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Original Article

Novel *INF2* mutations in an Italian cohort of patients with focal segmental glomerulosclerosis, renal failure and Charcot-Marie-Tooth neuropathy

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ABSTRACT

Background. Mutations of *INF2* represent the major cause of familial autosomal dominant (AD) focal segmental glomerulosclerosis (FSGS). A few patients present neurological symptoms of Charcot-Marie-Tooth (CMT) disease but the prevalence of the association has not been assessed yet.

Methods. We screened 28 families with AD FSGS and identified 8 *INF2* mutations in 9 families (32 patients overall), 3 of which were new. Mutations were in all cases localized in the diaphanous-inhibitory domain (DID) of the protein.

Results. Clinical features associated with *INF2* mutations in our patient cohort included mild proteinuria (1.55 g/L; range 1–2.5) and haematuria as a unique symptom that was recognized at a median age of 21.75 years (range 8–30). Eighteen patients developed end-stage renal disease during their third decade of life; 12 patients presented a creatinine range between 1.2 and 1.5 mg/dL and 2 were healthy at 45 and 54 years of age. CMT was diagnosed in four cases (12.5%); one of these patients presented an already known mutation on exon 2 of *INF2*, whereas the other patients presented the same mutation on exon 4, a region that was not previously associated with CMT.

Conclusions. We confirmed the high incidence of *INF2* mutations in families with AD FSGS. The clinical phenotype was mild at the onset of the disease, but evolution to ESRD was frequent. The incidence of CMT has, for the first time, been calculated here to be 12.5% of mutation carriers. Our findings support *INF2* gene analysis in families in which renal failure and/or neuro-sensorial defects are inherited following an AD model.

Keywords: focal segmental glomerulosclerosis, *INF2* gene, mutation analysis, proteinuria

INTRODUCTION

Segmental glomerulosclerosis is a histology form of kidney injury characterized by focal and segmental areas of glomerular sclerosis and tubulointerstitium fibrosis (FSGS) [1]. Primary forms of FSGS usually occur as isolated proteinuria in children and adults, and may evolve to end-stage renal failure (ESRD) [2]. Primary FSGS should not be confused with secondary glomerulosclerosis, which is a common finding in several renal disorders with progressive decline of renal function and often coincides with end-stage renal lesions. However, an isolated defect with fusion of podocytes could represent the unique pathology feature in early phases of primary FSGS [3]. This diagnosis can be done in ~10% of renal biopsies performed at any age for evaluation of proteinuria, with a slight prevalence in males and with a higher frequency in the African-American population [4]. Overall, FSGS is the most frequent cause of nephrotic syndrome in children and is becoming a leading cause also in adults. It accounts for up to 20% of causes of ESRD [5].

Starting from 1998, studies on familial FSGS/nephrotic syndrome have led to the identification of several genes that are essential for podocyte development and function. Their alteration is almost invariably associated with proteinuria and glomerulosclerosis: an updated list includes 13 genes [6], and mutations in 7 of these are a cause of autosomal dominant (AD) FSGS, e.g. *ACTN4* [7], *TRPC6* [8], *CD2AP* [9, 10], *WT1* [11], *LMX1B* [12], *ARHGAP24* [13] and *INF2* [14]. Recent literature suggests that mutations of *INF2* are more frequent than mutations in other genes, and based on these findings, *INF2* sequencing analysis has been included in the molecular flow chart to approach diagnosis in families with AD FSGS. *INF2* encodes for a member of the formin family of actin-regulating proteins [15, 16] that plays a key role to maintain plasticity of podocyte. More recently, an association has been shown between *INF2* mutations and Charcot-Marie-Tooth (CMT) [16], which is characterized by a demyelinating peripheral neuropathy. Up to now, 60 families, for a total of 205 patients, have been reported to carry an *INF2* mutation; in a few cases, the association of FSGS with CMT has been demonstrated but the real incidence of neuro-sensorial defects is still unknown. In this study, we report 9 new families with a total of 32 patients carrying *INF2* mutations, 3 of which are described here for the first time. All but two patients presented proteinuria and signs of renal involvement while CMT was detected in four patients. The aim of the present study is to supply a detailed map of *INF2* mutations and the relative clinical phenotypes associated with them.

MATERIALS AND METHODS

Clinical data

We recruited 28 families with FSGS and autosomal dominant inheritance. One hundred and one patients were studied with multiple molecular tests relative to genes involved in AD FSGS. Each family was selected on the basis of presence of affected subjects in at least two generations and biopsy-proven FSGS in at least one member. Clinical data relative to each patient were examined for excluding secondary causes that include evaluation of extra-renal signs of renal disease, autoimmunity and metabolic causes; urine and blood samples were routinely collected and analysed by local laboratories. Asymptomatic individuals were examined and tested for proteinuria. One index case from each FSGS family was selected and analysed for *INF2, ACTN4*, *TRPC6*, *CD2AP* and *WT1*. In case of *INF2* mutations, all the available family members were tested for the same mutation and the same individuals were evaluated for the presence of neurologic alteration of CMT. Informed consent was obtained from all participants. This study was approved by The Giannina Gaslini Institute ethical committee and complies with the Declaration of Helsinki guidelines.

Mutational analysis

Genomic DNA was extracted from peripheral blood by standard methods. Gene annotation was performed using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) and the University of California Santa Cruz genome browser (http://genome.ucsc.edu/). Molecular analysis was performed by direct sequencing as previously described [17, 18]. Exons were amplified by PCR using specific flanking intron primers (sequence available on request) designed by Exon Primer tools (http://ihg.helmholtz-muenchen.de) and subjected to automated sequence analysis by dye-terminator reactions (Automated Sequencer ABI 377xl; Applied-Biosystem, Milan, Italy). Data were analysed using the Sequencher software version 5.0 (Genecodes Corp.).

All new non-synonymous variants were evaluated with the PolyPhen-2 software (http://genetics.bwh.harvard.edu/pph2/index.shtml) and the SIFT software (http://sift.jcvi.org/) to examine the predicted damaging effect of the amino-acid substitution to the function of inverted formin 2 isoform 1 (Uniprot ID: Q27J81).

Data about *INF2* gene variations were obtained from NHLBI ESP Exome Variant Server database (http://evs.gs.washington.edu/EVS/) and matched with both our results and other already existing results.

RESULTS

We identified 9 families out of 28 with AD familial nephrotic syndrome with mutations of *INF2* (pedigrees are presented in Figure 1). Of the remaining 19 families, 4 presented mutations in one of the other genes already described as responsible of familial FSGS: *CP2AP* [10], *TRPC6* [8, 19], *WT1* [11], *LMX1B* [12] and one family was associated with mutations in *COL4A4* (Gianluca Caridi, personal communication); in the remaining 14 families, no gene alteration was identified. Overall, 32 patients were identified who carried 8 *INF2* variants, 4 of which have previously been reported while 3 (p.Val105Gly, p.Leu162Arg, p.Tyr168del) were new (see Table 1 and Figure 2). All the above mutations segregated with symptoms of active disease, with the exception of a man of 54 (Fam GE 06, p.Arg214Cys) and a woman of 45 years of age (Fam NA01, p.Arg218Gln) who were normal and are currently monitored

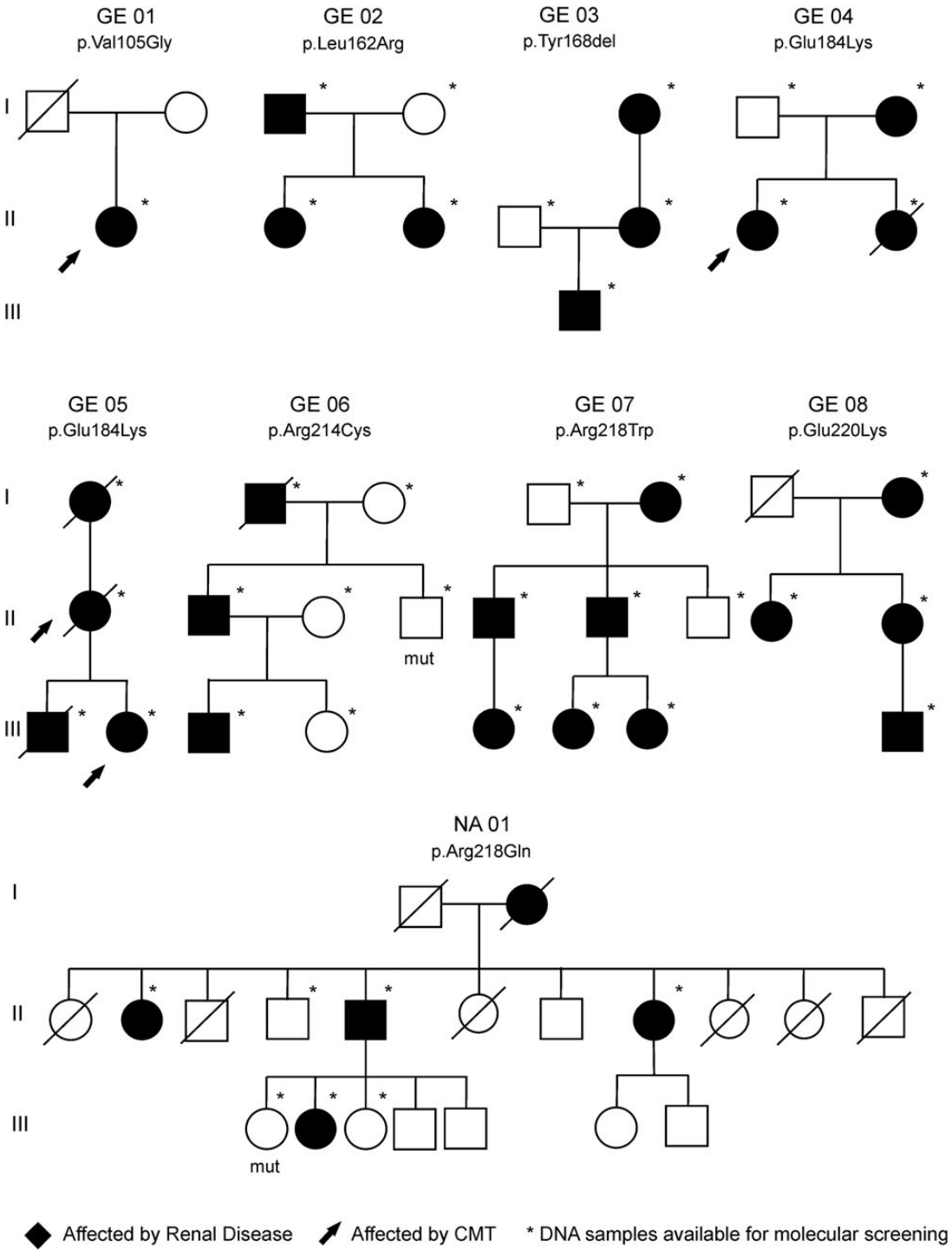

FIGURE 1: Pedigree diagrams and segregation of *INF2* mutations in our cohort. The figure shows the pedigrees of the *INF2* mutated families; black filled individuals are affected by renal disease; black arrows indicate the presence of Charcot-Marie-Tooth. All affected individuals carried the mutation with the exception of two asymptomatic individuals labelled as *'mut'* (Fam GE 06; NA 01).

for proteinuria. None of the new variants described here were present in 100 controls of the same ethnicity, place of origin, sex and age; moreover, they were not reported in the public NCBI databases and in both the 1000 Genome and Exome Sequencing Projects (NHLBI ESP 6400).

As reported in Tables 1 and 2, all mutations described here were detected at exons 2, 3 and 4 that code for a highly conserved region at the NH2 terminus of the protein, also known as the diaphanous-inhibitory domain (DID), which functions as a regulator of polymerization and depolymerization of actin filaments [14, 16, 20]. Analysis by SIFT and Polyphen-2 predicted deleterious structural and functional effects. As previously reported by other authors [12, 14, 16, 21–28] and with the exception of Sanchez-Ares *et al.* [25]

Table 1. Clinical and genetic data of the nine families with *INF2* mutations

cDNA Mut	Prot Mut	Exon	No. Fam	No. Mut	FSGS	FSGS+ CMT	Healthy	Age at onset median (range)	Proteinuria (g/L) median	ESRD	Polyphen-2/ SIFT score
c.314T>G	p.Val105Gly	2	GE 01[a]	1	0	1	0	17,2	1.1	1	1/0
c.485T>G	p.Leu162Arg	3	GE 02	3	3	0	0	17.7 (8–30)	2.1	2	1/0
c.502_504del	p.Tyr168del	3	GE 03	3	3	0	0	23.7 (20–28)	2.5[b]	2	n.d/n.d
c.550G>A	p.Glu184Lys	4	GE 04	3	2	1	0	28.3 (27–30)	1.3	3	1/0.25
c.550G>A	p.Glu184Lys	4	GE 05	3	1	2	0	19 (17–22)	1.7	4	1/0.25
c.640C>T	p.Arg214Cys	4	GE 06	4	3	0	1	22.4 (15–26)	1.6	1	1 /0
c.652C>T	p.Arg218Trp	4	GE 07	6	6	0	0	26 (16–38)	1.2	1	1/0
c.653G>A	p.Arg218Gln	4	NA 01	5	4	0	1	23.3 (17–30)	1.0	3	1/0.06
c.658G>A	p.Glu220Lys	4	GE 08	4	4	0	0	21.4 (17–25)	1.5	1	1/0.01
			Tot.	32	26	4	2	21.75 (8–38)	1.55	18	

[a]No clinical data available about parents.
[b]Onset of a family member during pregnancy with value 6 g/L.

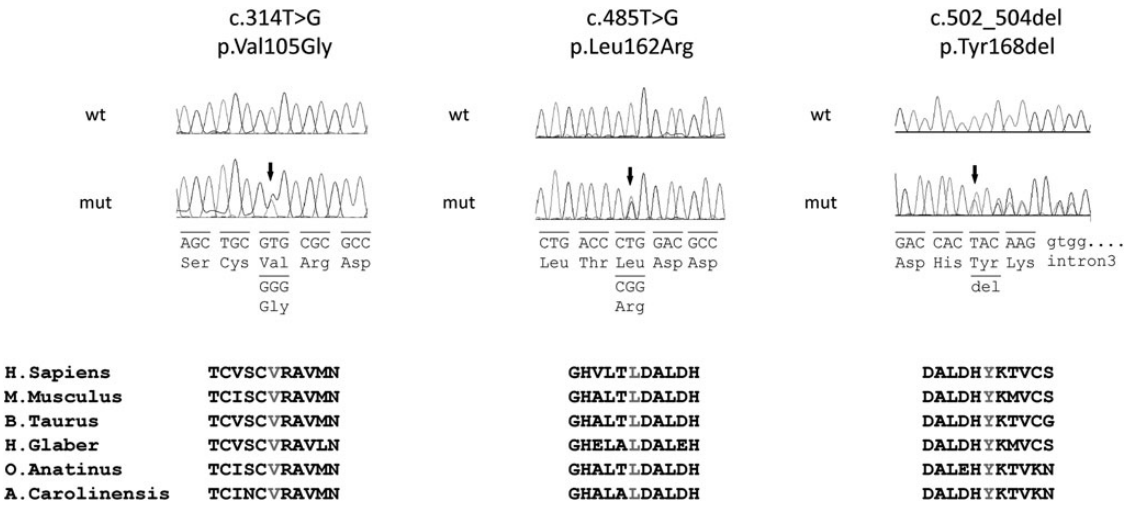

FIGURE 2: Electropherograms and evolutionary conservation of novel *INF2* mutations. Electropherograms of the new *INF2* mutations identify in our study. Arrows indicate the mutated nucleotide. Different species alignments of inverted formin 2 protein.

who described mutations in exon 6, all mutations of *INF2* associated with FSGS have been found in the region above (see Table 2).

Clinical details relative to our patient cohort are given in Table 1. The onset of the disease was at a median age of 21.8 years (range 8–38); as expected, mild proteinuria was the unique symptom in most cases (1.55 g/L; range 1–2.5) with the exception of one woman who developed nephrotic syndrome during pregnancy. In spite of mild proteinuria, 18 patients developed ESRD during the third decade of life; 12 other patients maintained a slight increase of creatinine (between 1.2 and 1.5 mg/dL) at a median age of 27.5 years. CMT was found in four patients (12.5%) belonging to three families (Fam GE 01, Fam GE 04 and Fam GE 05). They showed an association of FSGS with progression to ESRD with typical neurologic features of peripheral neuropathy that include distal muscle weakness with atrophy and distal sensory loss. One family (Fam GE01) of the CMT cohort carried a mutation never described before (p.Val105Gly) that is localized at exon 2. Interestingly, the other two families presented an already known variant at exon 4 (p.Glu184Lys) [14], which has never been associated with CMT. The two families above were unrelated and came from distinct geographic regions. Genetic analysis of *PMP22* was negative for deletions/duplications.

DISCUSSION

The results of our survey confirm the prevalence of *INF2* mutations over other genes in families with autosomal dominant FSGS. They also confirm that the association of FSGS with CMT can be detected in a minority of patients with mutations of *INF2*, 12.5% in our study cohort. Finally, we could describe three new *INF2* variants associated with FSGS (p.Val105Gly, p.Leu162Arg, p.Tyr168del) and the new association of FSGS-CMT with an already reported mutation (p.Glu184Lys).

Including the cases described here, 40 mutations of *INF2* have been reported so far in the literature. With the exception of one case [25], all mutations cluster in exons 2–3 and 4 of the gene in the DID located at the N-terminus of the protein, which

Table 2. Spectrum of the *INF2* mutations identified so far and clinical associated phenotype

No.	cDNA	Protein	Exon	No. Fam	No. Mut	FSGS	FSGS+CMT	Und.	Healthy	Reference
1	c.37G>A	p.Ala13Thr	2	1	2	1	0	0	1	[13][a]
2	c.125T>C	p.Leu42Pro	2	1	3	3	0	0	0	[13]
3	c.170T>C	p.Leu57Pro	2	1	1	0	1	0	0	[15]
4	c.205_216del	p.Leu69_Ser72del	2	1	1	0	1	0	0	[28]
5	c.206T>C	p.Leu69Pro	2	2	2	0	2	0	0	[16]
6	c.217G>A	p.Gly73Ser	2	1	4	4	0	0	0	[24]
7	c.227T>C	p.Leu76Pro	2	1	5	5	0	0	0	[14]
8	c.230T>C	p.Leu77Pro	2	1	2	0	2	0	0	[23]
9	c.230T>G	p.Leu77Arg	2	1	1	0	1	0	0	[28]
10	c.242T>C	p.Leu81Pro	2	1	6	6	0	0	0	[24]
11	c.305_314del	p.Val102_Cys104	2	1	1	1	0	0	0	[24]
12	c.310T>C	p.Cys104Arg	2	1	1	0	1	0	0	[15]
13	c.311G>T	p.Cys104Phe	2	1	1	0	1	0	0	[15]
14	c.312C>G	p.Cys104Trp	2	1	3	0	3	0	0	[15]
15	c.314T>G	p.Val105Gly	2	1	2	0	1	1	0	–[b]
16	c.317G>C	p.Arg106Pro	2	1	1	0	1	0	0	[15]
17	c.323T>A	p.Val108Asp	2	1	1	0	1	0	0	[16]
18	c.341G>A	p.Gly114Asp	2	1	1	0	1	0	0	[28]
19	c.383T>C	p.Leu128Pro	2	3	3	0	3	0	0	[15, 23]
20	c.385T>C	p.Ser129Pro	2	1	1	1	0	0	0	[24]
21	c.395T>G	p.Leu132Arg	3	2	4	0	4	0	0	[15]
22	c.415T>C	p.Cys151Arg	3	1	4	4	0	0	0	[24]
23	c.472C>G	p.His158Asp	3	1	9	9	0	0	0	[24]
24	c.485T>G	p.Leu162Arg	3	1	3	3	0	0	0	–[b]
25	c.490_498del	p.Ala164_Asp166del	3	2	5	1	4	0	0	[15]
26	c.494T>C	p.Leu165Pro	3	1	2	1	1	0	0	[15]
27	c.502_504del	p.Tyr168del	3	1	3	3	0	0	0	–[b]
28	c.529C>T	p.Arg177Cys	4	1	4	4	0	0	0	[24]
29	c.530G>A	p.Arg177His	4	3	9	7	0	0	2	[14, 21]
30	c.542T>G	p.Val181Gly	4	1	2	2	0	0	0	[24]
31	c.550G>A	p.Glu184Lys	4	3	9	6	3	0	0	[13][b]
32	c.550G>C	p.Glu184Gln	4	1	8	8	0	0	0	[21]
33	c.556T>C	p.Ser186Pro	4	2	28	18	0	5	5	[13]
34	c.577T>C	p.Tyr193His	4	1	1	1	0	0	0	[14]
35	c.593T>G	p.Leu198Arg	4	2	5	5	0	0	0	[13, 14]
36	c.605A>G	p.Asn202Ser	4	1	2	2	0	0	0	[21]
37	c.608C>A	p.Ala203Asp	4	1	2	2	0	0	0	[21]
38	c.640C>T	p.Arg214Cys	4	5	16	12	0	0	4	[14, 21, 24][b]
39	c.641G>A	p.Arg214His	4	4	26	20	0	2	4	[13, 21]
40	c.652C>T	p.Arg218Trp	4	4	14	13	0	0	1	[13, 14, 24][b]
41	c.653G>A	p.Arg218Gln	4	5	26	19	0	3	4	[13, 21, 24, 26][b]
42	c.658G>A	p.Glu220Lys	4	5	13	13	0	0	0	[13, 14, 22, 26][b]
43	c.734T>C	p.Pro245Leu	6	1	3	3	0	0	0	[25]
				72	240	177	31	11	21	

[a]Present in NHLBI ESP6400.
[b]Present study.

functions as a regulator of polymerization and depolymerization of actin filaments [14, 15, 20]. Prediction analysis of the effects of amino acid substitutions reported here supports abnormalities of the protein structure [14, 16, 21, 25] and functional defects with consequent anomalies of cytoskeleton physiology. In fact, *INF2* mutations at the DID alter the binding to CDC42, which is an actin-regulating protein.

Our observations confirm some previously reported characteristics relative to FSGS associated with *INF2* mutations but also suggest new clinical elements that are worth being reported here.

Confirmatory data are relative to the absence of nephrotic syndrome in practically all patients and the association with signs of advanced renal failure. In fact, proteinuria was mild during the course of the disease in all patients confirming the general concept that proteinuria is not a good surrogate biomarker of renal defect in glomerular pathologies and, in particular, in the presence of alterations of the cytoskeleton. Other findings obtained in patients with FSGS with mutations of other cytoskeleton genes (such as in the case of non-muscle myosin II) already showed mild proteinuria and renal failure as the prevalent phenotype [29].

New clinical elements relative to patients carrying INF2 mutations concern penetrance of renal and neuro-sensorial phenotypes and variable expressivity of the renal phenotype. As for the first aspect, we observed two subjects of 45 and 54 years of age, who carried an *INF2* mutation without any sign of renal involvement. Our percentage of healthy carriers of

INF2 mutations (6%) is in line with what is reported in the literature. Neuro-sensorial defects were present in 4 patients out of 32 and were always associated with proteinuria/FSGS. Data in the literature on the association of FSGS with CMT are scanty not allowing any comparison; the only author reporting the association of CMT with *INF2* mutations in a sufficient number of patients is Boyer *et al.*, [15] who described 12 unrelated index cases out of 16 with both CMT and FSGS. There is an obvious bias in recruiting patients in this report, because they were selected for the presence of neuro-sensorial problems out of a large neurologic database. Thus, they do not represent the whole population with *INF2* mutations and do not allow us to calculate the real prevalence of CMT. In the same way, Toyota *et al.* [26] described three unrelated patients and Rodriguez *et al.* [23] reported one family and one sporadic case of CMT and FSGS with mutations of *INF2*. More recently, Mademan *et al.* [28] described three cases with *de novo INF2* mutations and FSGS-CMT; this finding strengthens the role of *INF2* and excludes a causal association. In our study, we considered the incidence of CMT in association with *INF2* mutations of ∼10%. Since this percentage has been determined in an unbiased cohort of patients, it probably represents the first reliable report on the topic. Larger data sets are required to strengthen the power of this first observation.

CMT was associated with mutation in exons 2 and 4 of *INF2*. The later mutation, i.e. p.Glu184Lys in exon 4, was found in three patients from two unrelated families and represents the first example of *INF2* mutation localized outside the previously reported region [16, 26]. There are molecular reasons to consider why mutations associated with CMT are differently localized compared with those associated with a pure renal phenotype. In fact, *INF2* variants associated with CMT (with the unique exception of p.Glu184Lys) cluster in the same DID domain but in a region between nucleotides 300 and 500, whereas they involve a downstream region in those cases associated with FSGS. The two regions above code for different armadillo repeats of the molecule. The existence of mutations outside this gene region suggests the possibility of other molecular mechanisms to explain neurological defects. Anyway, neuro-sensorial phenotypes presented a variable penetrance; in fact, we observed CMT in 50% of patients belonging to the same family (i.e., 4 patients out of 8 carrying *INF2* mutation). This confirms what has been reported by Boyer *et al.* [16] and suggests the involvement of a specific genetic background or of environmental causes in the onset of the neurological phenotype.

A final clinical aspect is the renal phenotype and its intrafamilial variability. Lee *et al.* [22] presented a family (father and two sons) with the same *INF2* mutation and very different severity of the disease. We also observed marked clinical heterogeneity in some of our families. Our data confirmed that possible development of ESRD usually starts during the third decade, despite mild proteinuria being the predominant feature. Therefore, mild proteinuria is not synonymous of a mild phenotype. For this reason it is difficult to define with precision the onset of renal symptoms, since patients can present no or mild symptoms until ESRD development. A late expression of renal symptoms may explain the possibility of patients without urinary modification in the presence of *INF2* mutation (11.2% in our data set). Overall, difference in clinical expression of neurological and renal phenotypes still presents unresolved problems that suggest a prominent role of molecular or environmental modifiers.

A particular feature of patients with *INF2* mutations of our cohort is the age of onset of clinical symptoms with start at a mean age of 22 years. Only one patient presented mild proteinuria before puberty (8 years). Therefore, it is clear that under this age several other molecular defects are implicated in proteinuria. Testing *INF2* can be done in young children in the presence of proteinuria and/or renal failure with autosomal dominant inheritance.

In conclusion, we confirm here the high incidence of *INF2* mutations in families with AD FSGS and also point out how the phenotype is mainly characterized by mild proteinuria and renal failure. We may, for the first time, indicate that CMT is present in a minor proportion (∼12.5%) of carriers of *INF2* mutations and describe a first case of neuro-sensorial phenotype associated with mutations outside exons 2 and 3 of the gene. Taken together with data in the literature, our findings strongly support *INF2* analysis in all families in which renal failure and/or neuro-sensorial defects are inherited following an AD model.

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CONFLICT OF INTEREST STATEMENT

The results presented in this paper have not been published previously in whole or part, except in abstract format.

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