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Mutations in *PLCE1* are a major cause of isolated diffuse mesangial sclerosis (IDMS)

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Abstract

Background and objectives. Diffuse mesangial sclerosis (DMS) is a histologically distinct variant of nephrotic syndrome (NS) that is characterized by early onset and by progression to end-stage kidney disease (ESKD). Besides syndromic DMS, isolated (non-syndromic) DMS (IDMS) has been described. The etiology and pathogenesis of DMS is not understood. We recently identified by positional cloning recessive mutations in the gene *PLCE1/NPHS3* as a novel cause of IDMS. We demonstrated a role of *PLCE1* in glomerulogenesis. Mutations in two other genes *WT1* and *LAMB2* may also cause IDMS. We therefore determine in this study the relative frequency of mutations in *PLCE1*, *WT1* or *LAMB2* as the cause of IDMS in a worldwide cohort.

Methods. We identified 40 children from 35 families with IDMS from a worldwide cohort of 1368 children with NS. All the subjects were analyzed for mutations in all exons of *PLCE1* by multiplex capillary heteroduplex analysis and direct sequencing, by direct sequencing of exons 8 and 9 of *WT1,* and all the exons of *LAMB2.*

Results. The median (range) age at onset of NS was 11 (1–72) months. We detected truncating mutations in *PLCE1* in 10/35 (28.6%) families and *WT1* mutations in 3/35 (8.5%) families. We found no mutations in *LAMB2*.

Conclusions. *PLCE1* mutation is the most common cause of IDMS in this cohort. We previously reported that one child with truncating mutation in *PLCE1* responded to cyclosporine therapy. If this observation is confirmed in a larger study, mutations in *PLCE1* may serve as a biomarker for selecting patients with IDMS who may benefit from treatment.

Keywords: IDMS; *LAMB2*, mutation; *PLCE1, WT1*

Introduction

Diffuse mesangial sclerosis (DMS) is a clinico-pathological entity that was first described in 1985 [1]. The disease is characterized by onset of nephrotic syndrome (NS) in the first year of life and rapid progression to end-stage kidney disease (ESKD). Pathologically, findings include mesangial matrix expansion with no mesangial hypercellularity, hypertrophy of the podocytes in early disease, thickened basement membranes, diminished patency of capillary lumen and hypertrophied and vacuolized podocytes, which surround the glomerular tufts like a crown in advanced disease [2]. DMS has been described as part of syndromes such as Denys-Drash syndrome (DDS). DDS includes ambiguous genitalia, Wilm's tumor and DMS caused by mutation in the *WT1* gene [3]. Pierson syndrome is defined by the association of mental retardation, microcoria and DMS caused by mutation in *LAMB2* gene [4]. However, non-syndromic isolated DMS (IDMS) can also occur, and there have been suggestions in the past that it may be inherited in an autosomal recessive fashion, although few cases due to dominant *WT1* mutations have been reported in the literature [1,2]. We have recently identified *PLCE1* mutations as a novel cause of DMS [5].

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The *PLCE1* gene resides on chromosome 10q23. The gene encodes phospholipase C epsilon 1 (PLCε1). PLCε1 is a member of the phospholipase family of enzymes that catalyzes the hydrolysis of polyphosphoinositides to generate second messengers, such as inositol-1,4,5 trisphosphate and diacylglycerol [6]. These second messengers are involved in cell growth and differentiation [6]. PLCε1 is expressed in the podocyte of the glomerulus, neural tissue and skeletal muscle of mouse embryos, as well as the skin, skeletal muscle and heart of adult mice [7–9].

The *WT1* gene is on chromosome 11p13 and it encodes a zinc-finger nuclear transcription factor that regulates the expression of other genes by binding DNA [10]. It is expressed in podocytes and in the gonads. The locus for *LAMB2* is on chromosome 3p21 [4]. The gene encodes laminin β2, a component of laminin, an extracellular glycoprotein that is an essential component of the basement membrane [4,11–12]. Laminin β2 is widely expressed in the glomerular basement membrane where it plays a key role in anchoring and differentiation of podocyte foot processes [4]. It is also expressed in ocular tissue and skeletal muscle [4,13]. The mechanisms by which these three distinctly different genes cause DMS are unknown. There are, however, data to suggest that each of them may be important during critical stages of glomerulogenesis and mutation of the genes may cause disruption of the process [4,5,14,15].

NS due to DMS characteristically presents in the majority of cases within the first year of life and there is no clear-cut guideline for therapy. However, some reported cases with presentation in the first year of life have been found to be steroid resistant [16]. Interestingly, a child with *PLCE1* loss of function mutation and DMS histology responded to cyclosporine therapy [5]. The mechanism by which this occurs is unknown; however, this observation for the first time offers a possible biomarker for therapy responsive DMS.

To further define the relative frequencies of *PLCE1* and other genes (*WT1* and *LAMB2*) known to cause DMS, we performed mutational analysis of *PLCE1*, *WT1* and *LAMB2* in a worldwide cohort of children with IDMS.

Materials and methods

We identified 57 children with IDMS from a worldwide cohort of children with NS. We have previously reported mutations in eight of these children (six families) before [5,17].

Clinical data

Inclusion criteria were: (i) early-onset proteinuria, generalized edema and hypoalbuminemia; (ii) classical histologic findings of DMS as described in the introduction; (iii) absence of disease-causing mutations in *NPHS2* (podocin) and (iv) absence of disease-causing mutations in *NPHS1* (nephrin) in children aged 0–3 months. Exclusion criteria were: (i) presence of constellation of extra renal signs that may suggest Pierson's syndrome (DMS, mental retardation or ocular changes) or DDS (DMS, ambiguous external genitalia and Wilm's tumor) and (ii) other extra renal disease symptoms and signs.

Approval for the study was granted by the Institutional Review Board (IRB) of the University of Michigan Medical School, Ann Arbor. Informed consent was obtained from all of the study subjects and/or their parents. Clinical information was obtained from the study subjects. This included basic demographic data such as age, gender, race or ethnicity, history of parental consanguinity and family history of renal disease, specifically NS, age at onset of symptoms, age at onset of proteinuria, associated hematuria, generalized edema and elevated blood pressure. Also recorded were the presence of extra renal symptoms and signs such as ambiguous genitalia, history of Wilm's tumor, mental retardation, microcoria and visual disturbances. When applicable, age at onset of ESKD, commencement of renal replacement therapy, history of kidney transplantation and recurrence of primary disease in renal allograft were recorded. Serum creatinine, urine protein/creatinine ratio and glomerular filtration rate at the time of data acquisition were obtained.

Mutation analysis

Genomic DNA was extracted from whole blood samples using standard methods [18].

PLCE1: We used a two-step approach for mutation analysis in *PLCE1*. First, patients were screened by exon-PCR using multiplex capillary heteroduplex analysis (MCHA) that has a >95% detection rate [19]. Samples were run on a MegaBACE 1000 DNA analysis system (GE Healthcare). The results were analyzed using the Genetic profiler v.2.0 software (GE Healthcare). Secondly, samples with aberrant peak patterns on MCHA were directly sequenced by the dideoxy chain termination method using the capillary ABI sequencer at the University of Michigan sequencing core facility. Sequences were evaluated for mutations using the Sequencher^{TM} software (Gene Codes Corporation, Ann Arbor, MI, USA). All mutations were confirmed by sequencing of the complementary strand. Where parental samples were available, segregation analysis was carried out to confirm the mutation.

WT1: The mutation bearing exons 8 and 9 of the *WT1* gene were screened using exon flanking primers as previously described [17] and mutations were confirmed by sequencing of the complementary strands.

LAMB2: Exon flanking primers for all the 32 exons of *LAMB2* were developed in the laboratory of MZ. Bidirectional direct sequencing was carried out using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) on the ABI 3730 capillary sequencer (Applied Biosystem) in the same laboratory.

All the primer sequences and PCR conditions for *PLCE1*, *WT1* and *LAMB2* mutation analysis are available on request.

Results

A total of 57 children with DMS were identified from a cohort of 1368 children with NS. Fourteen of these children were found to have structural anomalies in at least two organ systems and were therefore excluded from further

analysis. One child with a *WT1* mutation was reported to have developed Wilm's tumor during subsequent follow-up and two children were found to have disease-causing mutations in nephrin (*NPHS1*) and were therefore excluded from the study. The remaining 40 children from 35 families are the subjects of this report.

Mutational analysis

Mutations were detected in *PLCE1* and *WT1* in 13/35 (37%) of the families studied.

PLCE1/NPHS3: A total of 10 different mutations were found in 10/35 (28.6%) families (14 children) (Table 1). Eight of the ten families are known to be consanguineous. In this cohort of 35 families, mutations in *PLCE1* cause IDMS in 10/35 (28.6%) of the families. Except in one family, all the mutations detected were homozygous loss of function (frameshift or stop codon) mutations. One child (A1407) has two compound heterozygous loss of function mutations (splice site) in exons 9 and 14. Four of these mutations have been previously reported [5]. Table 1 shows the clinical characteristics of the children with mutations. The six novel mutations are shown in Figure 1. We did not find any of the mutations in at least 75 healthy Turkish and Central European control individuals.

WT1: We found single heterozygous mutations in 3/35 (8.5%) of all the families with IDMS (Table 1). All the children from the three families are females with a 46XX karyotype. One of the three children died shortly after diagnosis. The other two children have been followed up for 2–4 years with serial ultrasound and they have not developed Wilms tumor or any other extra renal symptoms since diagnosis. Two of the three mutations have previously been reported in our study on the role of *WT1* in the etiology of steroid-resistant NS and NS in the first year of life (Table 1) [17,20]. The third mutation (Q369H) is a novel mutation that was not found in 100 healthy individuals.

LAMB2: We found no mutation in any of the exons of *LAMB2*.

Genotype phenotype correlations

Statistical evaluations of clinical characteristics of the study population are shown in Table 2. Information on consanguinity was available in 34/35 families; 11 families were known to be consanguineous.

Mutations were detected in *PLCE1* and *WT1* in 13/35 (37%) of the families studied.

There was no difference in age at onset of disease, gender distribution or response to therapy between the group of children with mutations and the group with no mutations in any of the three genes (Table 2). However, children from 8/13 families with mutations are from a consanguineous union compared with 3/21 for the group with no mutation ($P = 0.002$, Table 2).

The median age of onset of disease in children with *PLCE1* mutations was 9 months (range 1–48 months). The three patients with *WT1* mutations presented in the first year of life. The median age at onset of disease in all the children with no mutation in any of the three genes was 10 months (range 1–72 months); there was no difference in age of onset between the children with mutations and those with no mutations. Since in routine clinical practice, children with early-onset NS and/or DMS are not routinely offered immunosuppressive treatment, only 14 out of the 40 children in this cohort were treated. In this group, eight children had mutations (seven in *PLCE1* and one in *WT1*) and six children had no mutation. All the children were therapy resistant except one child (A38 II-4) with *PLCE1* loss of function mutation who responded to cyclosporine and angiotensin converting enzyme inhibitor (ACEI) therapy; this child was reported before [5]. This child has been followed for 12 years and he is now in complete remission with normal kidney function. At the time of the last follow-up, 25 of the 40 children are known to be in ESKD. They comprised 10 children with mutations and 15 with no mutations in any of the three genes. The children with no mutation tended to reach ESKD later than children with mutations (median age 24 versus 10 months Kruskal Wallis $P = 0.016$, Table 2). Sixteen of the forty children underwent kidney transplantation. Seven of these had mutations and nine had no mutation. None of the children are known to have recurrence of DMS in their renal allograft at the time of the last follow-up.

Discussion

In this study, we detected recessive *PLCE1* mutations as the most frequent (28.6%) and *WT1* mutations as the second most frequent (8.5%) cause of IDMS in a worldwide cohort of 36 families.

The etiology of non-syndromic DMS is largely unknown. However, it has been suspected for more than 20 years that it may be at least in part, an autosomal recessive genetic disease [1]. Recent identification of mutations in *PLCE1* as a cause of early-onset NS that may manifest with a DMS histopathologic feature by our group prompted us to perform mutation analysis in all genes that have been previously reported to cause DMS. As far as we know, the cohort of patients in this study represents one of the largest series of patients with IDMS ever reported in the literature. We found that 37.1% of the families in this series have IDMS that can be explained by homozygous mutations in *PLCE1* (28.6%) or dominant heterozygous mutations in *WT1* (8.5%). Of these two genes, mutations in *PLCE1* account for the majority of cases being responsible for 28.6% of all mutations. We found no mutations in *LAMB2* in this cohort. In families of subjects with *PLCE1* mutations, in whom parental DNA was available, segregation analysis was found to be consistent with an autosomal recessive mode of inheritance thus confirming our identification of *PLCE1* as a new cause of IDMS [5].

In this series, 8 of the mutations detected in *PLCE1* are loss of function (frameshift or stop codon) mutations. One patient had compound heterozygous loss of function (splice site mutations). We found no missense mutations in this series. Furthermore, apart from two families with the same mutation in exon 14, each family has its own private mutation with no clustering around any exons or specific region of the *PLCE1* gene. Thus there is no founder effect in this series. Mutations in *PLCE1* are not confined to any

Table 1. Mutations in *PLCE1* and *WT1* in 40 children with IDMS

Study number	Origin	Consanguinity	Gender	Age of onset (months)	Age ESKD (months)	Therapy	Renal transplant	Gene	Nucleotide mutation	Alteration in coding sequence	Exon
A38 II-1[a]	Israel	Y	F	4	10	SRNS	Y	*PLCE1*	1477C > T	R493X (H)	3
A38 II-4	Israel	Y	M	1	NA	SRNS/CYA-s	N	*PLCE1*	1477C > T	R493X (H)	3
F389 II-1[a]	Turkey	Y	M	48	60	SRNS	ND	*PLCE1*	3346C > T	R1116X (H)	10
F389 II-2[a]	Turkey	Y	F	24	24	None	Y	*PLCE1*	3346C > T	R1116X (H)	10
A1465 II-1	ND	Y	M	ND	ND	ND	ND	*PLCE1*	3338–3339delAA	K1113fsX1121 (H)	10
F331 II-3[a]	Turkey	Y	M	36	36	None	ND	*PLCE1*	3846delG	L1281fsX1308 (H)	14
A1466 II-1	ND	Y	M	ND	ND	ND	ND	*PLCE1*	3846delG	L1281fsX1308 (H)	14
A1676 II-1	USA (Hi)	N	F	ND	24	None	N	*PLCE1*	4192insC	L1396fsX1467 (H)	16
A1676 II-2	USA (Hi)	N	F	10	13	None	Y	*PLCE1*	4192insC	L1396fsX1467 (H)	16
A1274 II-1[a]	Turkey	Y	M	8	12	SRNS	Y	*PLCE1*	4846C > T	Q1616X (H)	21
A1274 II-3[a]	Turkey	Y	M	8	36	SRNS	Y	*PLCE1*	4846C > T	Q1616X (H)	21
A1391 II-1	Turkey	Y	M	11	11	SRNS	N	*PLCE1*	5410–5411delTT	1804fsX1819 (H)	24
A1630 II-1	Pakistan	Y	M	18	ND	SRNS	N	*PLCE1*	6182delT	F2060fsX2062 (H)	29
A1407 II-1	Latvia	N	M	2	8	None	Y	*PLCE1*	INV9 + 1G > T(h) INV14 + 1 G > T(h)		9 14
A651 II-1[a]	Serbia	N	F	<1	ND	None	N	*WT1*	1097G > A(h)	R366H (h)	8
A1277 II-1	Israel	Y	F	10	30	None	Y	*WT1*	1107G > C(h)	Q369R (h)	8
A794 II-1[a]	Austria	N	F	2		None	Y	*WT1*	1186G > A(h)	D396N (h)	9

None of the changes were present in >75 controls.

[a]Cases previously reported [5,17].

ND: no data; NA: not applicable. (Hi): Hispanic ethnicity; SRNS: steroid resistant; CYA-s: cyclosporine A sensitive; All mutations were homozygous (H) unless indicated as heterozygous (h).

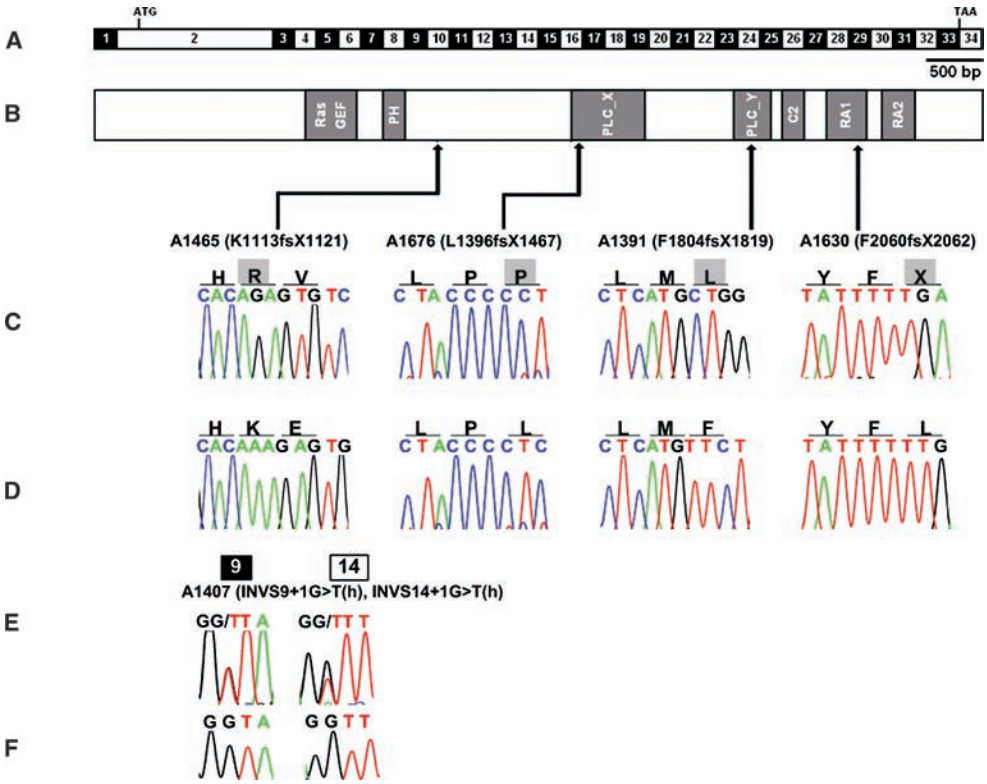

Fig. 1. New mutations of *PLCE1* in children with IDMS. (**A**) Exon structure of human *PLCE1* cDNA. (**B**) Position of protein domains in relation to the encoding exon positions in A. (**C**) Five new homozygous mutations detected in *PLCE1*. Family number and mutations are given above the nucleotide sequences. (**D**) Wild-type nucleotide and amino acid sequences. (**E**) Heterozygopus splice site mutations in exons 9 and 14 in A1407. (**F**) Wild-type nucleotide sequence for exons 9 and 14.

Table 2. Clinical and demographic characteristics of children with non-syndromic DMS

Parameter	Mutation	No mutation
Age at onset median (range) months	9.0 (1–48)	11.5 (1–72)
Gender (m:f)	1.2:1	1:1.1
Consanguinity[a,b]	8/13 (61.5%)	3/21 (14.3%)
Age ESKD median (range) months[c]	10.5 (8–60)	24 (11–84)
Response to therapy	1/8	0/6

[a]Information available in 34 families.
[b]$x^2 = 9.32$, $P = 0.002$.
[c]Kruskal Wallis, $P = 0.016$.

part of the world, as we were able to detect mutations in families from Europe, North America, Asia and the Middle East, with the majority of the cases ascertained from Turkey (Table 1).

One of the mutations in *WT1* Q369H is a novel mutation that was not found in 100 healthy individuals. This mutation is in exon 8, which encodes for the zinc finger domain of the *WT1* protein. The glutamine residue in position 369 is conserved in evolution down to the fish *tetraodon*. The other two mutations have been previously reported in children with DDS or IDMS [10]. We found only three patients with *WT1* mutation in this study; this is consistent with a recent review of the literature by Niaudet *et al*. where only about 17% of patients with IDMS were found to have mutations in *WT1* [21]. Apart from an isolated case from a Japanese study, virtually all cases of IDMS due to mutations in *WT1* are found exclusively in exons 8 and 9, which encodes for zinc fingers 2 and 3, respectively [17,20–24]. It is therefore unlikely that we have missed cases of IDMS due to mutations in other exons of *WT1*.

The mechanisms by which mutations in *PLCE1* cause DMS are still under investigation. However, there are data to suggest that PLCε1 may serve as an assembly scaffold for organization of a molecular complex involved in glomerular development at the capillary loop stage of glomerulogenesis [5]. Evidence for this is the finding that PLCε1 interacts with GTPase-activating protein 1 (IQGAP1) which is found at the basal aspect of the developing podocyte where foot processes are forming and which is also known to interact with nephrin [5,25]. It has been observed that patients with *WT1* mutations have loss of downregulation of *PAX2* gene by *WT1*, which leads to persistent expression of *PAX2* in podocytes [14,15]. This mechanism is further supported by findings of a severe congenital NS phenotype in *PAX2* transgenic mice [26]. The majority of the children in this study presented with NS within the first year of life although some presented later on in life (Tables 1 and 2). Mutations in *PLCE1* therefore expanded the genetic causes of NS in the first year of life [20]. The age at onset of symptoms was not different between patients with *WT1* mutations and patients with *PLCE1* mutations (Table 2). Neither is there a difference between patients with and without mutations

(Table 2). Age of onset therefore does not appear to be a discriminant factor for the presence of mutations. However, children without mutations in *PLCE1*, *WT1* and *LAMB2* seem to have slower progression of disease and reached ESKD at an older age (Table 2). Although we did not detect any patients with missense mutations in *PLCE1*, it is plausible to speculate that missense mutations in *PLCE1* may be associated with milder disease course or other histologic variants such as focal segmental glomerulosclerosis (FSGS) [5]. Since 36% of consanguineous offsprings with IDMS did not have mutation in *PLCE1*, *WT1* or *LAMB2*, it is likely that known genes and other unidentified mutated genes account for the remaining cases. Only 14 children were placed on corticosteroid or cyclosporine therapy in this series. We did not find any child with therapy response apart from the child with a *PLCE1* mutation that we previously reported [5]. This may be due to the fact that children with DMS and early-onset NS are not routinely offered treatment. There is a need for further study to screen children with IDMS due to mutations in *PLCE1* for therapy response. The mechanism of response to steroid and other immunosuppressive agents in children with mutations is not clear. There is a need for further study to explain this. However, if more children with mutations in *PLCE1* are found to be therapy responsive, there may be a case for controlled treatment with corticosteroids and other modalities of treatment in a subset of children with IDMS.

In conclusion, *PLCE1* is a major cause of IDMS in this worldwide series. As there is a possibility that the disease may be potentially amenable to treatment, further study is needed. We would suggest screening for mutations in *PLCE1* in children with IDMS.

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Conflict of interest statement. None declared.

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