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Clinical and pathologic features of focal segmental glomerulosclerosis with mitochondrial tRNA$^{Leu(UUR)}$ gene mutation

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Background. Several families have been described in which an A to G transition mutation at position 3243 (A3243G) of the mitochondrial DNA (mtDNA) is associated with focal and segmental glomerulosclerosis (FSGS). However, the prevalence, clinical features, and pathophysiology of FSGS carrying mtDNA mutations are largely undefined.

Methods. Among 11 biopsy-proven primary FSGS patients of unknown etiology, we examined seven FSGS patients to determine whether any of the clinical and pathological features of FSGS were associated with an A3243G mtDNA mutation. In four subjects in whom the A3243G mtDNA mutation was discovered in blood leukocytes, as well as in urine sediments, we retrospectively reviewed the medical records and re-evaluated the renal biopsy specimen using light and electron microscopy. We further screened the patient's family members for the presence and degree of heteroplasmy for this mtDNA mutation and obtained medical histories that were consistent with mitochondrial cytopathy.

Results. The four individuals identified with the A3243G mtDNA mutation were female. Proteinuria was diagnosed in these individuals during a routine annual health checkup in their teenage years. None of the patients showed any symptoms related to mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episode, whereas diabetes mellitus in two of the patients and a hearing disturbance in one patient became manifest within a 3- to 13-year follow-up period. Strict maternal transmitted inheritance was confirmed by pedigree studies in all of these patients. Steroid therapy was ineffective in all four patients. In two of these patients, renal function declined slowly to end-stage renal failure. Histologic examination of biopsy specimens revealed that glomeruli were not hypertrophied, while electron microscopic examination identified severely damaged, multinucleated podocytes containing extremely dysmorphic abnormal mitochondria in all patients.

Conclusions. FSGS may belong to the spectrum of renal involvement in A3243G mtDNA mutation in humans. Severely injured podocytic changes containing abnormal mitochondria may explain the pathogenesis of FSGS in association with the A3243G mtDNA mutation.

Key words: DNA mutation, A3243G transition mutation, podocyte, MELAS, proteinuria, kidney glomeruli, FSGS.

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An A to G transition at position 3243 in mitochondrial DNA (mtDNA) has been described mainly in association with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) or progressive external ophthalmoplegia (PEO) [1]. Although the central nervous system and striated muscle seem to be especially vulnerable in these syndromes, patients may also have atypical presentations suggestive of an involvement of other organs or tissues. Diabetes mellitus, hearing loss, and cardiomyopathy are well known as uncommon manifestations of the A3243G mutation [1, 2]. Renal tubular dysfunctions, including de-Toni-Debré-Fanconi syndrome, or renal tubular acidosis, have also been described as rare symptoms in late stages of MELAS [3].

Recently, focal and segmental glomerulosclerosis (FSGS) has been attracting attention as an unusual phenotype of mitochondrial cytopathy carrying the A3243G mutation. In some patients, FSGS was eventually found during a follow-up period for MELAS [4], whereas in other patients, FSGS was diagnosed even before characteristic signs and symptoms of MELAS became apparent [3]. On the other hand, Jansen et al have described four cases of progressive nondiabetic kidney disease in association with the A3243G mutation, in which none of these patients expressed MELAS or PEO during a follow-up period for over 5 to 17 years. In addition, biopsy-proven FSGS was confirmed in two of these patients [5]. Kurogouchi et al have also reported a case of mitochondrial cytopathy manifested as FSGS, but not as MELAS [6].

All of these reports suggest that FSGS may be a new phenotypical expression in the broad clinical spectrum associated with the A3243G mtDNA mutation.

However, hitherto, the pathological changes in kidney glomeruli in FSGS patients carrying the A3243G mtDNA mutation have not been studied as in much detail as the mtDNA mutation in the striated muscle and the central nervous system of MELAS patients. Both the prevalence and the clinical features of this putative atypical phenotype of mitochondrial cytopathy remain to be clarified. The present study reviews our experience in diagnosing four FSGS patients possessing the A3243G mtDNA mutation and their treatment period of 3 to 15 years. We have also studied the pathological changes of the affected kidneys in these patients in detail, and found severely damaged podocytic changes containing extraordinary dysmorphic mitochondria, which may participate in the development of FSGS.

METHODS

Patients and histologic studies

Between 1978 and 1997, out of 4227 renal biopsied patients, 68 patients were histopathologically diagnosed with FSGS, based on the criteria of Churg, Habib, and White [7]. Among these patients, 46 patients were nephrotic. Out of the 22 non-nephrotic patients, four patients were diagnosed with secondary FSGS caused by hypertensive nephrosclerosis. Four patients were diagnosed with reflux nephropathy, 2 patients were massively obese, and 2 patients had hypoplastic kidneys. The other 10 patients in this FSGS group were diagnosed pathologically with FSGS without nephrotic syndrome of unknown etiology. Since 7 out of these 10 patients kept visiting our out-patient clinic, we performed mtDNA mutation analyses in these patients after obtaining informed consent. Diseases known to be associated with FSGS, including sickle cell disease, human immunodeficiency virus infection, intravenous drug use, and urinary tract obstruction, were excluded in all seven patients. In addition, we screened several types of mtDNA mutations and identified an A3243G mtDNA mutation in four of these patients. We retrospectively reviewed the medical records of these four patients and re-examined in detail the renal biopsy specimens using light and electron microscopy, as described [7].

The histologic diagnosis of FSGS by kidney biopsy was based on the presence of areas of glomerular scarring and tuft collapse that were both focal (involving only a subpopulation of glomeruli) and segmental (sparing portions of involved glomeruli). Possible underlying causes of segmental glomerulonephritis [for example, immune complex deposition, antiglomerular basement membrane (anti-GBM) antibodies and vasculitis] and other potential causes of segmental glomerular pathology (for example, Alport's syndrome) were all ruled out to the fullest extent possible based on both clinical and histologic findings. Ancillary factors supporting the diagnosis of FSGS, including the detection of focal, segmental glomerular staining for immunoglobulin M, and/or C_3 by immunofluorescence microscopy, were confirmed. Glomerular volume was determined on light microscopic sections, as described previously [8]. Markedly deformed or globally sclerotic glomeruli were not included in the calculation.

Molecular studies

DNA was isolated from peripheral blood leukocytes, as well as from urine sediments obtained from 3 to 50 mL of voided urine, using a commercially available DNA isolation kit (Sepa Gene®; Sanko Pharmaceutical Co., Tokyo, Japan). Six types of mtDNA mutations, including A3243G, T3250C, A3252G, C3256G, T8993C, and T8993G [9], were screened in the extracted DNA samples. Polymerase chain reaction (PCR) amplification of the mtDNA fragments encompassing the 3243 point mutation was performed using the following two oligonucleotide primers: 5′-AGGACAAGAGAAATAAGGCC-3′ (3130 to 3149) and 5′-AAGAGCGATGGTGAGAGCTA-3′ (3555 to 3536). Cycling conditions applied for amplification were 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, for a total of 30 cycles. Approximately 1 μg of the PCR product was digested with 54 units of Apa I for 24 hours, and restriction fragments were separated by 3% agarose gel electrophoresis, followed by visualization with double-stranded DNA-specific dye, SYBR Green I (Molecular Probes, Eugene, OR, USA). With this method, the recognition site for the restriction endonuclease Apa I detected the A→G mutation at position 3243 of the mtDNA. Quantitative analysis of the mutated and wild-type mtDNA was done using an isotope-labeled primer, as previously described [10]. A good correlation was observed between the degree of heteroplasmy obtained with this method and that with the quantitation of the Apa I generated restriction fragments stained by SYBR Green I [11] using a fluoroimage analyzer (FLA 2000; $r = 0.90$, $P < 0.05$; Fujifilm, Tokyo, Japan).

Data analysis

Data were presented as mean ± SD and were analyzed by an unpaired Student t test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Identification of the mutation

Four FSGS patients had an A3243G mutation in the mtDNA obtained from both peripheral blood leukocytes and urinary sediments. The other three patients did not possess any of the mitochondrial mutations, in-

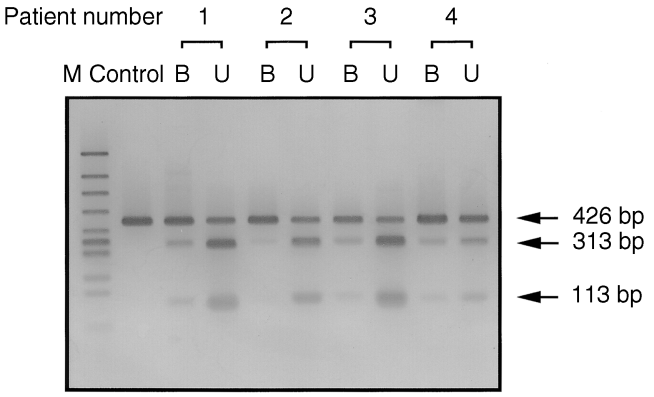

Fig. 1. Identification of an A to G mutation at position 3243 in mitochondrial DNA. DNA samples were isolated from peripheral blood leukocytes (B) and from urine sediments (U). Fragments of mitochondrial DNA encompassing position 3243 were obtained by PCR, digested with Apa I, separated using agarose gel (3%) electrophoresis, and stained with SYBR Green I. Affected subjects are heteroplasmic for the mutation. Urine sediment of normal adult was analyzed as control. M, molecular weight marker.

cluding A3243G, T3250C, A3252G, C3256G, T8993C, and T8993G [9]. Figure 1 shows the restriction fragment length polymorphism analysis of the digested PCR-amplified mtDNA fragment with Apa I, demonstrating that these subjects are heteroplasmic for this mutation. The mitochondrial mutation was also examined in some of the family members of these four patients, demonstrating that FSGS was cosegregated with the mutation in a fashion consistent with maternal transmission. In all of the subjects tested, the prevalence of this mtDNA mutation was higher in urine sediments than in peripheral blood leukocytes (Fig. 2). Moreover, we analyzed mtDNA mutations using urine sediments in 25 patients out of 46 nephrotic FSGS patients. In this comparative study, we did not find any type of mtDNA mutations, including A3243G.

Clinical history

The pedigrees of four patients within three families are shown in Figure 2. The clinical characteristics of patients with FSGS carrying the A3243G mutation are summarized in Table 1. All four patients were female and included two sisters (patients 2 and 3). All showed ambiguous onset of the disease since asymptomatic proteinuria was first detected during a routine annual health checkup when they were between 10 and 17 years old. Microhematuria was absent in all four patients. The mothers of patients 2, 3, and 4 had diabetes mellitus. The mother of patient 4 was also suffering from hearing disturbance, and a female cousin had chronic renal failure, diabetes mellitus, and hearing disturbance. The older brother of patient 1 had also been diagnosed with diabetes mellitus. At 24 years of age, nine years after patient 4 was found to have proteinuria, she noticed hearing disturbance. None of the patients nor family members showed stroke-like episodes or myopathy during the follow-up period. All four patients were rather short in stature for Japanese women (-2.98 to -0.38 SD of mean value),

At the time of renal biopsy, all patients showed normal blood pressure. None of the patients exhibited nephrotic syndrome, and the 24-hour urinary protein excretion ranged from 1.0 to 1.6 g. Urinary β_2-microglobulin (β_2m) concentration, which was examined in three patients, was within normal levels (Table 1). Renal function, defined by creatinine clearance level, was mildly impaired in all patients except patient 2. During a follow-up period, patients 2 and 4 showed a decline in renal function. At 35 years of age, 20 years after the onset of nephropathy, patient 4 reached end-stage renal failure and required regular dialysis treatment.

At the time of renal biopsy, none of the patients had overt diabetes mellitus, although a 75 g oral glucose tolerance test (75 g OGTT) was impaired in patients 2 and 4. Predonisolone steroid therapy was ineffective in all four patients. In addition, patient 4 developed overt diabetes mellitus following steroid therapy; thereafter, she needed insulin injections to maintain her blood glucose levels. Five years following the diagnosis of FSGS, patient 2 developed overt diabetes mellitus in the eighth month of her first pregnancy, but she had a full-term delivery without any complications. The other two patients (patients 1 and 3) did not develop overt diabetes mellitus during the follow-up period.

Histologic examination of renal tissues

All biopsy specimens were obtained by surgical needle biopsy. In patient 3, renal biopsies were performed twice in the nine-year follow-up period. The histologic changes are tabulated in Table 2. On light microscopy, the histology findings were compatible with FSGS [7] (Fig. 3), but at the time of diagnosis, all of the patients had only mild histologic changes: They showed between 0 and 20.0% obsolute glomeruli, 4.3 to 7.1% segmental sclerosis, only focal tubular interstitial atrophy or fibrosis, and no vascular changes. In the second biopsy of patient 3, the proportion of globally sclerotic glomeruli and segmentally sclerotic glomeruli increased to 53.3 and 20.0%, respectively, as did the interstitial fibrotic area (Table 2).

Then, the values of mean glomerular volume (MGV) in patients diagnosed with the FSGS carrying the A3243G mtDNA mutation were compared with MGV values of other FSGS patients. As shown in Figure 4, mean MGV in FSGS patients carrying the mtDNA mutation was $3.45 \pm 0.44 \times 10^6$ μm^3 ($N = 5$), which is not significantly different from those of the control population of minor glomerular abnormalities [8]. On the other hand, the MGV values were significantly increased in

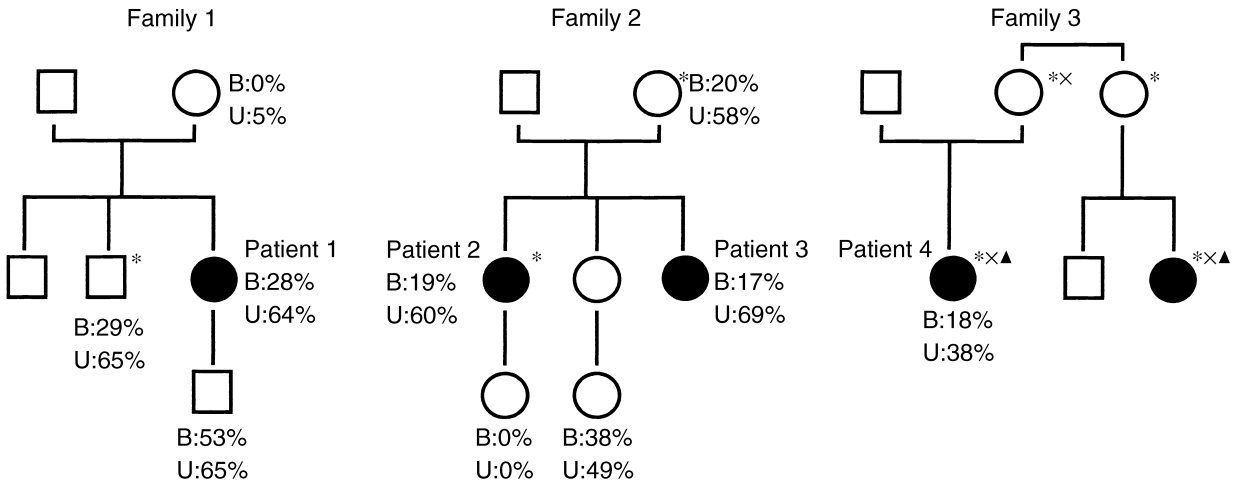

Fig. 2. Family trees of four patients diagnosed with focal segmental glomerulosclerosis (FSGS) and carrying the A3243G mutation. Percentages represent the population of mitochondrial mutation detected by restriction fragment length polymorphism analyses of the digested PCR-amplified mtDNA fragment with Apa I in peripheral blood leukocytes (B) and in urinary sediments (U). Symbols are: (●) proteinuria positive; (*) diabetes mellitus; (×) hearing disturbance; (▲) hemodialysis.

primary nephrotic FSGS ($4.96 \pm 0.84 \times 10^6$ μm^3, $N = 11$, $P < 0.01$) or secondary FSGS patients ($12.13 \pm 3.63 \times 10^6$ μm^3, $N = 6$, $P < 0.001$) compared with those in FSGS patients carrying the mtDNA mutation.

Each biopsy specimen was examined further in detail, and we found that glomerular podocytes were in the center of the pathological lesions. The podocytes were damaged (some of them severely). Typical podocytic changes consisted of cell body attenuation together with pseudocyst formation and foot process effacement (Fig. 5). Binucleated or multinucleated podocytes generally exhibited severe lesions in all four patients (Figs. 5–7A). Within the cytoplasm of the podocytes, a marked increase in the number of mitochondria with striking features were observed, including variation in size and shape, irregular outline, increase of cristae, and lamellar structures. Swollen mitochondria often lost the arrangement of cristae (Fig. 7B). Paracrystalline inclusions within mitochondria were not found. It should be noted that the degree of accumulation of abnormal mitochondria varied among the glomeruli and podocytes, and that these mitochondrial abnormalities were virtually absent in mesangial cells as well as in capillary endothelial cells. Changes in the GBM, including thickening and thinning of GBM, lamellation, and fragmentation of the lamina densa, suggesting Alport's syndrome, were absent in all cases. On the other hand, tubular epithelial cells showed an accumulation of abnormal mitochondria in both proximal and distal tubules (Fig. 8).

DISCUSSION

Our study examined four patients who were first diagnosed with FSGS, and thereafter a heteroplasmic A3243G mtDNA mutation was identified in peripheral blood leukocytes as well as in urine sediments. In these patients, typical manifestations of the MELAS syndrome were absent. Instead, diabetes mellitus in two patients and hearing disturbance in one patient became manifest during routine follow-up periods, which is compatible with mitochondrial cytopathy. The clinical features in these patients share several common characteristics with those described by Jansen et al, in which the authors describe four patients harboring the A3243G mtDNA mutation and manifesting FSGS with occasionally complicated hearing disturbance and diabetes mellitus, but not MELAS [5]. The similarities between our present study and that of Jansen et al include the following: (*1*) the onset of proteinuria is ambiguous at a young age (mean age of 16 years in the present report vs. 26 years in Jansen's report); (*2*) long-standing hypertension is absent in the early stages of the disease; (*3*) only hearing disturbance and diabetes mellitus are symptoms consistent with mitochondrial cytopathy; and (*4*) none of the probands nor their family members have had episodes of MELAS syndrome. Considering these similarities, it is tempting to suggest that the phenotypical expression of FSGS, which can be complicated by hearing disturbance and diabetes mellitus, may be classified as a new subtype of mitochondrial cytopathy harboring the A3243G mutation, as has been proposed by Jansen et al [5].

Pathological analyses of renal biopsy specimens in these four patients offer important insights into the mechanism of the development of FSGS in patients carrying the A3243G mtDNA mutation. The podocytes seen in our cases were essentially similar to those reported in idiopathic FSGS and demonstrated cell body attenuation, pseudocyst formation, and foot process ef-

Table 1. Clinical features of focal segmental glomerulosclerosis (FSGS) patients at the time of renal biopsy and clinical outcome

Patient number	Height *cm* (SD)	Age at biopsy	Sex	Onset of proteinuria *year*	Blood pressure *mm Hg*	Proteinuria *g/day*	C_{Cr} *mL/min/1.73 m²*	Total protein *g/dL*	Total cholesterol *mg/dL*	75 g OGTT	U_{β_2m} *mg/L*	Duration of follow-up *years*	Outcome of kidney
1	146 (−2.38)	22	F	10	110/70	1.6	74.3	5.8	179	Normal	109	3	Stable
2	150 (−1.58)	32	F	17	125/67	1.2	70.7	6.5	209	NT	NT	6	Stable
3[a]	156 (−0.38)	18	F	14	110/70	1.0	89.0	6.6	216	IGT	NT	11	Renal function↓
		27			120/80	1.7	60.3	6.4	258	NT	130		
4	143 (−2.98)	25	F	15	136/85	1.5	61.2	6.2	200	IGT	170	13	Dialysis

Abbreviations are: NT, not tested; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test; C_{Cr}, creatinine clearance; U_{β_2m}, urinary β_2-microglobulin.

[a] Note, a renal biopsy was performed twice

facement [12]. Frequent occurrence of binucleated and multinucleated podocytes, the hallmark of severely damaged podocytes [13], was also observed (Figs. 5–7A). However, within the podocytes, accumulation of numerous dysmorphic mitochondria was prominent in FSGS patients with the A3243G mtDNA mutation (Fig. 7B). It should be noted that in these specimens, abnormal mitochondria were exclusively accumulated in podocytes, whereas both mesangial cells and capillary endothelial cells in glomeruli appeared intact. Since podocytes are highly differentiated terminal cells that do not undergo cell division in the postnatal period [14], it is suggested that podocytes may be susceptible to the accumulation of mutated mitochondria such as in neural and muscle cells. Then, because of the accumulation of these abnormal mitochondria, mitochondrial protein synthesis and energy supply may be impaired [15], leading to the induction of podocytic dysfunction followed by development of FSGS.

Of interest is the differences of MGV values in FSGS specimens with the A3243G mtDNA mutation in comparison with other types of FSGS specimens (Fig. 4). The MGV values obtained from patients diagnosed with FSGS containing the mtDNA mutation were close to those of the control population group [8], whereas primary nephrotic or secondary FSGS patients exhibited significantly greater MGV values. Recently, Fogo et al have suggested that glomerular hypertrophy can play a crucial role in the pathogenesis of human FSGS by demonstrating that glomerular size in patients with primary FSGS is significantly larger than that of cases with minimal lesion [16]. In light of Fogo's hypothesis, the absence of glomerular enlargement in patients of FSGS carrying the mtDNA mutation and given that MGV did not change significantly during the clinical and histologic progression of FSGS in patient 3 suggests that at least glomerular hypertrophy may not be involved in the pathogenetic mechanisms in developing or progressing mitochondrial cytopathic FSGS.

Our diagnostic setup, consisting of DNA samples prepared from the sediments of voided urine followed by the detection of the A3243G mutation using PCR techniques, is an effective way to identify the mutation of mtDNA. In our study of all of the FSGS patients harboring the A3243G mutation, the proportions of mutant DNA in urine sediments were higher than those in peripheral blood leukocytes. We even experienced an individual in whom peripheral blood leukocytes did not yield evidence of the mtDNA mutation, although the mutation was detectable in urine sediments (the mother of patient 1). The reason for this higher ratio of mutant-to-normal type mtDNA in urine sediments has not been fully analyzed. However, urine sediments contain twice as many renal tubular epithelial cells than leukocytes [17]. As shown in the present study, tubular epithelial cells tend

Table 2. Histopathological findings

Patient number	Number of glomeruli	Global sclerosis	Segmental sclerosis	Tubulointerstitial changes	Vascular changes
1	14	0 (0%)	1 (7.1%)	Focal	—
2	20	0 (0%)	1 (5.0%)	Focal	—
3[a]	23	3 (13.0%)	1 (4.3%)	Focal	—
	15	8 (53.3%)	3 (20.0%)	Diffuse	—
4	20	4 (20.0%)	1 (5.0%)	Focal	—

[a]Note, histopathological findings were examined twice

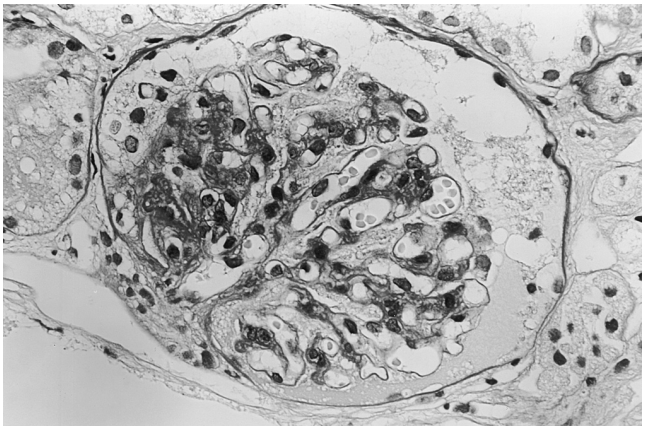

Fig. 3. Light microscopic view of a kidney biopsy specimen obtained from patient 4 revealing a segmentally sclerotic lesion (×400).

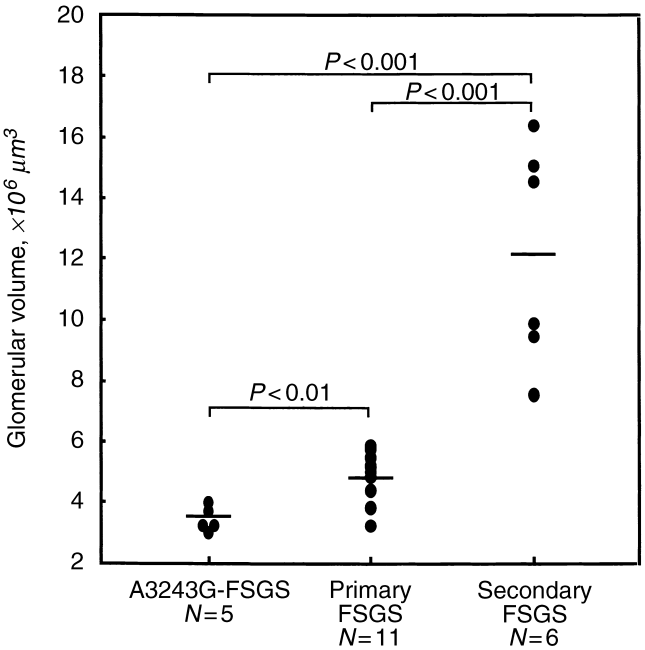

Fig. 4. Mean glomerular volume (MGV) in FSGS patients carrying the A3243G mtDNA mutation compared with those patients with primary FSGS or secondary FSGS. The horizontal bars indicate the median values. In patients with FSGS harboring the A3243G mtDNA mutation (A3243G-FSGS), the values were calculated using five biopsy specimens obtained from four patients. Primary FSGS patients were all nephrotic ($N = 11$), whereas patients with secondary FSGS ($N = 6$) were diagnosed with hypoplastic kidney ($N = 2$), reflux nephropathy ($N = 3$), and massive obesity ($N = 1$), respectively.

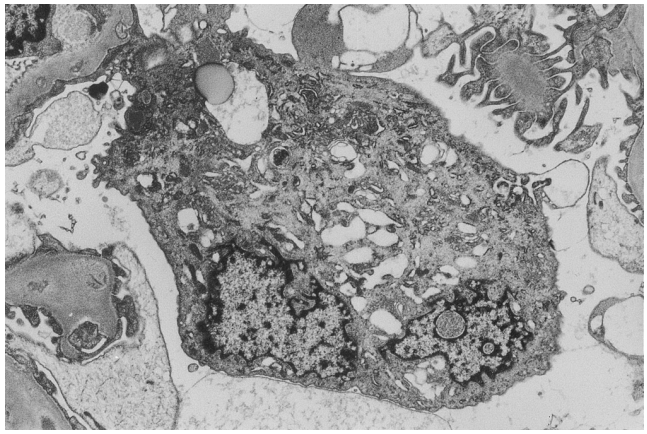

Fig. 5. Electron microscopic view of a podocyte in kidney tissue obtained from patient 1. Binucleated podocyte with vacuolar degeneration and widespread foot process effacement can be seen in this specimen (×5000).

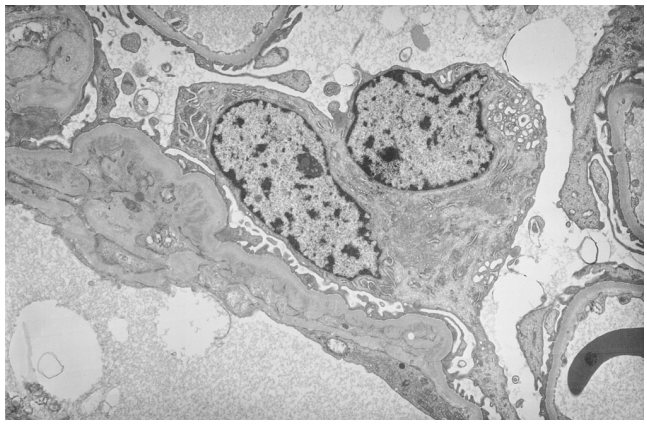

Fig. 6. Electron microscopic view of a podocyte in kidney tissue obtained from patient 2. Binucleated podocyte with dark staining absorption droplets can be seen in this specimen (×4000).

to accumulate abnormal mitochondria (Fig. 8), whereas heteroplasmic leukocytes possessing a high mitotic index tend to be selected over the course of many generations of cell division [18], suggesting that the tubular epithelial cell component of urine sediments may contribute to increases in the amount of mutated mtDNA in urine sediments. Although podocytes have recently been iden-

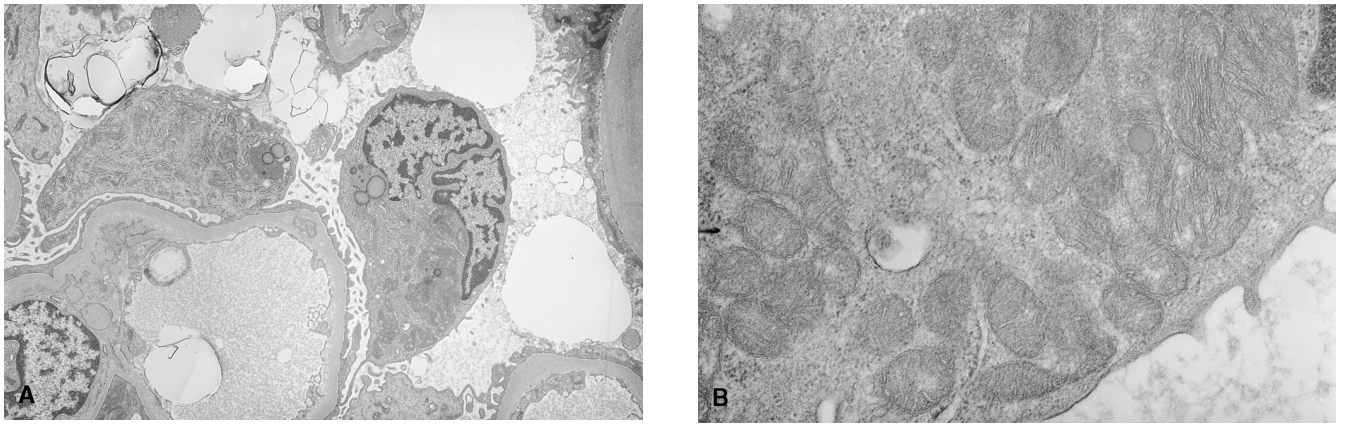

Fig. 7. Electron microscopic view of podocytes in renal glomerulus in patient 2. Binucleated podocyte with foot process reaching to the GBM is observed in this specimen (×4000; *A*). Higher magnification of the abnormal podocyte reveals a markedly increased number of abnormal mitochondria of varied sizes and shapes. Swollen mitochondria show disarrangement of cristae (×30,000; *B*).

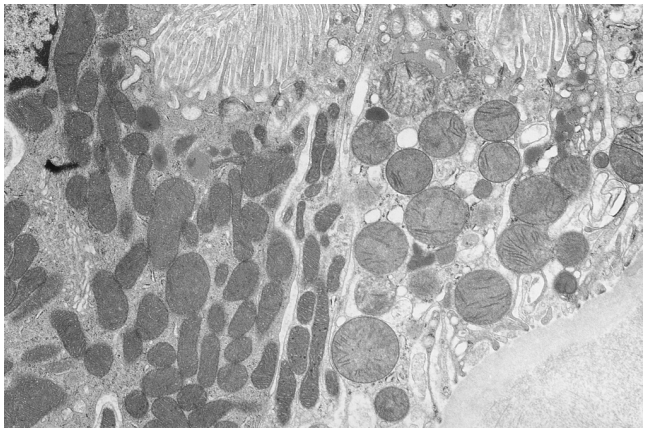

Fig. 8. Electron microscopic view of renal tubular epithelial cells in patient 2. Proximal tubular epithelial cells with microvilli had increased the number of dysmorphic mitochondria containing disoriented cristae. Note that abnormal mitochondria are prominent on the right side of the cell (×8000).

tified in human urine [19], it seems unlikely that the small amount of epithelial cells will contribute largely to the values of heteroplasmy. Taken together, it is reasonable to conclude that the detection of mutated mtDNA using urine sediments is an extremely useful method to detect mutated mitochondria. However, it is noteworthy that the ratio of mutant-to-normal mtDNA in urine sediments seems unlikely to correlate with the severity of FSGS. Patient 4 experienced severe renal failure but demonstrated the lowest ratio of mutated mtDNA in urine sediments among the patients tested, whereas the son of patient 1 had no symptoms of FSGS but showed a high percentage of mutated mtDNA in urine sediments (Fig. 2). Thus, given the discrepancy between the clinical features and the ratio of mutant-to-normal mtDNA in urine sediments, it is clear that the ratio of mutated mtDNA detected in urine sediments cannot predict the onset nor the progression of FSGS.

In conclusion, we reviewed the clinical features of four cases of familial FSGS harboring the A3243G mtDNA mutation and demonstrated that non-nephrotic FSGS is the characteristic feature at least in the early phase of the disease. We also suggested the causative role of the accumulation of abnormal mitochondria in podocytes. The overall incidence of A3243G mtDNA mutation in patients with asymptomatic proteinuria or in nephrotic FSGS patients is unknown. Since individuals with A3243G mtDNA mutation are often asymptomatic or oligosymptomatic, it can be assumed that the underlying mitochondrial cytopathy is present in a certain population of individuals with asymptomatic proteinuria as well as of familial or sporadic FSGS. We suggest that the recognition of this mitochondrial mutation will be clinically important in FSGS patients in order to avoid useless administration of steroids, which may potentially accelerate the onset of diabetes mellitus.

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APPENDIX

Abbreviations used in this article are: FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; Leu, leucine; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; mtDNA, mitochondrial deoxyribonucleic acid; PCR, polymerase chain reaction; PEO, progressive external ophthalmolplegia; RFLP, restriction fragment length polymorphism; SD, standard deviation.

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