Original Paper

Apolipoprotein E Mutation and Double Filtration Plasmapheresis Therapy on a New Chinese Patient with Lipoprotein Glomerulopathy

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Key Words

Apolipoprotein E • Missense mutation • Double filtration plasmapheresis • Lipoprotein glomerulopathy

Abstract

Background/Aims: Lipoprotein glomerulopathy (LPG) is a rare hereditary disease. In this study, we investigated the apoE mutation and the role of double filtration plasmapheresis therapy (DFPP) on a new Chinese patient with LPG. **Methods:** Renal biopsy was performed on this patient to allow a definitive diagnosis. The mutations in the coding sequence of apoE and the hereditary pedigree of this patient were investigated by DNA sequencing. The patient was treated with DFPP, and clinical parameters before and after DFPP were compared. **Results:** Two missense mutations were found in this patient: Cys112Arg and Arg25Cys. Arg25Cys was previously designated as APOE Kyoto. Family genotyping showed that Cys112Arg and Arg25Cys mutation were transmitted through his father and his mother, respectively. The patient's parents are healthy so far to date. Possibly there was a dose effect on apoE mutation induced LPG. Furthermore, DFPP treatment was first used on this patient and led to dramatic changes: Proteinuria and apo E values declined, and hemoglobin level increased significantly. **Conclusion:** APOE Kyoto mutation was found in a new Chinese patient with LPG, accompanied by Cys112Arg. More cases and further functional experiments are needed to investigate the role of these two mutations together in LPG. DFPP is an effective therapeutic modality for improving NS in patients with LPG.

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Introduction

Lipoprotein glomerulopathy (LPG) is a rare hereditary disease characterized by intraglomerular lipoprotein thrombi and abnormal lipid metabolism. It was first reported in 1989 [1], and to date less than 100 cases have been found. Most of the patients are from East Asian countries, especially from Japan and China. Genetic studies show that LPG is an autosomal dominant, with low penetrance. Abnormalities in the apolipoprotein E (apoE) gene have been suspected in the pathogenesis of LPG. No specific symptoms or signs were found in the affected patients, presenting with nephritic syndrome (NS), hypertriglyceridemia, elevated plasma apoE levels and gradual progress to renal failure. The abnormal lipid metabolism was often modified when NS was present. In fact, lipid profile is sometimes normal in LPG, but that matter may be different from the mechanism of NS. So the misdiagnosis was not rare, and the incidence of this disease should be underestimated. Renal biopsy is necessary for the diagnosis of LPG. In addition, it was not rare that glomerular mesangial proliferation lesions and basement membrane “double track” changes induced by mesangial insert was shown, but intraglomerular lipoprotein thrombi were essential. In this situation, the patients were more easily misdiagnosed without the evidence of apoE staining. As a result, many patients might be misdiagnosed or never diagnosed without the evidence of renal biopsy. The higher prevalence of LPG in Asians (especially in Japanese and Chinese) than that in Europeans and Americans maybe because the high threshold for biopsy. Renal biopsy is more and more common to be used as a routine screening method for urinary abnormalities in China. In 2005, Zhang et al summarized the characteristic of LPG in Chinese. They thought the Chinese LPG patients presented some common features, including increased kidney size, familial history of kidney disease, anemia, microscopic hematuria and elevated serum level of apoE [2].

In this study, we investigated a new Chinese patient who was diagnosed as LPG via renal biopsy. The clinical and pathological characteristics and the mutation of apoE gene were reported. Moreover, the effects of double-filtration plasmapheresis (DFPP) therapy on LPG were discussed for the first time.

Subjects and Methods

Ethics statement

This study was approved by the institution review board of Union Hospital, Tongji Medical College, Huazhong University of Science & Technology. We initiated this research in accordance with the defined protocols in which the design and performance of current study involving human subjects were clearly described. Written informed consent was obtained from all participants after the procedure had been fully explained.

Subject

A 23-year-old male who presented with NS was hospitalized at Union Hospital, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China. Reviewing the medical history of this patient, a mild edema of lower limbs was found 9 months ago. Then the edema gradually worsened and accompanied with foamy urine and fatigue. A 24-hour urine collection revealed proteinuria of 4.37 g/day and the serum albumin was 29.6 g/L. The secondary causes of NS were excluded, including systemic lupus erythematosus, systemic vasculitis, Henoch-Schonlein purpura, diabetes, multiple myeloma, hepatitis, hypertension, etc. The patient had been treated with glucocorticoid (1mg/kg/d) for 2 months, accompanied with supportive therapies such as angiotensin-converting enzyme inhibitors (ACEI) / angiotensin receptor blocker (ARB) with up-titration of dosage and rapid-lowering therapy, but the multiple proteinuria was persistent. Then Mycophenolate Mofetil (1.5g/d) was added but there was no improvement of the disease. The patient was referred to our hospital and the refractory reason was further explored by a renal biopsy.

Renal histological examination

Percutaneous renal biopsy was performed under ultrasonographic guidance. Formalin-fixed tissue was embedded in paraffin using routine procedures. Sections of 1 µm in thickness were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS),

periodic acid-methenamine silver (PAMS) and Masson's trichrome-elastica for light microscopic pathological diagnosis. Immunofluorescence staining was performed on 3 µm cryostat sections by using fluorescein isothiocyanate-(FITC) labelled rabbit anti-human immunoglobulin (Ig) G, IgA, IgM, complement (C) 3, C4 and C1q antibodies (DAKO Denmark A/S, Denmark). Apo A, apoB and apo E were analyzed with the indirect immunofluorescence technique using antisera monospecific antibodies to each (DAKO Denmark A/S, Denmark). Transmission electron microscopy was performed to observe the ultra-microstructures of the renal specimen which was cut into ultra-thin sections of 60-68 nm.

Mutation screening of apoE gene

The mutations in the coding sequence of apoE and the hereditary pedigree of this patient was further investigated. For the study protocol, informed written consent was obtained from the LPG patient and his parents. Genomic DNA was isolated from the whole blood of the patient and his parents by a routine method. The sequence of apoE gene was obtained from the UCSC Genome Browser (<http://genome.ucsc.edu>). Four exons of the apoE gene were amplified by polymerase chain reaction (PCR) with four sets of primers as shown in Table 1. The PCR mixture consisted of 0.1 µmol primers, 10 µmol dNTP, 2.5 U of Taq DNA polymerase and 380 ng of genomic DNA in a total 50 µl volume. The thermal cycle profile was 35 cycles of denaturation at 94 °C for 30 seconds, annealing (temperature showed in Table 1) for 35 seconds and a final extension of 1 minute at 72 °C. DNA sequencing was processed in both directions and the mutation identification was also performed by an ABI-PRISM3730 automated sequencer (Sangon biotech company, Shanghai, China). Genomic DNA from healthy volunteer was used as control.

Treatment and study protocol

DFPP procedure. A single dual-lumen catheter was placed into the right internal jugular vein for vascular access. Heparin plus citrate was given as an anticoagulant to prolong activated clotting time by twofold. A blood purification machine PEM10 (Excorim, Sweden) was used for DFPP. The filters were purchased from Kawasumi Laboratories, Inc., Tokyo, Japan, including the Plasma Separator Plasmacure PE-08 (primary filter, surface area 0.8 m², pore size 0.3 µm) and the Plasma Fractionators Evaflux 2A20 (secondary filter, surface area 2.0 m², pore size 0.01 µm). Blood flow rate was set at 100-180 ml/min, and the plasma flow rate was set at 20-40 ml/min. In brief, whole blood was separated into blood cells and plasma by the primary filter. Then high molecular weight substances and low molecular weight substances in plasma were divided in the secondary filter. The plasma fraction, containing low molecular weight substances such as albumin, was returned to the patient with the supplement solution together. The plasma fraction, containing high molecular weight substances associated with diseases, such as apoE, was discarded. The above steps were repeated until the processed plasma was up to twofold of the plasma volume (PV). PV was calculated using the Modified Retzlaff et al's equation [3]:

$$\text{Male: PV(ml)} = (23.7 \times L + 9.0 \times W - 1709) \times [100 - (0.91 \times \text{HCT})] / 57.2$$

$$\text{Female: PV(ml)} = (40.51 \times L + 8.4 \times W - 4811) \times [100 - (0.91 \times \text{HCT})] / 61.78.$$

Where L is the height (m), W is the weight (kg), and HCT is the hematocrit (%). During one session of DFPP, the amount of plasma discarded was about 5%-10%, and 10-20 g human albumin was infused as a supplemental solution. DFPP was performed once every 3 days, 3 times in total.

Table 1. ApoE gene primer records

Primer name	Primer sequence (5'-3')	Annealing temp. (°C)	Product size (bp)
APOE-1F	GGGAGCCCTATAATTGGACA	57	339
APOE-1R	TGGAGTCCTGCTATGGCTTAC		
APOE-2F	AAGGCTTGAAGGCTAACC	58	232
APOE-2R	CAGGAGGTTGAGGTGAGGAT		
APOE-3F	ATGGCTCCAAAGAAGCATTG	57	375
APOE-3R	GGCAGAATGAAACCTGGACC		
APOE-4F	CTGGCTCATCCCCATCTCG	61	1016
APOE-4R	GCCAGTATGTGGGCAGAAAGAG		

Fig. 1. Pathological findings of this LPG patient. Panel A shows mesangial hypercellularity and mesangium expansion. The glomerular capillaries are significantly dilated by thrombi of foamy material that stained weakly with periodic acid-Schiff (PAS stain, 400 x). Panel B shows thrombi in the highly dilated capillary lumen stained with trichrome. Red blood cells are squeezed to the edge of capillaries (Masson stain, 400 x). Panel C shows segmental layering of basement membrane, which is induced by mesangial insert (PASM-Masson stain, 400 x). Panel D shows diffuse moderate mesangial staining for apoB (Immunofluorescence stain, 400 x). Panel E shows diffused strong mesangial staining for apoE (Immunofluorescence stain, 400 x). Panel F shows capillary lumen occluded by various foaming lipid thrombi under electron microscope (x10000).

Laboratory investigations

Urine and venous blood samples were collected on the day before DFPP, after the first time of DFPP, after three times of DFPP and upon 3-month follow up, respectively. 24-hours proteinuria, serum creatinine, serum albumin, serum globulin, IgG, IgM, IgA, C3, C4, C-reactive protein (CRP), haemoglobin (HGB) and red blood cell (RBC) were analysed according to standard laboratory procedures. As for lipid profiles measurement, the venous blood was collected in tubes containing 0.1% of ethylene diamine tetraacetic acid (EDTA), and was separated by centrifugation (2000 *g* x 15 min). Total cholesterol and triglyceride values were determined enzymatically. High-density lipoprotein (HDL) and low-density lipoprotein (LDL) were isolated from plasma by sequential ultracentrifugation. Apo A1, apoB, apo E and lipoprotein (a) were measured by the immunoturbidimetric assay.

Results

Pathological findings

Upon renal biopsy, one cortex and one cortex-medulla tissue was collected. A total of 20 glomeruli without lobulated lesion was found under light microscope, including 2

global scleroses and 1 segmental sclerosis. The size of glomeruli was generally enlarged with hypercellularity (80-120 cells/glomerulus) and increased mesangial matrix. The most notable feature was that the glomerular capillaries were significantly dilated by thrombi of foamy material that was stained weakly with hematoxylin-eosin, periodic acid-Schiff (Figure 1A), and trichrome (Figure 1B). Mesangium insert and double contours of the capillary walls were found in several areas (Figure 1C). Cell swelling and granular degeneration was widespread in the epithelial cells of tubules. Interstitial fibrosis was graded semiquantitatively on a scale of 1+, based on the percentage of the cortical area affected (<5: 0; 6-25: 1+; 26-50: 2+; and >50%, 3+). No obvious lesions were found in the small arteries and arterioles. Immunofluorescence demonstrated apo B (Figure 1D) and apoE (Figure 1E) distributed in the glomerular capillaries in a diffuse and global pattern. The intensity of apo B staining was moderate (2+), and apoE was strong (3+). There was 1+ to 4+ crumbly staining for immunoglobulin (Ig) M in some mesangium and capillaries, and absent staining for IgG, IgA, complement (C) 3, C4 and C1q. Ultrastructural examination revealed dilated capillary lumen were occluded by multiple lipid vacuoles (Figure 1F). The glomerular basement membranes showed segmental separation and irregular thickening with the foot process of podocytes wide fusion. No electron-dense was found in the glomeruli.

Fig. 2. Mutation Detection of apoE on this LPG patient and his parents. Panel A shows the electrophoretogram of all exons of apoE gene. M: Mark; E1: exon 1 (339bp); E2: exon 2 (232bp); E3: exon 3 (375bp); E4: exon 4 (1016bp). Panel B shows a 'C' to 'T' transition in exon 3 in the samples of the LPG patient and his mother, which substituted arginine for cysteine (Arg25Cys). Panel C shows a 'T' to 'C' transition in exon 4 in the samples of the LPG patient and his father, which substituted cysteine for arginine (Cys112Arg). P: patient; F: father; M: mother. Panel D shows the family pedigree for the patient, which indicated the apoE Kyoto (Arg25Cys) genotype in this LPG patient (arrows) and his mother. Men are represented by squares, and women by circles. The shading indicates patients with LPG.

Table 2. Changes in laboratory data before and after the treatment of DFPP

	Before DFPP	After DFPP-1	After DFPP-3	After 3- months	Normal value
Proteinuria(g/24h)	4.37	0.01	0.01	0.09	<0.15
Serum albumin(g/L)	29.6	21.7	21.6	36.6	35-50
Serum globulin(g/L)	17.41	8.35	6.55	23.55	23-32
IgG(g/L)	0.96	0.57	0.48	5.51	7.51-15.60
IgM(g/L)	0.69	0.04	0.08	0.81	0.46-3.04
IgA(g/L)	0.48	0.08	0.07	0.54	0.82-4.53
C3(g/L)	1.12	0.29	0.23	0.86	0.79-1.52
C4(g/L)	0.24	0.03	0.02	0.14	0.16-0.38
CRP(mg/L)	7.05	2.69	2.12	5.66	<8
RBC($\times 10^{12}$ /L)	2.98	3.37	3.6	2.76	4-5.5
HGB(g/L)	90	107	114	84	120-160
Total Cholesterol(mM)	4.03	0.88	0.55	3.88	<5.2
Triglyceride(mM)	1.74	0.24	0.32	1.12	<1.7
HDL-C(mM)	1.66	0.45	0.20	1.54	1.16-1.42
LDL-C(mM)	1.67	0.41	0.17	1.31	2.7-3.1
LP(a)(mg/L)	27	13	14	28	0-300
Apo E(mg/dl)	5.6	1.2	1.9	4.4	2.7-4.5
Apo A1(mg/dl)	1.51	0.32	0.20	1.48	1.2-1.76
Apo B(mg/dl)	0.56	0.11	0.08	0.69	0.66-1.07

IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; C3: complement 3; C4: complement 4; CRP: C-reactive protein; RBC: red blood cells; HGB: hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LP(a), lipoprotein (a); Apo E, apolipoprotein E; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B.

Gene mutation analysis

To verify LPG, the coding region of the apoE gene was sequenced, and two point mutations were found in this patient's DNA sample. As showed as in the Figure 2B, there was a 'C' to 'T' nucleotide transition for the amino acid in exon 3 was found, which substituted arginine for cysteine (Arg25Cys). This missense mutation was originally described in a Japanese man with LPG and was designated as *apoE* Kyoto in 1999 [4]. In addition, a 'T' to 'C' nucleotide transition for the amino acid was found in exon 4, which substituted cysteine for arginine (Cys112Arg, Figure 2C). This single nucleotide variation (SNV, rs429358) was reported before as a strong risk factor for Alzheimer's disease (AD) and several chronic neurodegenerative diseases. Moreover, the patient's father also showed the same Cys112Arg mutation in exon 4, and his mother showed the same Arg25Cys mutation in exon 3 (Figure 2D). It indicated that two mutations of apoE in the LPG patient were inherited from his parents, respectively.

DFPP treatment and follow-up

Before DFPP treatment, this LPG patient presented NS (multiple proteinuria and serious hypoalbuminemia) with mild anemia (HGB 90g/L), and serum creatinine in the normal range (81 μ mol/L). No obvious abnormality of lipid was found in this patient, except a mild increased serum apoE and triglyceride. The levels of IgG and IgA were decreased, but those of C3, C4 and CRP were normal. After a single course of DFPP, clinical data changed dramatically. Proteinuria declined significantly, from 4.37g/24 h to 0.01g/24h. The HGB level increased from 90g/L to 114g/L, and apo E values decreased from 5.6 mg/dl to 1.9 mg/dl. After DFPP treatment, this patient continually received fenofibrate at a dose of 200mg/day. Changes of laboratory values before/after treatment and in 3-months follow-up are summarized in Table 2.

Discussion

This patient was first diagnosed as primary NS, after excluding the common secondary causes such as systemic lupus erythematosus, systemic vasculitis, Henoch-Schonlein purpura, diabetes, multiple myeloma, hepatitis, hypertension, etc. No obvious abnormality of lipid metabolism was found in this patient. So he was potentially misdiagnosed patient without the result of pathological evidence. We performed renal biopsy on this patient, because he was not responsive to the routine treatment of NS, including glucocorticoids and immunosuppressants. Renal biopsy is necessary in NS patients who had poor treatment outcomes. The pathological results of this patient also explained why the effect of routine treatment was not satisfying. NS was not induced by the cell proliferation, matrix expansion and immune complex deposit usually present. On kidney biopsy, lipoprotein thrombi within the lumina of severely dilated glomerular capillaries were the distinct finding for this patient, and the lipoprotein thrombi was found apoA/B/E positive on immunofluorescence. However, no thrombi in arterioles had been found.

The pathogenesis of LPG is not fully understood, although it is likely that mutated apoE contributes to it. A total of 13 apoE mutations associated with LPG have been reported so far [5-7]. Many of the apoE mutations occur in the LDL receptor binding site, involving 140-150 amino acids in patients with LPG. It's possible that binding of the mutated apoE to the LDL-receptor may be abnormal because of an altered three-dimensional structure of mutant protein [4, 8, 9]. In this LPG patient, we found two missense mutations of apoE gene: Cys112Arg in exon 4 and Arg25Cys in exon 3. Cys112Arg was reported before as a pathogenic allele of apoE gene associating with increased risk of AD [10], cognitive ageing [11], dementia of AD (DAD) in Down Syndrome (DS) [12], serum CRP [13], the prevalence of low HDL-cholesterol level and abdominal obesity [14], etc. Arg25Cys is a typical mutation of apoE that is etiologically related to LPG named as apoE Kyoto. Recently, Hu et al presented 35 LPG patients carrying the apoE Kyoto allele in southwest China, making it the most common mutation related to LPG [15]. Meanwhile, the family study showed that this patient's mother was a heterozygous carrier of apoE Kyoto and his father was a carrier of Cys112Arg. It was proved that the mutated genes of this patient were inherited from both of his parents. Above all, his parents are healthy so far to date and haven't shown any symptoms of diseases.

A similar situation was reported by Matsunaga et al [4] and Rovin et al [16]: the male LPG patient carried an Arg25Cys mutation in apoE, but heterozygous female carriers were clinically unaffected. Rovin et al remarked that the apoE mutation appears to be sufficient to lead to glomerular lipoprotein deposition but not to clinical LPG [16]. Therefore, it's possible that there was a dose effect on apoE mutation induced LPG. That is, co-occurring of two mutations (two chromosomes respectively carry a mutation) induces the relatively obvious clinical manifestations. This case we reported should belong to the compound heterozygous pathogenic: both Arg25Cys and Cys112Arg are pathogenic mutations. It's just a hypothesis due to the limited sample size. We have no evidence to conform that these mutations independently or together contribute to the pathogenesis of LPG. Further functional experiments need to be performed in the future. For example, site directed mutagenesis of apoE gene in mice can be a prospective way to verify the suspicious etiological factor of LPG.

However, it's notable that, although a total 13 apoE mutations have been identified as the causes of LPG, there are still some conflicting views. Chen et al [17] examined the 5.5 kb genomic DNA encompassing the entire apo E locus and adjoining flanking regions in 17 Chinese LPG patients. Their results suggested that there were no apoE gene mutations present in these LPG patients, including previously reported apoE mutations associated with LPG. Therefore, apart from apoE mutation, other genetic and/or epigenetic factors might also be considered to be involved in the pathogenesis of LPG. So apoE gene mutation might not the only cause of LPG.

No specific treatment for LPG has been established yet. Many therapeutic trials including glucocorticoids, immunosuppressants, anti-platelet drugs, fibrinolytics, ACEI and ARB have been performed. However, few trials have shown favorable effects. Most of LPG patients show abnormal metabolism of lipid, such as hypertriglyceridemia and increase level of serum apoE, so lipid-lowering therapy was often used as the primary treatment on LPG. Hydroxymethyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors Statin was reported ineffective against proteinuria [18]. Probucol might be useful for early-stage LPG, but not effective in patients with nephrotic LPG [19]. Now there are many reports showing the effect of fibrates for LPG. Hagiwara [20] and Arai [21] found the use of bezafibrate was effective on two LPG cases, even with normolipidaemia and normal ApoE level. Ieiri [22] reported that the combination of four lipidlowering agents, fenofibrate, nicositol, ethyl-icosapentate and probucol, markedly decreased proteinuria in a LPG patient with NS. Intensive lipid-lowering therapy with bezafibrate and ethyl-icosapentate was also reported to be effective in LPG patients by Kinomura [18]. Taken together, these findings suggest that early intervention by intensive lipid-lowering therapy including fibrates could decrease proteinuria and prevent progressive renal failure as well as NS in the present type of LPG. This patient was also treated with fibrates after DFPP treatment, and no progression of disease was found in the 3-month follow-up time.

Apart from pharmacotherapy, blood purification treatment also can be used on LPG, and immunoadsorption is the commonest choice. Zhang reported 13 cases of LPG which were successfully treated by staphylococcal protein A immunoadsorption, and no severe complications were found [23]. DFPP is a modified form of plasma exchange (PE), which removes macromolecules by a semi-selective method. DFPP has the advantages of a reduced need of supplement solutions and an increased volume of plasma processed resulting in higher removal of pathogenic antibodies [24]. No DFPP therapy was reported on LPG. We performed DFPP on this patients three times. It's surprising that just a single DFPP dramatically decreased the nephrotic range proteinuria to normal. It is thus suggested that DFPP could effectively eliminate the lipoprotein thrombi in the glomerular capillaries. Obviously, DFPP is not an etiological treatment, and lipoprotein thrombi will deposit in the kidneys again (the remission period depends on the speed of abnormal lipid synthesis). So far there is no effective etiological therapy. In other hand, like in other renal diseases, proteinuria should be a risk factor hastening the progression of LPG. So DFPP is a rational choice to decrease proteinuria and delay the progression of end-stage renal disease (ESRD) in LPG patients. Repeated DFPP might be an acceptable treatment during the relapse of proteinuria. Clinical results from this patient reveal that the short-time effect of DFPP is dramatic, but the long-time effect is still unclear and need to be followed over time. Intensive lipid-lowering therapy is suggested to prevent the deposit of lipoprotein thrombi in the remission stage after DFPP treatment.

Conclusion

We reported a new Chinese LPG case and found two missense mutations (apoE Kyoto and Cys112Arg) in the apoE gene. These mutations together might trigger the initiation and progression of LPG. This hypothesis should be investigated by further functional experiments. Furthermore, we demonstrated, for the first time, that DFPP was a highly effective therapy on LPG, at least in short term. Intensive lipid-lowering therapy and repeated DFPP treatment should be a prospective way to decrease the high risk of ESRD induced by multiple proteinuria in LPG patients.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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