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MUTATIONAL ANALYSIS OF THE *PLCE1* GENE IN STEROID-RESISTANT NEPHROTIC SYNDROME

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ABSTRACT

Background: Mutations in the *PLCE1* gene encoding phospholipase C epsilon 1 (PLCε1) have been recently described in patients with early-onset nephrotic syndrome (NS) and diffuse mesangial sclerosis (DMS). In addition, two cases of *PLCE1* mutations associated with focal segmental glomerulosclerosis (FSGS) and later NS onset have been reported.

Methods: In order to better assess the spectrum of phenotypes associated with *PLCE1* mutations, we performed mutational analysis in a worldwide cohort of 139 patients (95 familial cases belonging to 68 families and 44 sporadic cases) with steroid-resistant NS presenting at a median age of 23.0 months (range 0-373).

Results: We identified homozygous or compound heterozygous mutations in 33% (8/24) of DMS cases. *PLCE1* mutations were found in 8% (6/78) of FSGS cases without *NPHS2* mutations. Nine were novel mutations. No clear genotype-phenotype correlation was observed, with either truncating or missense mutations detected in both DMS and FSGS, and leading to a similar renal evolution. Surprisingly, 3 unaffected and unrelated individuals were also found to carry the homozygous mutations identified in their respective families.

Conclusion: *PLCE1* is a major gene of DMS and is mutated in a non-negligible proportion of FSGS cases without *NPHS2* mutations. Although we did not identify additional variants in 19 candidate genes (16 other PLC genes, *BRAF*, *IQGAP1* and *NPHS1*), we speculate that other modifier genes or environmental factors may play a role in the renal phenotype variability observed in individuals bearing *PLCE1* mutations. This observation needs to be considered in the genetic counselling offered to patients.

KEY WORDS: *PLCE1***,** nephrotic syndrome, diffuse mesangial sclerosis**,** focal segmental glomerulosclerosis, hereditary glomerular disease

INTRODUCTION

Nephrotic syndrome (NS) results from disruption of the renal filtration barrier composed of interdigitating podocyte foot processes linked by the slit diaphragm, and endothelial cells separated from podocytes by the glomerular basement membrane. It is characterized by massive proteinuria, hypoalbuminemia, hyperlipidemia, edema and podocyte foot process effacement by electron microscopy. NS is classified as steroid-sensitive (SSNS) or steroid-resistant (SRNS) and mutations in several genes, mostly coding for podocyte proteins, have been identified as causing SRNS in humans. Although kidney biopsy discloses focal and segmental glomerulosclerosis (FSGS) lesions for most patients with SRNS,[1] a smaller proportion of children may present a particularly severe renal histological pattern named diffuse mesangial sclerosis (DMS) with an early presentation and a poor renal prognosis. DMS is characterized by mesangial expansion and sclerosis that evolves toward obliteration of the capillary lumen and contraction of the glomerular tuft. This type of renal histology has been described as part of syndromes such as Denys-Drash syndrome or Pierson syndrome, caused by mutations in the *WT1* and *LAMB2* genes, respectively. More recently, *PLCE1* mutations have been found as a novel cause of DMS.[2] Involvement of the *PLCE1* gene (MIM*608414) in NS has been found using a combination of homozygosity mapping and cDNA microarrays from rat glomeruli. In the original series described by Hinkes et al.,[2] truncating *PLCE1* mutations were identified in 12 siblings from six families diagnosed with early-onset NS and DMS while a homozygous missense mutation was identified in two siblings with a later onset of disease and FSGS (Nephrotic syndrome, type 3; NPHS3 - MIM#610725); until now, these individuals were the only reported cases of FSGS secondary to *PLCE1* mutations. Subsequently, mutations in *PLCE1* have been demonstrated as a major cause of isolated DMS, identified in 28.6% of 35 families in a worldwide cohort.[3] Although *PLCE1* mutations typically lead to a severe phenotype, full and sustained treatment responses

have been reported in two individuals with truncating mutations[2] and a pathogenic homozygous mutation have also been found in a phenotypically completely normal adult.[4] This suggests that other factors may modify the effect of *PLCE1* mutations.

PLCE1 gene encodes the phospholipase C epsilon 1 (PLCε1). Members of the phosphoinositide-specific phospholipase C (PLC) family catalyze the hydrolysis of membrane phospholipids to generate the second messenger molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) that initiate intracellular pathways of cell growth and differentiation.[5] Several distinct PLC enzymes have been identified in a variety of mammalian tissues and are divided into four classes: PLC*β*, PLC*γ*, PLC*δ* and PLCε. Phospholipase catalytic domains (PLC_X and PLC_Y) and Ca^{2+} lipid-binding domain (C2 domain) typically characterized all PLCs. PLCε1, which is highly expressed in podocytes,[2] is the most recently identified member of the PLC family; it contains, in addition to conserved PLC domains, a RasGEF_CDC25 (guanine nucleotide exchange factor for Ras-like small GTPases domain) and two C-terminal Ras-binding (RA) domains, RA1 and RA2 (RasGTP binding domain from guanine nucleotide exchange factors). While PLC*β*, PLC*γ*, PLC*δ* are differentially regulated by heterotrimeric G-proteins, tyrosine kinases and calcium, it has been shown that PLCε1 is regulated by H-Ras (via RA1 and RA2 domains) and also interacts with IQGAP1 (IQ motif-containing GTPase-activating protein) and BRAF.[2 5-7]

In order to better define the phenotype spectrum of patients bearing *PLCE1* mutations, we performed mutational analysis and clinical follow-up of a large worldwide cohort of patients with either familial or sporadic SRNS. In addition, we aimed to determine the role of candidate genes, including other PLC genes, as modifiers of disease in families for which both symptomatic and asymptomatic members had homozygous *PLCE1* mutations.

MATERIALS AND METHODS

Patients

A total of 139 patients were included in the present study, all of which presented SRNS. In accordance with criteria established by the International Study of Kidney Disease in Children (ISKDC), NS was defined as the association of proteinuria > 40 mg/m^2/hr, hypoalbuminemia < 2.5 g/dL, edema, and hyperlipidemia.[1] Steroid- resistance was defined as lack of response to four weeks of treatment with 60mg/m^2 per day of prednisone.[8] None of the included patients presented relapse after transplantation, if performed. Ninety-five patients, belonging to 68 families (53 consanguineous families, 15 non-consanguineous multiplex families), were classified as familial cases, defined as the presence of one (or more) affected individual(s) in consanguineous families and two or more affected children in non-consanguineous families. Forty-four patients presented with sporadic SRNS. Mutations in *NPHS2*, encoding podocin, were excluded for all patients. Mutations of the exons 8 and 9 of the *WT1* gene, encoding the Wilm's tumor 1 protein, were excluded in all phenotypically female patients. Mutations in *NPHS1*, encoding nephrin, were excluded in children which presented NS at less than one year of age without DMS on kidney histology. Clinical data, including ethnic origin, type of renal histological lesions and age at onset of NS and ESKD, were recorded. Informed consent was obtained for all participating families and the study was approved by the Comité de Protection des Personnes «Ile de France II».

PLCE1 molecular analyses

Genomic DNA specimens were collected with informed consent and isolated from peripheral blood using standard procedures. Linkage of familial cases to the *PLCE1* locus was assessed

using four polymorphic microsatellite markers spanning 5.5 Mb and flanking the locus (*D10S185*, *D10S1680*, *D10S574*, *D10S1726*). When results were compatible with linkage to *PLCE1* locus, and for all sporadic cases, the complete coding sequence and exon-intron boundaries of *PLCE1* gene were amplified by PCR (33 exons, GenBank accession number NM_016341.3); subsequently, both strands were sequenced using a Big Dye terminator cycle sequencing kit and analyzed with an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). PCR and sequencing primers are available upon request. Sequence chromatograms were analysed using the Sequencher® software (Gene Codes Corporation, Ann Arbor, MI, USA). Segregation analysis of mutations in families was performed by direct DNA sequencing. Prediction of the functional impact of missense *PLCE1* variants was obtained with the PolyPhen software (http://genetics.bwh.harvard.edu/pph/). The absence of these mutations among at least 50 normal individuals 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.

Mutational analyses of possible modifier genes, including other phospholipases C

In order to identify modifier genes in families with asymptomatic individuals who carried the same *PLCE1* homozygous mutation as the affected patients, a total of 19 candidates genes were evaluated, including 16 genes encoding other PLC (*PLCB1*, *PLCB2*, *PLCB3*, *PLCB4*, *PLCD1*, *PLCD3*, *PLCD4*, *PLCG1*, *PLCG2*, *PLCH1*, *PLCH2*, *PLCL1*, *PLCL2*, *PLCXD1*, *PLCXD3* and *PLCZ1*) and 3 genes encoding proteins that interact directly or indirectly with PLCε1 (*BRAF, IQGAP1*, *NPHS1*) (Table 1). Firstly, a whole-genome wide search for linkage was performed using a 250k SNP array (GeneChip® Human Mapping 250K Nsp Array from Affymetrix, Santa Clara, CA). The potential role of one of the genes cited above as a

modifier of disease was excluded if the asymptomatic mutated patient shared the same haplotypes as at least one of the affected mutated patients. Then, for genes not excluded by this method, microsatellite markers were used to test each locus of the remaining genes: *PLCB2* (*D15S994, D15S968, D15S1006*), *PLCB4* (*D20S889, D20S115, D20S186*), *PLCH1* (*D3S1594, D3S1279, D3S1268*), *PLCL1* (*D2S117, D2S311, D2S374, D2S318, D2S115*), *PLCL2* (*D3S3701, D3S2338, D3S1293*) and *BRAF* (*D7S2560, D7S684, D7S2513, D7S661*). Finally, the complete coding sequence and exon-intron boundaries of the genes not excluded by the whole genome wide scan or by microsatellite markers analysis were sequenced as described above (*PLCH1*, 23 exons, GenBank accession number NM_014996.2; *PLCL1*, 5 exons, GenBank accession number NM_006226.3; *BRAF*, 18 exons, GenBank accession number NM_004333.4) in one asymptomatic mutated patient and one affected mutated patient of each families.

Statistical analysis

All values are expressed as means ± standard error or median (range). Comparisons between two continuous variables were performed using the Mann-Whitney test. All tests were two sided. *P*-values <0.05 were considered significant. Statistical analyses were performed using GraphPad Prism software version 5.01.

RESULTS

Patients characteristics

Patients characteristics are summarized in Table 2. Most of familial cases originated from Middle-East (33.8%) while most of sporadic cases were from Europe (66.7%) (mainly from France). Among familial cases, renal histology disclosed FSGS, DMS and other renal lesions (mesangial proliferation, membranoproliferative glomerulonephritis and terminal kidney) in 50, 10 and 4 families, respectively (renal biopsy not performed in 4 families). Among sporadic cases, FSGS was present in 28 cases, DMS in 14 cases and mesangial proliferation in two cases. Mean age at onset of NS was 46.2 ± 5.7 months. Seventy-six patients reached ESKD at a mean age of 79.7 ± 7.1 months.

Identification of *PLCE1* Mutations

Microsatellite markers analysis excluded linkage to the *PLCE1* locus in 43 families. Therefore, results were compatible with linkage to the *PLCE1* locus in 25 families as evidenced by homozygosity for the polymorphic markers flanking the *PLCE1* locus. Disease-causing mutations were found in 12 families, comprising a total of 18 patients (Table 3). Among sporadic cases, homozygous *PLCE1* mutations were identified in 3 patients. Most were homozygous truncating mutations (nonsense or frameshift mutations), although compound heterozygote missense mutations (p.E1386V/p.P1890L) were detected in two siblings and a homozygous missense mutation (p.H1407D) was detected in one sporadic case. The p.E1386V was predicted to be a possibly pathogenic mutation (Polyphen score 1.94) and involved a very highly conserved amino-acid from fishes to humans. The p.P1890L and p.H1407D were predicted to be probably pathogenic mutations with a Polyphen score of 2.55 and 2.50, respectively, involving amino-acids conserved from C-elegans to humans, and

included in either the C2 domain or the PLC_X catalytic domain of the protein. Nine of these mutations have not been previously reported (Figure 1).

Phenotypic Spectrum of *PLCE1* Mutations

Among the 12 families bearing *PLCE1* mutations, renal biopsy disclosed FSGS in 6 and DMS in 5 (not performed in one family). This translates in a 12% (6/50) and 50% (5/10) *PLCE1* detection rate among families with FSGS and DMS, respectively. All the 3 sporadic cases presented DMS on renal histology, meaning that *PLCE1* mutations were detected in 21% (3/14) of sporadic DMS cases, but in none (0/28) of the sporadic FSGS cases. Globally, *PLCE1* mutations were identified in 13% of our whole cohort (15/112), 33% (8/24) of DMS cases and 8% (6/78) of FSGS cases. When kidney histology of several affected patients of the same family was available, all siblings had the same type of renal lesions.

Patients with *PLCE1* mutations presented NS at a mean age of 22.8 mo ± 5.0 leading to ESKD at a mean age of 39.1 mo ± 6.3 (two patients with FSGS did not reach ESKD after a short follow-up of 1 year and one patient without renal biopsy died at 11 months of NS complications). This contrasts with non mutated patients which presented NS at a mean age of 50.9 mo ± 6.7 (p=0.12) leading to ESKD at a mean age of 92.6 mo ± 8.4 ($p<0.01$). In patients bearing *PLCE1* mutations, no clear-cut genotype-phenotype correlations were observed. Truncating (mean age at onset 24.6 mo ± 5.8, mean age at ESKD 37.1 mo ± 6.7) and missense mutations (mean age at onset 12.3 mo ± 1.5, mean age at ESKD 49.0 mo ± 19.9) lead to similar renal presentation and evolution (p=0.80 and p=0.41, respectively) and were detected in both FSGS and DMS histological forms. However, renal prognosis was worst in patients with DMS compared to those with FSGS (mean age at onset of NS 15.3 mo

± 6.0 vs 30.5 mo ± 7.7, p=0.15 and mean age at ESKD 20.9 mo ± 5.8 vs 57.2 mo ± 7.3, p<0.01).

Identification of asymptomatic individuals bearing homozygous *PLCE1* mutations

Surprisingly, segregation analysis revealed that 3 unaffected individuals belonging to 3 unrelated families were also found to bear, in the homozygous state, the mutations identified in their respective affected relatives (Figure 2). In family E, 3 out of six siblings of a consanguineous family from Pakistan presented SRNS with FSGS on renal histology at ~ 5 years of age and reached ESKD by the age of 6. Another brother had a past history of intermittent mild proteinuria but was free of renal manifestations at 12 years of age. The p.F839fs889X mutation was identified in these 4 patients. In family O, the index case was born from consanguineous parents of Turkish origin. She was diagnosed with SRNS and DMS at the age of 11 months and found to bear the p.R2150X mutation. Among his 2 siblings, 1 brother was homozygous for the mutation and had no proteinuria at 10 years of age. In family A**,** 2 out of 3 children of consanguineous parents from Pakistan developed SRNS at 5 months and 3 years of age with FSGS on renal biopsy and reached ESKD when they were 1.4 and 4 years old, respectively. The p.R321X mutation was identified. Segregation analysis revealed that the father, who was also born from consanguineous parents, was homozygous for the mutation. Although he had presented hypertension and mild proteinuria as a teenager, his renal evaluation, at 46 years of age, was normal.

Search for modifier genes

To understand this phenomenon, we ought to identify mutations or variants in other genes that could modulate the clinical phenotype. We hypothesized a protective effect arising from other members of the phospholipase C family in the asymptomatic individuals. Alternatively,

another hypothesis was that additional mutations or variants in *BRAF*, *IQGAP1* or *NPHS1* genes in symptomatic patients could explain the phenotypic difference. Analysis of whole genome DNA arrays in family E excluded the role of 13 of the 19 candidate genes as a modifier of the disease, since the asymptomatic mutated individual shared the same haplotypes as at least one of his affected siblings at these loci. Then, microsatellite markers analysis of the remaining genes (*PLCB2*, *PLCB4*, *PLCH1*, *PLCL1*, *PLCL2* and *BRAF*) in family O showed that both the affected and the clinically unaffected case shared the same haplotypes for *PLCB2*, *PLCB4* and *PLCL2* genes, excluding them as being potentially involved in the phenotypic variability in the family. Therefore, the direct DNA sequencing of the 3 remaining genes was performed in one affected case and one asymptomatic case of family E, O and A, but did not allow identification of mutations or variants.

DISCUSSION

In 2006, positional cloning uncovered mutations in *PLCE1* causing early-onset NS. Since then, 17 different mutations have been reported (Figure 1), among which only one was associated with FSGS. We therefore aimed to better assess the phenotype spectrum of patients bearing *PLCE1* mutations in a large worldwide cohort of patients with SRNS.

Among familial forms of SRNS, *PLCE1* mutations were found in 12% of families with FSGS and half of families with DMS. Although a significant proportion (21%) of the DMS sporadic cases had *PLCE1* mutations, none of the 28 sporadic cases with FSGS had mutations in this gene. If we had included all children with SRNS without any prior genetic testing, the detection rate of *PLCE1* mutations in FSGS cases would have been lower, as *NPHS2* mutations would have been identified for a significant proportion of them. In contrast, the high rate of *PLCE1* mutations found in DMS cases in our study is probably not overestimated

and is concordant with what has been previously published.[3] Indeed, in our cohort, none of the patients presented signs of Denys-Drash or Frasier syndrome, which could have suggested a mutation in other DMS-associated genes such as *WT1* or *LAMB2*. In addition, *WT1* mutations were excluded in all phenotypically female patients and *NPHS2* mutations have never been found as a cause of DMS.[9] Similar to previous studies,[2-4 10] mutated individuals presented with relatively early NS (from 3 months to 6 years of age) leading to ESKD before the age of 7 years. In addition, Gbadegesin et al. did not identify *PLCE1* mutation in a cohort of 231 affected individuals with FSGS and later onset of NS (mean 26 years, range 1-66) originated from 69 unrelated families (10 were sporadic cases).[11] This confirms that *PLCE1* is a major gene of DMS and that all subjects with this type of histological lesions should be screened for mutations in this gene. On the other hand, *PLCE1* mutations seem to account for a small, although not negligible, proportion of FSGS cases for which *NPHS2* mutations have been ruled out. Considering that the mean age of FSGS patients with *PLCE1* mutations in our cohort was ~ 2.5 years, *PLCE1* genetic testing should probably be performed only in those with an early presentation, particularly if they are considered as familial cases.

Among the initial cohort reported by Hinkes et al.,[2] all patients with homozygous truncating *PLCE1* mutations had DMS as opposed to two patients with missense mutations who exhibited FSGS with a later onset of NS. In a follow-up study, this group reported 10 truncating but no missense *PLCE1* mutations in a cohort of 35 families with DMS.[3] In contrast, we observed either truncating or missense mutations in both FSGS and DMS patients and no clear phenotype-genotype correlation. For example, the p.R1246X mutation was found in the homozygous state in patients with FSGS (families H and I) or DMS (patients J and K), with age at NS onset varying between 6 months and 5 years. In addition, in the present study, missense mutations did not lead to a milder course of disease; however,

missense mutations were identified in only few cases, thereby precluding a definite conclusion. Nevertheless, our data showed the clinical heterogeneity associated with mutations in the *PLCE1* gene.

In this regards, we identified three unaffected individuals carrying the same homozygous mutation as their affected relatives, a finding particularly unusual for disease of recessive inheritance. One other previous case report of a family of 3 affected siblings with DMS also showed that the asymptomatic father, who was himself born from consanguineous parents, had the same homozygous *PLCE1* mutation as his affected children.[4] These data are also reminiscent of the unusual progression of two patients with DMS in the original description of *PLCE1* mutations who seemed to respond to steroid or cyclosporin therapy.[2] The above observations raise the possibility of oligogenic inheritance, with either a third deleterious mutation in a second gene explaining the appearance of a renal phenotype in patients bearing two *PLCE1* mutations or the presence of a protective modifier allele, which could compensate for the *PLCE1* dysfunction in asymptomatic individuals. Although oligogenic inheritance has been suggested in several cases of renal diseases,[12-14] it is in only very rare cases that asymptomatic individuals presented two pathogenic mutations in one gene in contrast to affected individuals bearing an additional third mutation in a second gene.[15] Indeed, oligenic inheritance has been clearly demonstrated in some cases of Bardet-Biedl syndrome (BBS), a genetically heterogeneous disorder characterized by pigmentary retinal dystrophy, polydactyly, obesity, developmental delay and renal defects. In a study of 163 BBS families, Katsanis et al. found the presence of "three mutant alleles" (either two *BBS2* mutations and one *BBS6* mutation or the opposite) in four pedigrees. More interestingly, they detected two *BBS2* mutations but not *BBS6* mutations in unaffected individuals in two pedigrees, suggesting that BBS may not be a single-gene recessive disease but a complex trait requiring a total of 3 mutations in two different genes to manifest the phenotype. On the other hand,

existence of protective modifier gene has been demonstrated in a recent study on autosomal recessive spinal muscular atrophy (SMA).[16] The detection of asymptomatic individuals (all females) carrying the same mutations in the *SMN1* (survival motor neuron 1) gene as their affected siblings has suggested the influence of modifier genes. In this study, higher expression of plastin 3, encoded by *PLS3*, were found in unaffected *SMN1*-deleted females compared to their SMA-affected counterparts. Overexpression of PLS3 rescued the axon length and outgrowth defect associated with down-regulation in motor neurons of SMA mouse embryos and in zebrafish, thereby explaining the absence of a neurological phenotype in some patients bearing *SMN1* mutation.

Effect of modifiers genes could also explain the absence of obvious renal phenotype in the *Plce1* knock-out mouse model, developed on a mixed C57/B6 X 129/S6 genetic background.[2] [17] Indeed, contrary to the zebrafish knock-out model in which embryos injected with Plce1 morpholino show edema at D4 of development and foot process effacement by electron microscopy,[2] *Plce1*$^{-/-}$ mice do not exhibit any renal phenotype. However, as C57/B6 mice are resistant to the development of glomerulosclerosis,[18] it might be of interest to assess the presence of a renal phenotype in *Plce1* knock-out mice from a different genetic background or under nephrosis-promoting conditions.

Based on the preceding observations, we ought to identify mutations or variants in other genes that could explain the absence of renal phenotype in some patients with *PLCE1* mutation. We first speculated a compensatory protective effect arising from other members of the PLC family in the unaffected individuals since the common role of these proteins is to initiate intracellular signaling through the generation of the second messenger molecules IP3 and diacylglycerol. Interestingly, some mouse models of PLC genes develop a renal phenotype, including increased kidney weight, polycystic kidneys, renal dysplasia,

glomerulosclerosis or signs of glomerulonephritis (Table 1). Moreover, PLC-γ1 has been shown to play a key-role in the podocyte slit diaphragm. PLC-γ1 binds to phosphorylated nephrin, the main component of the slit, leading to PLC-γ1 activation and subsequent increase in IP3, and triggering of Ca2+ intracellular response.[19] In our study, we did not identify a PLC candidate that was mutated in a recessive or dominant fashion in the affected individuals and not in the asymptomatic relative, or the reverse. We then used the same approach for candidate genes that are known to interact directly or indirectly with PLCε1 such as *IQGAP1*, *BRAF* and *NPHS1*. IQGAP1 is a known scaffold protein of the mitogen-activated protein (MAP) kinase pathway[20] and interacts with PLCε1,[2] nephrin[21] and BRAF.[22] BRAF, which has been involved in various cancers,[23] belongs to the RAF family of genes and binds H-Ras.[24] It has also been implicated in the MAP kinase pathway and is considered as a third interaction partner of PLCε1,[7] in addition to H-ras and IQGAP-1. In the present study, *IQGAP1*, *BRAF* and *NPHS1* genes did not appear to explain the phenotypic variability observed in our patients. However, our approach cannot exclude an effect of different PLC or PLC partner genes in the three different families. Finally, it has to be stressed that, to date, only very few homozygous *PLCE1* mutations detected in asymptomatic individuals or patients that responded to therapy have been reported and, surprisingly, all of them are males. This is reminiscent of the unaffected *SMN1*-deleted individuals, which were all females,[16] and suggests the existence of gender-specific modifiers of disease.

In conclusion, *PLCE1* mutations are frequently found in familial and sporadic DMS cases. Although *PLCE1* mutations had been rarely associated with FSGS, we showed that mutations in this gene are detected in a non-negligible proportion of familial FSGS cases without *NPHS2* mutations. In this study, no clear genotype-phenotype correlation was present. Although variants in other phospholipases C genes do not seem to explain the absence of

renal manifestations in some cases with *PLCE1* mutations, we speculate that other modifier genes or environmental factors may play a role in the renal phenotype variability observed in individuals bearing *PLCE1* mutations. More importantly, the clinical heterogeneity associated with *PLCE1* mutations substantially increases the complexity of genetic counselling, including prenatal diagnosis, and needs to be considered in the discussion about renal prognosis with patients.

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Competing Interest

None declared.

Table 1.Candidate genes tested in order to evaluate the presence of a modifier effect

Gene code	Gene full name	Gene mapping	Mouse model/ RP*	Unigen EST (kidney)**
Phospholipase genes				
PLCB1	phospholipase C, beta 1	20p12	Yes/Yes[¶]	23
PLCB2	phospholipase C, beta 2	15q15	Yes/No	14
PLCB3	phospholipase C, beta 3	11q13	Yes/No	14
PLCB4	phospholipase C, beta 4	20p12	Yes/No	28
PLCD1	phospholipase C, delta 1	3p22-p21.3	Yes/No	47
PLCD3	phospholipase C, delta 3	17q21.31	Yes/No	51
PLCD4	phospholipase C, delta 4	2q35	Yes/No	18
PLCG1	phospholipase C, gamma 1	20q12-q13.1	Yes/Yes[§]	32
PLCG2	phospholipase C, gamma 2	16q24.1	Yes/Yes[†]	65
PLCH1 ou PLCL3	phospholipase C, eta 1	3q25.31	No	0
PLCH2 ou PLCL4	phospholipase C, eta 2	1p36.32	No	9
PLCL1	phospholipase C-like 1	2q33	Yes/No	23
PLCL2	phospholipase C-like 2	3p24.3	Yes/No	23
PLCXD1	phosphatidylinositol-specific phospholipase C, X domain containing 1	Xp22.33; Yp11.32	No	18
PLCXD3	phosphatidylinositol-specific phospholipase C, X domain containing 3	5p13.1	No	18
PLCZ1	phospholipase C, zeta 1	12p12.3	No	0
Non-phospholipase genes				
BRAF	v-raf murine sarcoma viral oncogene homolog B1	7q34	Yes/No[‡]	32
IQGAP1	IQ motif containing GTPase activating protein 1	15q26.1	Yes/No	150
NPHS1	nephrosis 1, congenital, Finnish type (nephrin)	19q13.1	Yes/Yes[λ]	18

[*]Existence of a mouse model (yes/no) and presence of a renal phenotype (RP) in a mouse model (yes/no)

[**]Approximate abundance of Expressed Sequence Tags (ESTs) in kidneys, inferred from EST sources and provided in the Unigene databank. The number indicates number of transcripts per million. For comparison, *PLCE1* EST in kidneys = 9 transcripts per millon.

[¶]The $Plcb1^{Tg(LGB)56Mbal}$ transgenic mouse model presents increased kidney weight.[25]

[§]In the $Plcg1^{tm1Ine}$ knock-out mouse model, extensive chimerism (>70% agouti coat color) results in lethality. Chimeras with a 30-70% agouti coat color display: enlarged, polycystic kidneys, renal dysplasia (at 2 weeks), dilated renal tubules (at >2 weeks), and occasional glomerulosclerosis in high % chimeras with severe renal dysplasia.[26]

[†] The $Plcg2^{Ali5}$ chemically induced (ENU) mouse model presents signs of glomerulonephritis.[27]

[‡]Prenatal/perinatal lethality is present in the knock-in and knock-out braf mouse model, precluding a complete assessment of the renal phenotype.[28-30]

[λ]*Nphs1* null mice are massively proteinuric at birth with death occurring in the first 24h. [31]

Table 2. Patients characteristics (*n*=139)

	***n* (%)**[†]		
	All cohort	Familial cases	Sporadic cases
Number of cases	139	95	44
Type of families			
n		68	
Consanguineous family		53 (77.9)	NA
Non-consanguineous multiplex family		15 (22.1)	NA
Ethnic origin			
n	134	68 families	39
North Africa	31 (23.1)	21 (30.9)	5 (12.8)
Middle-East	36 (26.9)	23 (33.8)	4 (10.3)
Europe	46 (34.3)	13 (19.1)	26 (66.7)
Other	21 (15.7)	11 (16.2)	4 (10.3)
Renal histological lesions			
n	129	64 families	44
FSGS	98 (76.0)	50 (78.1)	28 (63.6)
DMS	25 (19.4)	10 (15.6)	14 (31.8)
Other	6 (4.7)	4 (6.3)	2 (4.5)
Age at onset of NS (months)			
n	127	86	41
Median (range)	23.0 (0-373)	24.0 (0-373)	23.0 (0-81)
Mean ± SE	46.2 ± 5.7	55.7± 8.1	26.4 (3.4)
ESKD			
n	131	88	43
Number of patients reaching ESKD	76 (58.0)	47 (53.4)	29 (67.4)
Age at onset of ESKD (months)			
n	75	46	29
Median (range)	68.0 (0.5-264)	81.0 (4-264)	60.0 (0.5-139)
Mean ± SE	79.7 ± 7.1	94.7 ± 10.4	55.9 ± 5.7

*****The number of available data are shown as ***n***.
[†]The denominator used to calculate percentages is the number of available data.
FSGS, Focal segmental glomerulosclerosis; DMS, diffuse mesangial sclerosis; NS, nephrotic syndrome; ESKD, End-stage kidney disease; SE, Standard Error; NA, not applicable

Table 3. Spectrum of *PLCE1* mutations and associated phenotypes

Family/ Individual	Individual	Familial / Sporadic	Parental consanguinity	Origin	Nucleotide alterations(s)	Predicted effect on protein	Exon	Age at onset of NS	Age at ESKD	Histology
A.	1	F	Yes	Pakistan	c.961C>T	p.R321X	2	3 yr	3 yr	FSGS
A.	2							5 mo	1.4 yr	FSGS
B.	1	F	Yes	Morocco	c.1020_1021 delGT	p.V340fs452X*	2	2 yr	2.5 yr	DMS
C.	1	F	Yes	Morocco	c.1148C>G	p.S383X	2	9 mo	None at 1.2 yr	FSGS
D.	1	F	Yes	Morocco	c.1477C>T	p.R493X*[¶]	3	3 mo	6 mo	DMS
D.	2							4 mo	8 mo	DMS
E.	1	F	Yes	Pakistan	c.2516insT	p.F839fs889X	8	5.5 yr	5.7 yr	FSGS
E.	2							5.8 yr	5.8 yr	FSGS
E.	3							4.8 yr	5.8 yr	FSGS
F.	1	F	Yes	Algeria	c.3058C>T	p.Q1020X	8	6 mo	Died at 11 mo	ND
G.	1	F	Yes	Turkey	c.3346C>T	p.R1116X[¶]	10	10 mo	1.2 yr	DMS
H.	1	F	Yes	Greece	c.3736C>T	p.R1246X	13	10 mo	3.8 yr	FSGS
H.	2							4 yr	6.5	FSGS
I.	1	F	Yes	Turkey	c.3736 C>T	p.R1246X	13	11 mo	None at 1 yr	FSGS
J.	1	S	No	Bosnia	c.3736C>T	p.R1246X*	13	6 mo	1.1 yr	DMS
K.	1	S	No	Serbia	c.3736C>T	p.R1246X	13	5 yr	5 yr	DMS
L.	1	F	No	USA	c.4157A>T c.5669C>T	p.E1386V het[§] p.P1890L het[§]	16 26	10 mo	7 yr	FSGS
L.	2							12 mo	4 yr	FSGS
M.	1	F	No	Morocco	c.4219C>G	p.H1407D[§]	16	1.3 yr	1.3 yr	DMS
N.	1	S	No	France	c.5744_5745delC	p.T1915fs1916X	26	5 mo	10 mo	DMS
O.	1	F	Yes	Turkey	c.6448C>T	p.R2150X	30	11 mo	2.7 yr	DMS

All the mutations cited above have been found in the homozygous or compound heterozygous state. Mutations numbering is based on the c-DNA reference sequence (GenBank accession number NM_016341.3).

*The clinical data of these patients have been previously published in Ismaili K *et al*., 2008.[10]

[¶] These mutations have been reported in Hinkes B *et al*., 2006. [2]

[§] The Polyphen score of the p.E1386V, p.1890L and p.H1407D variations is 1.94 (possibly damaging), 2.55 (probably damaging) and 2.50 (probably damaging), respectively.

F, familial case; S, sporadic case; NS, nephrotic syndrome; ESKD, end-stage kidney disease; FSGS, focal segmental glomerulosclerosis; DMS, diffuse mesangial sclerosis ND, not determined.

Figure 1. *PLCE1* mutations reported in the literature, including the mutations detected in the present study, with the associated renal histological lesions

a. Exon structure of the *PLCE1* gene, GenBank accession number NM_016341.3. The arrows indicate relative positions of mutations.
b. Positions of putative protein domains.
In red are the novel mutations found in our study. In black are the other mutations reported in the literature.[2-4 10] The p.P382fsX387 mutation has been reported in one patient for which renal histology was not available. [2] We also found the p.Q1020X mutation in one patient without kidney biopsy.[*]The p.R1246X mutation has been reported in DMS cases;[10] we also found the same mutation in both DMS and FSGS cases.
DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis.

Figure 1. *PLCE1* mutations reported in the literature and associated renal histological lesions

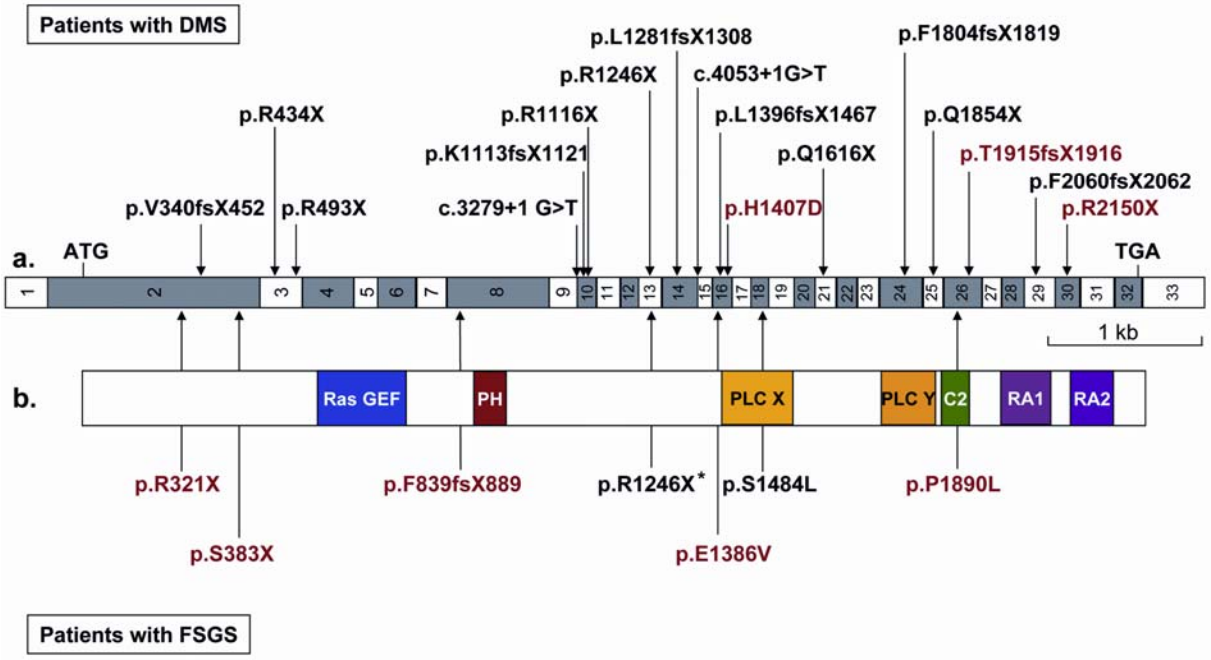
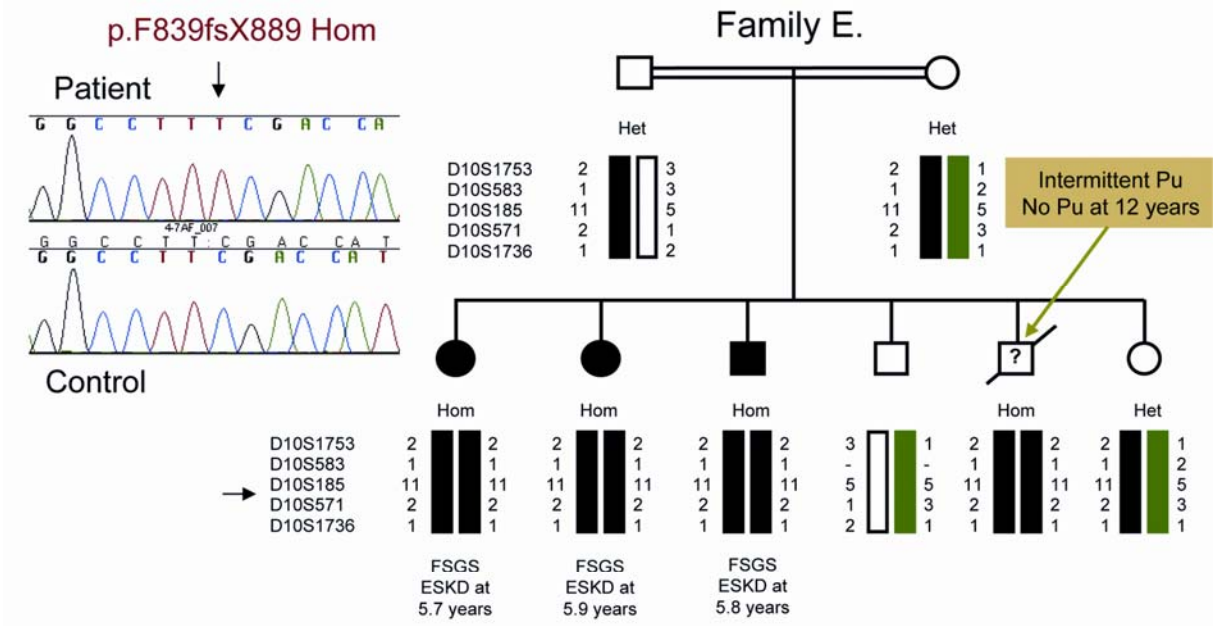


Figure 2. Identification of homozygous *PLCE1* mutations in 3 unrelated asymptomatic individuals.

The arrows indicate the position of the *PLCE1* gene.
Hom, homozygous; Het, heterozygous, Pu, proteinuria; FSGS, focal segmental glomerulosclerosis; DMS, diffuse mesangial sclerosis; ESKD, end-stage kidney disease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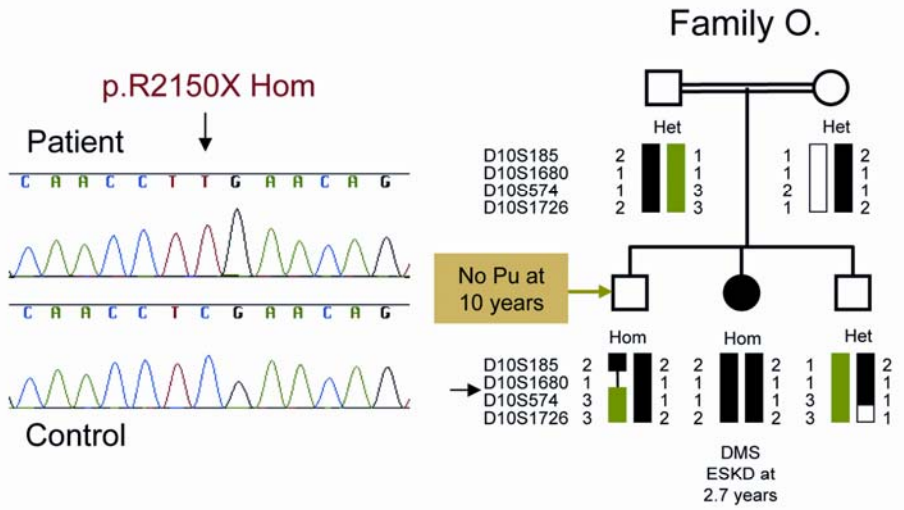
Patient
Control
Family E.
Het
Het
D10S1753
D10S583
D10S185
D10S571
D10S1736
Intermittent Pu
No Pu at 12 years
Hom
Hom
Hom
Hom
Het
FSGS
ESKD at
5.7 years
FSGS
ESKD at
5.9 years
FSGS
ESKD at
5.8 years


Family O.
p.R2150X Hom
Patient
Control
Het
Het
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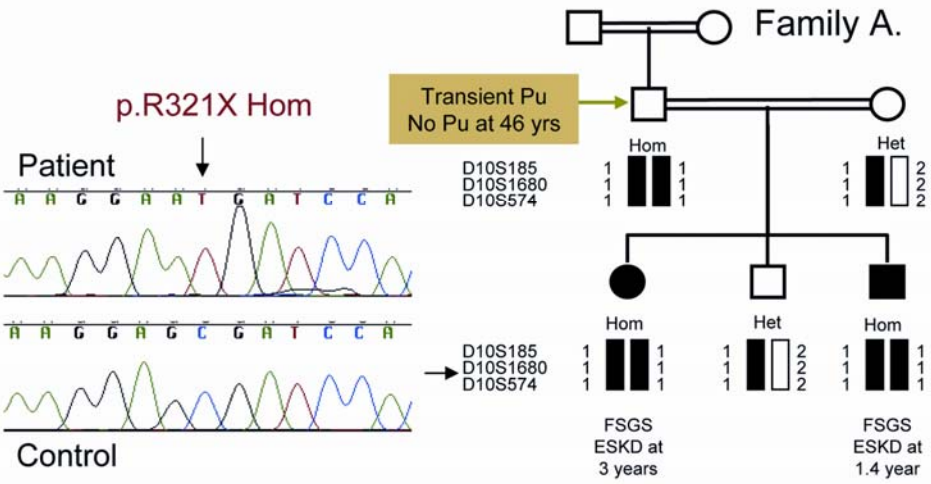
D10S1680
D10S574
D10S1726
No Pu at
10 years
Hom
Hom
Het
DMS
ESKD at
2.7 years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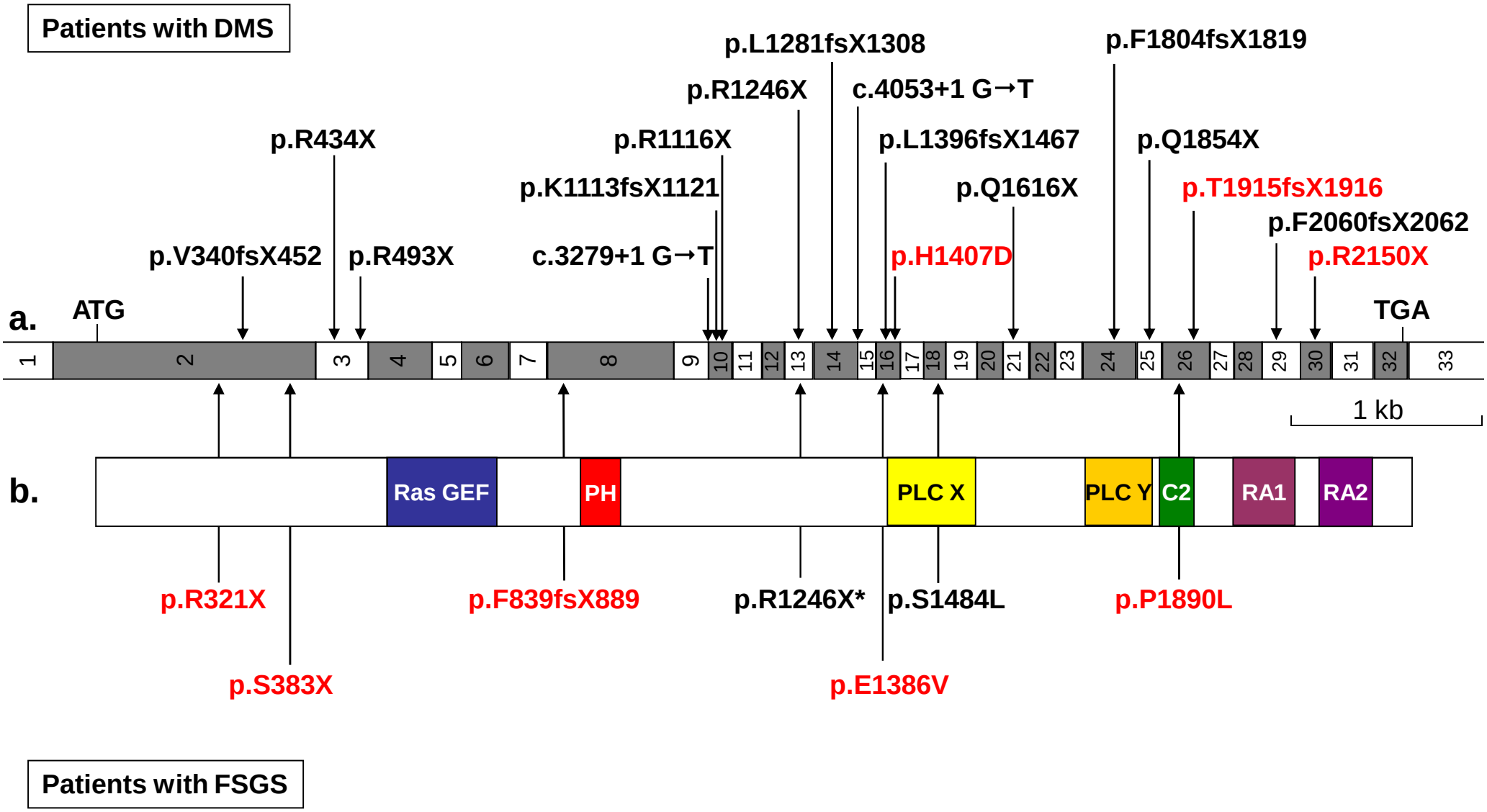
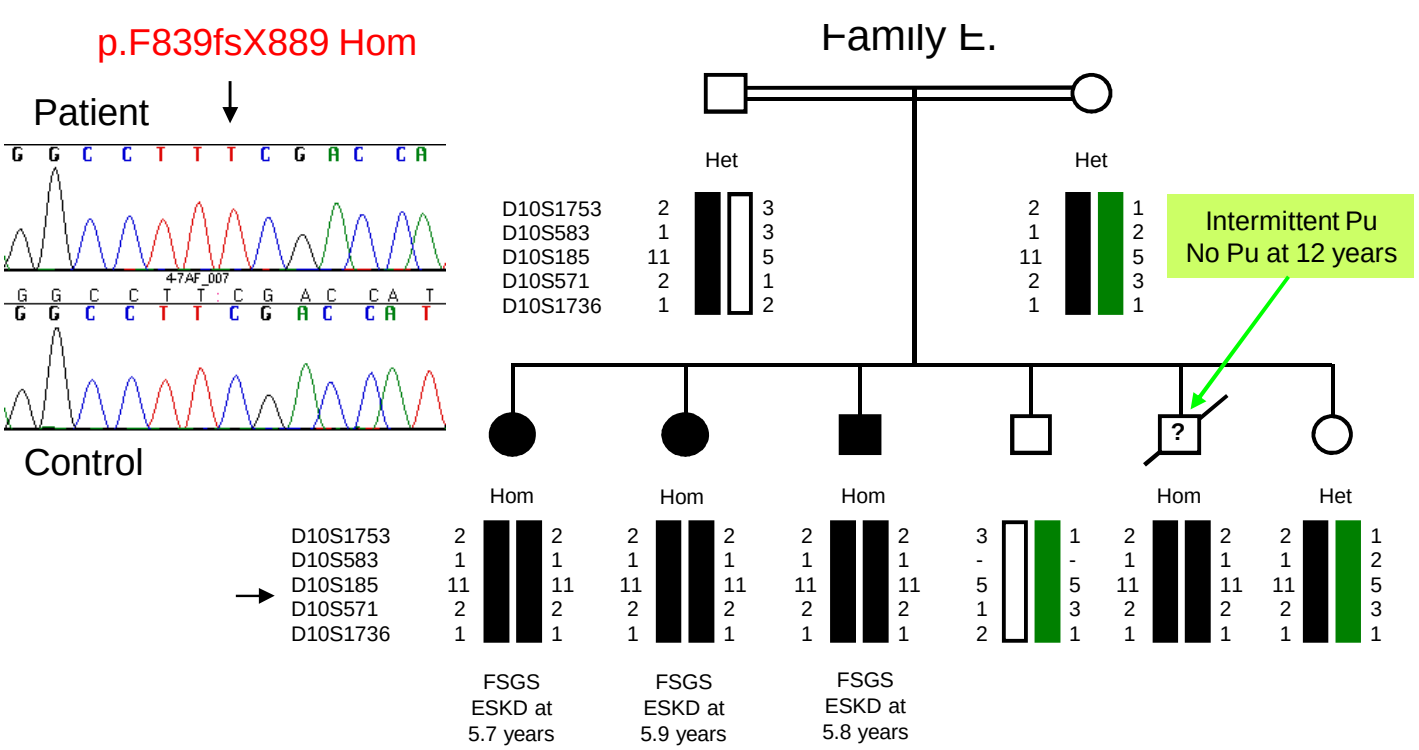
Patient
Control
Transient Pu
No Pu at 46 yrs
Hom
Het
D10S185
D10S1680
D10S574
Hom
Het
Hom
FSGS
ESKD at
3 years
FSGS
ESKD at
1.4 year

Figure 1. ***PLCE1*** **mutations reported in the literature and associated renal histological lesions**

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D10S583
D10S185
D10S571
D10S1736
Intermittent Pu
No Pu at 12 years
Hom
Hom
Hom
Hom
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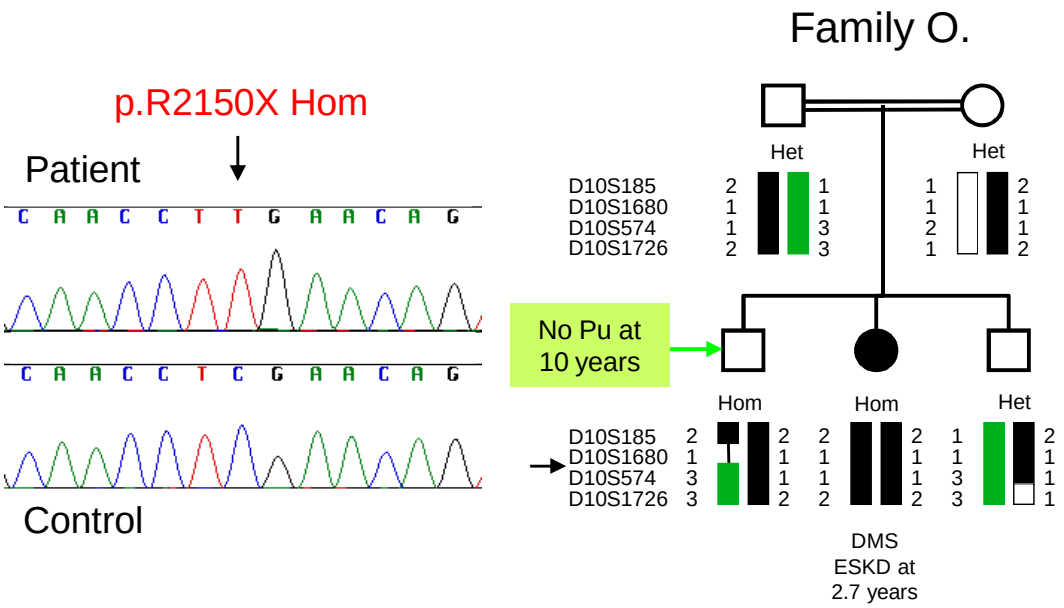
FSGS
ESKD at
5.7 years
FSGS
ESKD at
5.9 years
FSGS
ESKD at
5.8 years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Patient
Control
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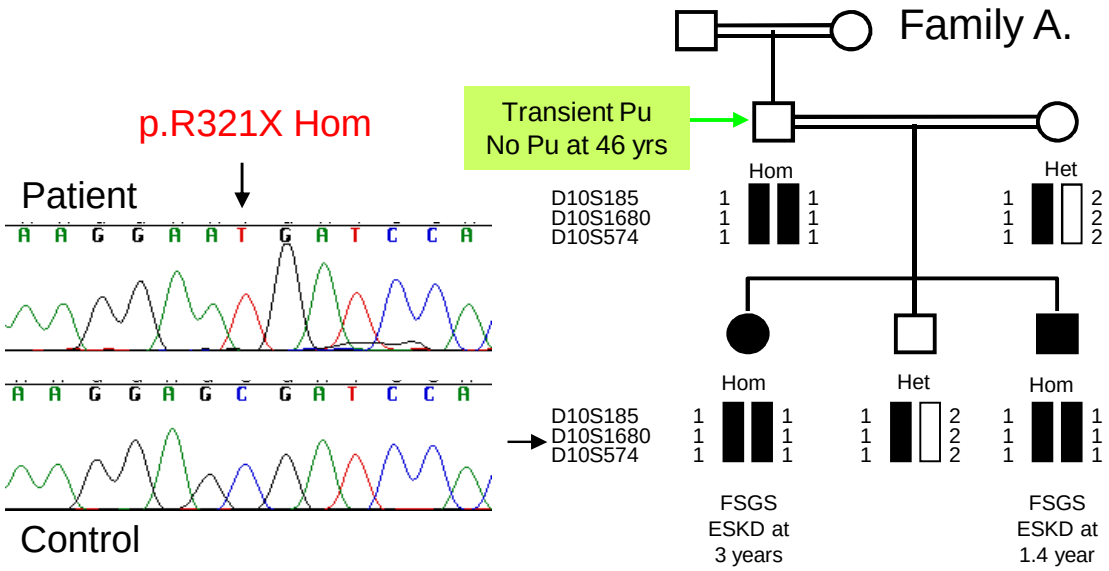
DMS
ESKD at
2.7 years


Family A.
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Patient
Control
Transient Pu
No Pu at 46 yrs
Hom
Het
D10S185
D10S1680
D10S574
Hom
Het
Hom
FSGS
ESKD at
3 years
FSGS
ESKD at
1.4 year