Mutations in *phospholipase C epsilon 1* are not sufficient to cause diffuse mesangial sclerosis

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Diffuse mesangial sclerosis occurs as an isolated abnormality or as a part of a syndrome. Recently, mutations in phospholipase C epsilon 1 (*PLCE1*) were found to cause a nonsyndromic, autosomal recessive form of this disease. Here we describe three children from one consanguineous kindred of Pakistani origin with diffuse mesangial sclerosis who presented with congenital or infantile nephrotic syndrome. Homozygous mutations in *PLCE1* (also known as *KIAA1516*, *PLCE*, or *NPHS3*) were identified following genome-wide mapping of single-nucleotide polymorphisms. All affected children were homozygous for a four-basepair deletion in exon 3, which created a premature translational stop codon. Analysis of the asymptomatic father of two of the children revealed that he was also homozygous for the same mutation. We conclude this nonpenetrance may be due to compensatory mutations at a second locus and that mutation within *PLCE1* is not always sufficient to cause diffuse mesangial sclerosis.

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Congenital nephrotic syndrome is defined as proteinuria leading to clinical symptoms presenting in the first 3 months after birth.[1] This age cutoff is arbitrary and not an adequate classification of early onset nephrotic syndrome. Genetically determined congenital or infantile nephrotic syndrome is classified into Finnish Type congenital nephrotic syndrome, focal segmental glomerulosclerosis, and diffuse mesangial sclerosis (DMS). Less frequently it may be caused by a mitochondrial disorder.[2] Finnish type congenital nephrotic syndrome is caused by mutations in NPHS1, which codes for the slit diaphragm protein nephrin.[3] Autosomal recessive focal segmental glomerulosclerosis is caused by mutations in NPHS2 which codes for podocin, but for it to present in the first 3 months of life (congenital focal segmental glomerulosclerosis) there is evidence that it requires a 'tri-allelic hit' with mutations of any three of the four alleles of NPHS1 and NPHS2.[4] DMS may occur as part of a syndrome or as an isolated abnormality. Denys–Drash syndrome (nephropathy, Wilm's tumor, and genital abnormalities) is caused by mutations of WT1[5] whereas Pierson syndrome (DMS with microcoria) is caused by mutations of lamininβ-2.[6] The genetic basis for Galloway–Mowat syndrome (DMS with neuronal migration disorder) is unknown. Some patients with nonsyndromic DMS inherited as an autosomal-dominant disease have mutations in WT1.[7,8] In 2006, Hinkes *et al.*[9] described mutations in phospholipase C epsilon 1 (*PLCE1*) as a cause of nonsyndromic, autosomal recessive DMS and in 2007 Gbadegesin *et al.*[10] found *PLCE1* mutations in 10 of 35 cases. The molecular mechanisms by which these mutations cause DMS are unknown.

We undertook genetic studies in a consanguineous kindred of Pakistani origin with three affected children to determine the genetic cause of DMS in this family. The pedigree structure (Figure 1) indicated that an autosomal recessive pattern was the most likely mode of inheritance.

CASE REPORTS

The family tree is shown in Figure 1. The index patient was individual 3. He presented at 14 months of age with

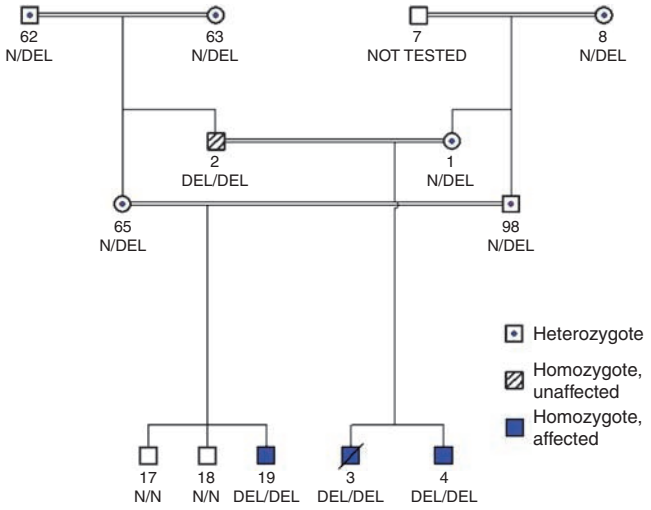

Figure 1 | Extract of the extended pedigree of the affected kindred.

nephrotic syndrome (periorbital edema, plasma albumin concentration 9 g/l (normal 34–45), urine protein/creatinine ratio 3904 mg/mmol (normal <23)) and renal impairment (plasma creatinine 94 μmol/l, estimated glomerular filtration rate 34 ml/min per 1.73 m^2.) His blood pressure was severely elevated at 140/80. The rest of the examination was normal. A renal biopsy showed features of DMS with extensive chronic tubulointerstitial nephritis (Figure 2a). A computed tomography scan of his head showed no evidence of structural abnormality. He received treatment with prednisolone, ciclosporin, an ACE inhibitor (enalapril), a calcium channel blocker (amlodipine), and a β-blocker (atenolol). In spite of this he progressed rapidly to end-stage renal failure and hemodialysis was commenced 1 month after initial presentation. At 3 months after the initial presentation he developed severe pancreatitis and died. His post-mortem head circumference was 46 cm, on the 9th centile for age.

Individual 19 is the cousin of the index patient. At 5 months of age his parents sought medical advice as they were concerned that he might have the same renal condition. Urinalysis showed 4+ proteinuria and he was referred to our hospital for further assessment. Clinical examination showed that his length (67 cm) and weight (7.8 kg) were on the 50th centile for age but his head circumference (41 cm) was on the 0.4th. His blood pressure was 80 mm Hg systolic. The rest of the examination was normal apart from a coronal hypospadias. Investigations showed a plasma creatinine concentration of 44 μmol/l (estimated glomerular filtration rate of 51 ml/min per 1.73 m^2), plasma albumin of 34 g/l and a urine protein/creatinine ratio of 1002 mg/mmol. A renal biopsy shortly after presentation confirmed the diagnosis of DMS (Figure 2b) Further investigation confirmed the presence of hemoglobin E, known to be present in the family. An MRI scan of his head showed no abnormalities and an ophthalmologic examination was normal. He has been treated with ACE inhibitors, but no glucocorticosteroids or other immunosuppressive drugs. His proteinuria has responded well to therapy. His urine protein/creatinine ratio was 42 mg/mmol and his estimated glomerular filtration rate 99 ml/min per 1.73 m^2 at age 61 months.

Individual 4 is the brother of the index patient. The pediatric renal unit was alerted to the fact that his mother was pregnant and he was first seen on the day after his birth. Clinical examination was normal. The urinary protein/creatinine ratio was 65 mg/mmol, rising to 154 mg/mmol on day 4 after birth. At 1 week of age his blood pressure was 80 mm Hg systolic and the urine protein/creatinine ratio was 158 mg/mmol. He was started on captopril with a reduction in protein/creatinine ratio to 68 mg/mmol. His urine protein/creatinine ratio peaked at over 2000 mg/mmol but has reduced to 307 mg/mmol at 33 months with an increased dose of ACE inhibitor. His estimated glomerular filtration rate is 108 ml/min per 1.73 m^2. His weight and length have grown along the 2nd centile and his head circumference along the 0.4th. A renal biopsy at 1 year of age confirmed the diagnosis of DMS with areas of chronic tubulointerstitial damage (Figure 2c).

Individual 2 is the father of two of the affected children. He was born in the United Kingdom after a pregnancy apparently complicated by polyhydramnios. As a child he had mild asthma but no other illnesses. He was on no regular medication. His height is 168.8 cm and his head circumference 56 cm, which is on the 25th centile of head circumference for height.[11] He is normotensive (BP 112/90) and has normal renal function (plasma creatinine 79 μmol/l) with no proteinuria (urine protein/creatinine ratio 5 mg/mmol).

RESULTS

Single nucleotide polymorphism (SNP) array analysis of three affected individuals revealed two long regions of homozygosity. The most promising region common to the three affected and none of the four unaffected individuals tested, was a 3.0 Mb (42.6 LDU) region located at chromosome 10q23. This region contained the *PLCE1* gene implicated in other DMS cases.[9] A 4 bp deletion, c.1303_1306delAGGA; p.R434X, was identified in exon 3 of the *PLCE1* gene (reference sequence ensemble transcript ID ENST00000371380). This mutation directly generates a premature translational termination signal at codon 434 (Figure 3).

Unaffected individuals 1, 8, 62, 63, and 98 were found to be heterozygous carriers of the mutation. Affected individuals 3, 4, and 19 were found to be homozygous for the deletion.

Unaffected individual 2 was also found to be homozygous for the deletion. Analysis with two alternative sets of PCR primers also indicated homozygosity for the deletion in individual 2 (data not shown) thus eliminating the possibility of a PCR primer binding site mutation causing artefactual amplification failure. Both parents of individual 2 were heterozygous carriers of the mutation. Multiplex ligation-

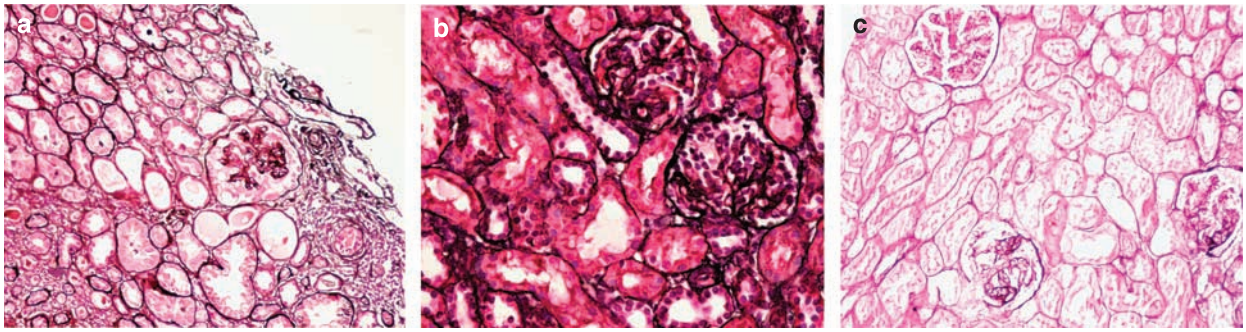

Figure 2 | Light microscopy of renal biopsies (silver stain). (**a**) Biopsy taken from individual 3 at 15 months of age. (**b**) Biopsy from individual 19 at 5 months of age. (**c**) Biopsy from individual 4 taken at 12 months of age. All biopsies show prominence of the mesangial matrix and podocytes with varying degrees of glomerular sclerosis and tubulointerstitial fibrosis. All were stained with Jones' silver stain. Original magnification $\times 200$.

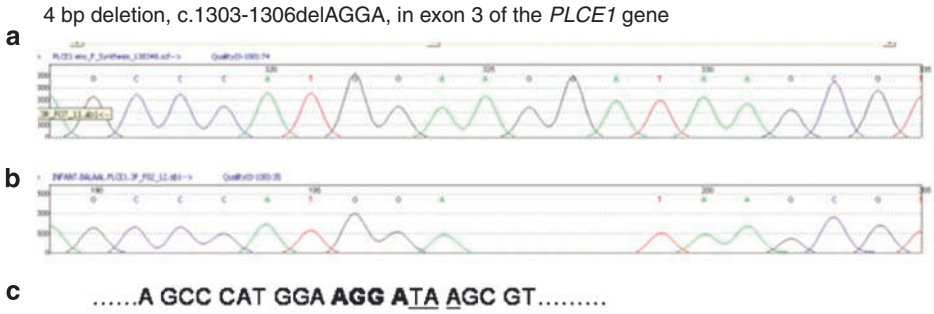

Figure 3 | DNA sequence analysis of exon 3 of the *PLCE1* gene. (**a**) Sequencing data for bases 1293–1313 of exon 3 of the *PLCE1* gene in a normal individual. (**b**) Sequencing data of an affected member of the pedigree showing deletion of bases 1303–1306. (**c**) Interpretation of sequence data indicating AGGA deletion (bold) and generation of the translational termination codon TAA (underlined).

dependent probe amplification (MLPA) analysis shows no evidence of a normal exon 3 sequence in individual 2. Methylation analysis of the *INPP5a* gene showed no methylation anomaly indicating normal biparental inheritance of chromosome 10. Repeat DNA samples from both peripheral lymphocytes and saliva of individual 2 were analyzed and found to be homozygous for the deletion in both samples. No evidence of mosaicism for the normal *PLCE1* exon 3 sequence was seen (data not shown).

Analysis of CA repeat polymorphisms confirmed homozygosity in the region of the *PLCE1* gene. Individual 2 was homozygous for CA repeats D10S2316, D10S1680, and D10S520 at 96, 95.6, and 96.4 Mb, respectively, on chromosome 10. These markers are highly polymorphic and very close to the *PLCE1* locus at 95.8 Mb (ENSEMBL, release 50). D10S2316 analysis was also performed on the three affected men who were all homozygous for the same allele as individual 2.

A total of 32 family members were analyzed for the *PLCE1* c.1303_1306del AGGA mutation. No other unaffected homozygotes were found.

DISCUSSION

We have described a consanguineous kindred in which three children are affected by DMS. All three are homozygous for a 4 bp deletion in exon three of the *PLCE1* gene. This deletion results in a premature translational stop codon. This is completely consistent with previous descriptions of mutations of this gene as a cause of DMS. Both Hinkes *et al.*[9] and Gbadegesin *et al.*[10] describe DMS patients with *PLCE1* mutations generating premature translational termination signals. The physiological substrates of *PLCE1* are unknown as are the mechanisms by which abnormalities of this protein lead to this devastating renal disease.

The finding of an apparently pathogenic homozygous mutation in a phenotypically completely normal adult suggests that other factors significantly modify the effect of abnormalities of *PLCE1*. This could include the possibility that mutations at another locus render mutations of *PLCE1* nonpathogenic. Possible candidates might include BRAF and IQGAP1, both of which interact with *PLCE1*.[12] Alternatively, a pathogenic mutation in one or both alleles at a different locus or an environmental challenge early in development may be necessary in addition to *PLCE1* mutations for a clinical presentation with nephrotic syndrome. Less likely explanations include the possibility that *PLCE1* mutations are not pathogenic and are an incidental finding in this family. This was discounted as this gene is known to be implicated in other families with DMS and was identified through homozygosity mapping in the three affected individuals.

Mosaicism for uniparental disomy in the unaffected homozygote causing reduction to homozygosity at 10q23 in the blood but not in kidney tissue was also postulated but excluded as far as possible by molecular investigation, which detected no evidence of mosaicism. The possibility was considered that the mutation may affect RNA splicing and cause varying degrees of skipping of part of exon 3 in different individuals and contribute to the nonpenetrance in individual 2. However *in silico* analysis using the splice site prediction programs 'fruitfly' (http://www.fruitfly.org/seq_tools/splice.html), 'SpliceSiteFinder' (http://violin.sickkids.on.ca/ali-cgi-bin/splicesitefinder.cgi.pl), and 'NetGene2' (http://www.cbs.dtu.dk/cgi-bin/nph-webface?jobid=netgene2) showed no evidence of this. Furthermore, the orthologous murine *PLCE1* sequence shows a high degree of conservation of the exon 3 amino-acid sequence indicating that it is likely to be important for *PLCE1* function (data not shown).

Although the exact mechanism to explain the nonpenetrance seen in the family is not known, variable expression is another possibility given that there have been so few families described and the natural history of the disorder is yet to be fully characterized. It remains a possibility that subclinical early disease followed by spontaneous remission would mean that individuals investigated for the first time as adults are no longer symptomatic. Interestingly a mouse model with homozgous deletion of *PLCE1* did not exhibit a nephrosis-like phenotype.[13]

Hinkes *et al.*[9] suggested that DMS caused by mutations of *PLCE1* might respond to treatment with steroids and/or ciclosporin. We treated the index patient with these agents with no beneficial effect. It is possible that he presented with disease that was too far advanced to respond to any such treatment. Indeed he progressed with extreme rapidity to end-stage renal failure. We cannot exclude the possibility that the nephrotoxicity of cyclosporin contributed to the rapid deterioration of his renal function. In either event we would recommend caution in using these agents on affected patients in future. Our patients have responded well to ACE inhibitors and we feel that these agents should be first-line treatment for affected patients.

We have described here, for the first time, a healthy adult individual with a homozygous truncating mutation of the *PLCE1* gene. We conclude that either changes in other genes might be required to render mutations of *PLCE1* deleterious, or, more likely, that one or more mutations at other loci can render an apparently pathogenic, truncating mutation of *PLCE1* benign. The discovery of more families with this DMS may help to further our understanding.

MATERIALS AND METHODS

SNP array analysis

Five individuals were genotyped for SNPs on the Illumina HumanHap 550K bead array; three affected (3, 4, and 19) and two unaffected (individuals 25 and 114 in the extended pedigree). The same three affected individuals and unaffected individuals 17, 18, and 25 were genotyped on the Affymetrix GeneChip 500K Mapping Array Set. The genotype data were analyzed using specifically designed software in the C programming language. This recorded the locations of tracts of continuously homozygous SNPs common to the affected individuals, with the same allele present at each locus (that is the same underlying haplotype).

The regions detected in this analysis were prioritized by length on the genetic scale using linkage disequilibrium units (LDU), from LDU maps produced by the LDMAP program[14] from genome-wide HapMap data.[15] This accounts for the association with LD structure and prioritizes regions of autozygosity over tracts of homozygosity associated with high LD.

PLCE1 sequence analysis

DNA was extracted from samples of peripheral blood (and saliva for individual 2) using standard techniques.[16] PCR amplification of *PLCE1* gene exons was performed before sequencing using the primers described by Hinkes *et al.*[9] Further sequencing of exon 3 was performed in individual 2 using the primers originally described by Hinkes *et al.* (viz E3F: 5′ttgcacttggagcatctgag and E3R: 5′tgaacttaattttccatcaggag) as well as two alternate sets of primers (E3alt1F: 5′ggttaatgcatgagccgagt; E3alt1R: 5′ttctcttaaagaagcgacttttactta and E3alt2F: 5′gtcatcttggcattttgtca; E3alt2F 5′tccttctctgtagtcaaagatagaaa) Reactions were in a 20 µl volume containing 50 ng DNA, 1 mM forward primer, 1 mM reverse primer, 2 µl 10 × reaction buffer (Qiagen, Crawley, UK), 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.5 U Taq polymerase (HotStar Taq; Qiagen). Cycling parameters were 94 °C for 12 min followed by 32 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. PCR products were sequenced bidirectionally using the ABI Big Dye terminator v 1.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) and the same primers as for PCR amplification. Results were analyzed with Mutation surveyor version 3.2 (Soft Genetics, State College, PA). Genotyping results for 12 individuals are shown in Figure 1. A further 20 individuals have been genotyped. They were either homozygous for the normal allele or heterozygous.

UPD analysis

Investigation for possible mosaic uniparental disomy of chromosome 10 in individual 2 was carried out by methylation analysis of the imprinted *INPP5a* gene based on the method of Zeschnigk *et al.*[17] Genomic DNA (2 µg) was bisulphite treated, followed by methylation-sensitive PCR amplification (msPCR) and size analysis using an ABI 3100 automated DNA analyzer. The methylated and nonmethylated alleles generate PCR products of different length thus enabling the resultant peak heights to be used as a measure of the relative proportions of the alleles present in the sample. Peak height ratios were compared with those of four normal control samples (data not shown).

MLPA analysis

Dosage analysis was carried out using MLPA probes designed specifically for normal sequence in exon 3, the probe binding site comprising bases 1279–1326 of *PLCE1* (reference sequence ensembl transcript ID ENST00000371380, base 1 being the first base of the ATG translation initiation codon). (Probe sequence: PLCE1_3L: GGGTTCCCTAAGGGTTGGACCAGCCTCCGAGACAGCCCATGGA; PLCE1_3R AGGATAAGCGTTGGTCCATGCTTACCTACCGTGTTC TAGATTGGATCTTGCTGGCAC). The probes were diluted to 1.33 fmol/µl, and 1.5 µl of this was used for MLPA in conjunction with a commercially available MLPA probe mix (CFTR-P10, 91; MRC-Holland, Amsterdam, the Netherlands) to provide controls.

The standard protocol of MRC-Holland[18] was used reducing the initial DNA denaturation volume to 3.5 µl to allow for the extra 1.5 µl of *PLCE1* probe mix.

Microsatellite analysis

Microsatellite analysis was carried out by PCR amplification of DNA using primers for polymorphic $(CA)_n$ repeat sequences in the region of the *PLCE1* gene. One primer from each pair was labeled with a fluorophore. 50 ng of DNA was amplified using HotStarTaq polymerase (Qiagen) under the conditions recommended by the manufacturer with additional magnesium to a final concentration of 2 mM. PCR amplification conditions were as follows: 95 °C for 10 min followed by 32 cycles of 94 °C for 30 s; 55 °C for 30 s; 72 °C for 30 s followed by 7 min at 72 °C. PCR products were run on an ABI 3100 automated sequencer for separation, visualization and sizing.

DISCLOSURE

All the authors declared no competing interests.

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