Positions of mutations were numbered with the A of the ATG-translation initiation codon in the reference cDNA sequence being 1. A haplotype analysis was performed for the five families and the single sporadic case with p.R177H, p.R214C, and p.R218Q mutations using six microsatellite markers surrounding the gene locus: D14S1010, D14S260, D14S292, D14S1007, D14S105.25, and D14S105.50. The two last ones were designed for this study, flank the gene, and are located at a distance of 71 and 105 kb, respectively (primer sequences are reported in Supplemental Table 1).

Structural Model

Three-dimensional models of the human INF2 (amino acids 1 through 330) were designed using the Phyre threading program (www.sbg.bio.ic.ac.uk/phyre/),[20] based on primary sequence conservation and known protein structures. Phyre aligns the primary sequence of the human INF2 to the mDia1 structure (PDB entry: 2BNX). We manipulated the models using the program PyMOL (PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA). Altered residues identified in this study, residues important for the DID–DAD interaction, and the DID subdomain interacting with IQGAP1 were mapped onto this structural model.

Statistical Analysis

All values are expressed as mean ± SD and median (range). Comparisons between two continuous variables were performed using the Mann-Whitney U test. For categorical variables, testing for difference in proportions was performed using the using the χ^2 or the Fisher exact test. All tests were two sided. Only $P < 0.05$ was considered significant. Statistical analyses were performed using SPSS 11.0.

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DISCLOSURES

None.

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