Mitochondrial tRNA\textsuperscript{Leu(UUR)} mutation in a patient with steroid-resistant nephrotic syndrome and focal segmental glomerulosclerosis

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Abstract

Background. The heterogeneity of mitochondrial cytopathies is characteristic for this group of disorders, which preferentially affect the muscle and nerve system. The A3243G transition in the tRNA\textsuperscript{Leu(UUR)} gene has been associated with slowly progressive forms of focal segmental glomerulosclerosis (FSGS). Here we present a patient who developed a severe nephrotic syndrome during her first pregnancy, which persisted after delivery, and proved resistant to immunosuppressive therapy. A sister of our patient had developed diabetes mellitus. We analysed the DNA for the presence of the mitochondrial DNA (mtDNA) A3243G transition.

Methods. DNA was isolated from peripheral blood leukocytes and urine sediments. Polymerase chain reaction was performed to amplify the mtDNA. Restriction enzyme analysis was used to detect the presence of the A3243G transition. Quantitative analysis of the A3243G mutation was done using the pyrosequencing technique.

Results. Quantitative analysis revealed a proportion of mutated mtDNA of 30% in the leukocytes and 68% in the urine sediments of the proband. On further analysis, we also found the transition in the mother, the diabetic sister and the daughter of the proband.

Conclusion. MtDNA abnormalities can cause a steroid-resistant nephrotic syndrome, histologically characterized by FSGS. Physicians should be especially mindful of mitochondrial abnormalities when hearing loss, diabetes mellitus or neuromuscular disorders are present in the patient or family members.

Keywords: A3243G mtDNA transition; glomerulosclerosis; heteroplasmy; pregnancy; proteinuria

Introduction

Focal segmental glomerulosclerosis (FSGS) is a common cause of the nephrotic syndrome in adults. FSGS is a description of a histological lesion and not a disease entity. In recent years, much progress has been made in unravelling the pathogenesis of (various forms of) focal glomerulosclerosis. It has been demonstrated that mutations in podocytic proteins such as podocin and \(\alpha\)-actinin 4 are responsible for autosomal-recessive and autosomal-dominant forms of focal glomerulosclerosis [1]. Mutations in mtDNA have also been associated with focal glomerulosclerosis.

Although mitochondrial cytopathies preferentially affect the muscle and nervous system, this group of disorders is characterized by their phenotypic heterogeneity. This large variety in clinical symptoms is due mainly to the co-existence of wild-type and mutated mitochondrial DNA (heteroplasmy), unequally distributed between cells and organs [2].

A typical example of a mitochondrial cytopathy and its variety of symptoms is the A3243G transition in the tRNA\textsuperscript{Leu(UUR)} gene. \textit{In vitro} studies have shown that the A3243G transition is responsible for a mitochondrial respiratory chain defect and that it disturbs the protein synthesis in the mitochondrion [3].

This mitochondrial DNA (mtDNA) mutation is associated mainly with the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [4]. Occasionally, renal tubular dysfunction and FSGS have been associated with MELAS [5]. Several other clinical presentations...
associated with the A3243G mutation include chronic progressive external ophthalmoplegia (CPEO), diabetes mellitus, (cardio)myopathy, hearing loss and dystonia [6,7].

More recently, this mutation was associated with FSGS in patients who were not diagnosed as having a MELAS phenotype [8–15]. In most patients, the clinical picture was characterized by the absence of a nephrotic syndrome and slowly progressive renal failure.

We present a patient with a full blown, steroid-resistant nephrotic syndrome, histologically characterized by FSGS, with evidence of abnormal mitochondria in the podocyte and the detection of mutated mtDNA in urine and blood.

Methods

Molecular genetic study

DNA was isolated from peripheral blood leukocytes using a salting-out method. Also, urine sediments were used to isolate DNA. After centrifugation of the urine for 10 min at 3000 r.p.m., the pellet was washed with phosphate-buffered saline. DNA was extracted using a commercially available DNA isolation kit (Puregene™ DNA isolation kit; Gentra Systems, MN).

Screening for the A3243G mutation was performed by polymerase chain reaction (PCR) using the following primers: 5'-CCACCTAGTTATATAAACAC-3' and 5'-ATTAGAATGGGTACAATGAGGA-3'. The PCR conditions were 92°C for 30 s, 55°C for 30 s and 72°C for 60 s, for a total of 35 cycles leading to a PCR product of 162 bp. PCR products subsequently were digested overnight with ApaI restriction enzyme. The presence of the A3243G mutation results in an additional restriction site at bp 104 of the PCR product. The restriction fragments were analysed on a 1% agarose gel containing ethidium bromide to visualize the DNA.

Positive DNA samples were analysed quantitatively using Pyrosequencing™ technology (Pyrosequencing, Upsala, Sweden). Pyrosequencing was performed according to the protocol of the manufacturer. PCR of a mtDNA fragment containing the 3243 position was performed using the following primers: universal primer (biotinylated), 5'-GGGACACCGCTGATCTTTA-3'; forward primer, 5'-GACGGGACACCGCTGATCGTTTACAACTTAGTA-3'; reverse primer, 5'-ATTAGAATGGGTACAATGAGGA-3'. PCR was carried out in a 50 µl volume containing 0.02 µM forward primer, 0.2 µM reverse primer and 0.2 µM of the biotinylated universal primer. PCR conditions were 92°C for 30 s, 55°C for 30 s and 72°C for 30 s, for a total of 40 cycles. Single-stranded template DNA, which in the present assay is the forward strand of the fragment, was purified using streptavidin-coated Sepharose beads. The actual pyrosequencing was performed on the PSQ96 platform using sequence primer 5'-TATGCGATTACATGAGGA-3'.

In a pyrosequence reaction, the four different deoxy-nucleotide triphosphates (dNTPs) are added separately one after the other. The incorporation of dNTP is accompanied by release of pyrophosphate (PPi). This PPi is involved in a light-producing reaction of which the amount of light produced is proportional to the number of nucleotides incorporated (Figure 3C). The light is detected by a charge coupled device camera and seen as a peak in a pyrogram. Apyrase, a nucleotide-degrading enzyme, continuously degrades ATP involved in the light-producing reaction, and unincorporates dNTPs. This switches off light production and regenerates the reaction solution. Because the forward strand is used as template in the sequencing reaction, the change detected in the present assay concerns a T to C exchange. In fact, the amount of dTTP and dCTP incorporated at position 3243 during the sequencing reaction was determined in this way and from this the percentage of heteroplasmy was calculated.

Results

Clinical history of the proband

Our patient was referred to our out-patient clinic at the age of 31 years because of a nephrotic syndrome that persisted after pregnancy. Dipstick-positive proteinuria was noted in the first trimester of pregnancy. At the 27th week, she developed pre-eclampsia and a Caesarean section was performed because of fetal distress. Six weeks after pregnancy termination, she visited the out-patient clinic.

Her medication consisted of enalapril 5 mg twice daily, and bumetanide 1 mg twice daily. Her medical history was unremarkable. The family history was negative for renal diseases, deafness or diabetes. At physical examination, blood pressure was 132/80 mmHg. There was moderate oedema. Abdominal ultrasound revealed two normal sized kidneys. Laboratory investigation revealed a nephrotic syndrome and normal renal function: serum creatinine 73 µmol/l, serum albumin 26 g/l and serum cholesterol 11.3 mmol/l. Proteinuria ranged from 8.2 to 15.8 g/10 mmol creatinine, and urinary cholesterol 11.3 mmol/l. Proteinuria was noted from the first trimester of pregnancy that persisted after pregnancy. Dipstick-positive proteinuria was noted in the first trimester of pregnancy.

The selectivity index calculated as clearance of IgG/clearance of transferrin was 0.19. Estimated creatinine clearance according to the Cockcroft and Gault formula was 101 ml/min. The urinary sediment revealed only sporadic erythrocytes, and many oval fat bodies.

Because of the persistence of the nephrotic syndrome, a renal biopsy was performed, ~6 months after pregnancy. On light microscopy, characteristic lesions of focal glomerulosclerosis were present in five of eight glomeruli (Figure 1A). There was no evidence of mesangial cell proliferation. We observed focal tubulo-interstitial infiltrate and tubular atrophy. Arterioles and small arteries showed moderate hyalinosis, both subendotheli ally and in the media, in a spot-like distribution, consistent with loss of smooth muscle cells. On immunofluorescence, we observed focal segmental coarse granular deposits of IgM and C3 consistent with FSGS. In addition, there were granular depositions of IgA in the mesangium and to a lesser extent in the capillary walls. Electron microscopy showed diffuse podocytic foot process...
effacement (Figure 1B). The endothelium was often swollen and vacuolated. Paramesangial deposits were noted. In view of the clinical presentation and histological picture, we favoured a diagnosis of idiopathic focal glomerulosclerosis occurring in a patient with IgA deposits of undetermined significance (see Discussion).

Treatment consisted of enalapril 20 mg, losartan 25 mg, bumetanide 1–2 mg and atorvastatine 20 mg. Blood pressure was relatively well regulated, with values varying between 138/78 and 156/84 mmHg. Since proteinuria persisted at levels above 8 g per day, and renal function deteriorated with a rise in serum creatinine to levels of 112 μmol/l, immunosuppressive therapy was instituted. However, successive treatment with prednisone, cyclophosphamide and finally plasmapheresis were all unsuccessful (Figure 2).

Steroid treatment was complicated by the development of diabetes mellitus, which required oral antidiabetic therapy. At that time, the patient informed us that her sister had in the meantime developed insulin-dependent diabetes mellitus. Because of our interest in the genetics of steroid-resistant focal glomerulosclerosis, we performed an analysis for mtDNA mutations in DNA recovered from the blood and the urine.

Because of the abnormality found in the mtDNA, podocytes were visualized at greater magnification by electron microscopy. The pictures revealed abnormal mitochondria in the visceral epithelial cells (Figure 1C). At present, the patient is seen regularly at the outpatient clinic, with moderate proteinuria and slowly progressive renal failure (Figure 2). In view of the observed mitochondrial mutation (see below), the patient was referred to the oto-rhino-laryngologist who found a hearing loss of 35 dB. Echocardiography disclosed concentric left ventricular hypertrophy, with a left ventricular mass index of 140 g/m2 (normal <110 g/m2). There was no evidence of asymmetrical hypertrophy of the interventricular septum.

Fig. 1. Light and electron microscopic pictures of the renal biopsy taken from the proband. (A) Light microscopy, silver staining. Shown is a glomerulus with peri-hilar segmental sclerosis, hyalinosis and an adhesion, consistent with an FSGS lesion. Arterioles and small arteries show moderate hyalinosis, subendothelially and in the media, with a spot-like distribution, consistent with loss of smooth muscle fibres. (B) Electron microscopy, original magnification 3000×. Glomerular segment with extensive foot process effacement of the podocytes. Most podocytes showed condensation of the actin cytoskeleton and mild vacuolation of the cytoplasm. Multinucleated podocytes were not encountered. The endothelium was swollen and vacuolated. The GBM has normal structure and width. Electron-dense deposits were observed in the paramesangial regions (not shown). (C) Electron microscopy, original magnification 10000×. Mitochondria in podocytes show abnormal morphology. Cristae appear irregular and frequently a large central vacuole with irregular outline is present. There appears to be transformation of mitochondria into lysosome-like vesicles, suggesting degradation of mitochondria.

Fig. 2. Time course of serum creatinine (A) and proteinuria (B) in the proband. Because of a rise in serum creatinine and persistent proteinuria >8 g/day, treatment was instituted. Successive treatment with prednisone, cyclophosphamide and plasmapheresis (PF) as indicated was unsuccessful.
Molecular genetic study

We isolated DNA from peripheral blood leukocytes and urine sediment and tested the DNA for the presence of the A3243G transition in the tRNALeu(UUR) gene. We found the mutation in both DNA samples after digesting the PCR fragment with ApaI. Quantitative analysis revealed a proportion of mutated mtDNA of 30% in the leukocytes and 68% in the urine sediments.

We also tested DNA of the maternal relatives of the patient. The results are shown in Figure 3. The mother of the proband revealed no mutation in her leukocytes, but a positive proportion of 62% in the urine sediments. To our knowledge, the mother has no clinical symptoms related to the A3243G mutation. The sister of the proband, who developed insulin-dependent diabetes mellitus, was also found positive for both DNA samples. A proportion of 25% was found in the leukocytes and of 28% in her urine sediments. Finally the daughter of the proband, who has normal renal function and no proteinuria, revealed the mtDNA mutation in a proportion of 51% in her leukocytes and 52% in her urine sediments.

Discussion

To our knowledge, our case report is the first to document the development of FSGS and a severe steroid-resistant nephrotic syndrome in an adult patient with the mitochondrial A3243G transition. Mutated mtDNA amounted to 30% in leukocytes and 68% in cells derived from urine sediments. Abnormal mitochondria were present in the podocytes. The mutation was also found in the family members, notably in a sister with diabetes mellitus.

Admittedly, we must consider whether this patient had IgA nephropathy in view of the deposits observed by immunofluorescence and electron microscopy. However, we feel that in our patient the clinical presentation and histological data favour a diagnosis of primary FSGS co-occurring with the incidental finding of IgA deposits. Specifically, we did not observe mesangial proliferative lesions; the patient never had episodes of macroscopic or microscopic haematuria.

It is well known that IgA deposits of ++ to +++ intensity can be found incidentally in biopsies of otherwise healthy cadaveric donors (reported incidence 7–9%), and even in living donors without urine abnormalities. Furthermore, many patients have been reported who presented with a glomerular disease and unexpectedly displayed mesangial IgA deposits, the most characteristic being patients with minimal change disease [16]. In a recent study, 18 patients with FGS and IgA deposits were described, clearly demonstrating that these patients presented with a clinical picture and a prognosis that closely resembled that of patients with typical primary FSGS [17].

The interest of nephrologists in mitochondrial diseases has been fostered by publications concerning
the involvement of the kidney in mitochondrial cytopathies [5,8]. tRNA mutations affect multiple pathways and can be traced to the destabilization of structural features that destroy the native tRNA conformation required for protein synthesis efficiency, aminocyclation, post-transcriptional modification and processing. The A3243G mutation alters the A14 nucleotide that is highly conserved in tRNALeu. The mtDNA A3243G transition has been associated with the MELAS syndrome, and more recently with maternally inherited diabetes mellitus, hearing loss, cardiomyopathy and chronic progressive external ophthalmoplegia [6,7]. It has become evident that the mutation can also cause isolated renal disease in patients without signs of MELAS even after prolonged follow-up [8–15].

In case reports and small studies, >30 patients have been documented with the mtDNA A3243G transition and FSGS [8–15]. Clinically, most patients had non-nephrotic proteinuria (<3 g/day) and a progressive deterioration of renal function. In only two adult patients was proteinuria in the nephrotic range. One male patient was diagnosed at the age of 18 years with diabetes mellitus, which was very poorly controlled in the following years [13]. At the age of 23 years, he had developed a severe renal insufficiency (serum creatinine 522 μmol/l) and proteinuria of 3.5 g/day. Renal biopsy disclosed numerous globally sclerotic glomeruli, an increase of mesangial matrix, thickened glomerular basement membrane on electron microscopy, and no foot process fusion. Also, abnormal mitochondria were not found in the podocytes. These data strongly suggest that diabetic nephropathy was a major cause of the renal disease. The second patient was a 50-year-old female, with a 4 year history of diabetes, who was referred with severe renal failure (creatinine clearance 15 ml/min), polycystic kidneys and a proteinuria of 3 g/day, below the nephrotic range [12].

Although our patient was not aware of any hearing loss, an abnormal audiogram was noted. Hearing loss was also frequently noted in the described patients and, in most, hearing loss preceded the development or discovery of proteinuria [11,12,14]. Since hearing loss was often familial, it is not a surprise that many patients had been diagnosed with Alport’s syndrome [8,9]. Diabetes mellitus was also a frequent finding; however, in most patients, diabetes mellitus became manifest only during follow-up, after the discovery of proteinuria or renal disease [10,12,15].

Information on renal biopsy findings is somewhat limited. In the available biopsies there was evidence of FSGS with various percentages of globally sclerotic glomeruli [9–14]. In isolated cases, hyalinosis of the arterioles with evidence of smooth muscle cell necrosis and the presence of multinucleated podocytes has been reported [10,15]. Most characteristic was the finding of abnormal mitochondria in podocytes and tubular cells, reported in eight out of 10 patients studied by electron microscopy [9–13].

Our patient disclosed the typical lesions of FSGS, and abnormal mitochondria were observed in the podocytes, although we must admit that these abnormalities were identified only when we specifically looked at higher magnification after discovery of the mtDNA mutation. We did not observe multinucleated podocytes. Notably, as described by others, we also observed some abnormalities of the vessel wall, with hyalinosis in a spot-like pattern, consistent with loss of smooth muscle cells [10,15]. These findings of vessel wall abnormalities have not been emphasized thus far; however, they might suggest that dysfunction of the mitochondria in the vascular endothelial or smooth muscle cells could result in early vascular damage.

We analysed the presence of the A3243G transition in blood leukocytes and urine sediments. Patients harbouring this mutation usually have higher percentages of mutated mtDNA in muscle or urine sediments than in cells derived from rapidly dividing tissues such as bone marrow. Since urine sediments contain podocytes but also tubular epithelial cells and cells of the lower urinary tract, the measured heteroplasmic load does not represent the actual load in the podocytes.

Chinnery et al. found no relationship between the level of A3243G in blood and the frequencies of any of the clinical features they assessed, such as recurrent stroke-like episodes, CPEO, diabetes mellitus, pigmentary retinopathy, deafness, dementia, ataxia and myopathy [7]. In contrast, they found a correlation between the levels found in muscle and certain clinical features, particularly if the amount of mutated mtDNA exceeded the 15% level. For example, the observed frequency of recurrent strokes rose from zero in individuals with 21–60% A3243G in muscle to 94% for individuals with 91–100% A3243G in muscle.

Although the literature data are limited, it is notable that the majority of patients with renal diseases and an A3243G mutation are female. Furthermore, in the female patients, the disease was often more severe. This suggests that the phenotypic (renal) expression of this mitochondrial cytopathy is influenced by hormonal factors. However, the preponderance of females is as yet unexplained.

Should we screen all patients with primary FSGS for mitochondrial mutations? At present, the evidence in favour of such a strategy is lacking. In fact, two Japanese studies did not find the A3243G transition in 25 and 17 patients with primary non-familial FSGS, respectively [10,11]. Clinicians should be suspicious of patients with steroid-resistant FSGS who present with some particular features such as deafness, diabetes, neuromuscular symptoms, cardiomyopathy or a family history positive for any of these. In conclusion, the mtDNA A3243G transition can be associated with FSGS and severe nephrotic syndrome. Clinicians should be aware of the clinical heterogeneity of mitochondrial cytopathies, which will allow earlier detection of these disorders. It is advised that patients with mtDNA mutations be followed closely to detect the development of associated conditions such as diabetes mellitus and cardiomyopathy.
Acknowledgements. We would like to thank M. H. Siers, Department of Anthropogenetics, University Medical Centre Nijmegen, The Netherlands, and Professor L. A. Monnens and Dr E. N. Levchenko, Department of Pediatric Nephrology, University Medical Centre Nijmegen, The Netherlands, for their contribution in discussions, mutation analyses and providing DNA. This project was supported by a grant from the Dutch Kidney Foundation (project number C.98-1764).

Conflict of interest statement. None declared.

References


Received for publication: 20.4.04
Accepted in revised form: 22.9.04