J Hum Genet (2001) 46:330–334

ORIGINAL ARTICLE

Naoko Iwasaki · Tetsuya Babazono · Ken Tsuchiya
Osamu Tomonaga · Akitake Suzuki · Michiko Togashi
Noriko Ujihara · Yumiko Sakka · Hirohide Yokokawa
Makiko Ogata · Hiroshi Nihei · Yasuhiko Iwamoto

Prevalence of A-to-G mutation at nucleotide 3243 of the mitochondrial tRNA$^{Leu(UUR)}$ gene in Japanese patients with diabetes mellitus and end stage renal disease

Received: November 29, 2000 / Accepted: February 27, 2001

Abstract The A-to-G mutation at nucleotide 3243 of the mitochondrial *tRNA*$^{Leu(UUR)}$ gene (mt.3243A>G) is associated with both diabetes mellitus and myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Recently, this mutation was found in three diabetic subjects with progressive kidney disease, suggesting that it may be a contributing factor in the development of kidney disease in patients with diabetes. The aim of this study was to evaluate the contribution of this mutation to the development of end stage renal disease (ESRD) in patients with diabetes. The study group consisted of 135 patients with diabetes and ESRD. The control group consisted of 92 non-diabetic subjects with ESRD who were receiving hemodialysis. The mt.3243A>G mutation was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). We found the mt.3243A>G mutation in eight patients (8/135; 5.9%), all of whom were initially diagnosed with type II diabetes. Five of the eight patients were subsequently also diagnosed with MELAS. We did not find the mutation in any of the 92 non-diabetic subjects with ESRD. The prevalence of this mutation was 6.5-fold higher in patients with diabetes and ESRD than in those with diabetes alone (8/135 vs 5/550, respectively; $\chi^2 = 13.704$; $P = 0.0002$). The mt.3243A>G mutation may be a contributing genetic factor in the development of ESRD in Japanese patients with diabetes.

Key words Diabetes mellitus · Mitochondria · MELAS · End stage renal disease · Genetics · Nephropathy · Susceptibility gene · Hemodialysis

N. Iwasaki (✉) · T. Babazono · O. Tomonaga · M. Togashi · N. Ujihara · Y. Sakka · H. Yokokawa · M. Ogata · Y. Iwamoto
Diabetes Center, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
Tel. +81-3-3353-8111ext. 27113; Fax +81-3-3358-1941
e-mail: niwasaki@dmc.twmu.ac.jp

K. Tsuchiya · H. Nihei
Kidney Center, Tokyo Women's Medical University, Tokyo, Japan

A. Suzuki
Second Department of Histology, Tokyo Women's Medical University, Tokyo, Japan

Introduction

Mutations in the maternally inherited mitochondrial genome have been found to be associated with a subtype of diabetes mellitus designated maternally inherited diabetes and deafness (MIDD) (van den Ouweland et al. 1992). The most common mitochondrial mutation found in patients with diabetes is an A-to-G transition at nucleotide 3243 of the mitochondrial *tRNA*$^{Leu(UUR)}$ gene (mt.3243A>G). This mutation was found in approximately 0.9% of Japanese diabetic patients (Otabe et al. 1994). The mt.3243A>G mutation is also the cause of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (Ciafaloni et al. 1992) and has been found in patients with progressive kidney disease, diabetes, and deafness (Jansen et al. 1997). In addition, we have seen several diabetic patients with this mutation who showed evidence of proteinuria prior to the diagnosis of diabetes mellitus or diabetic retinopathy, suggesting that it may be a contributing factor in the development of kidney disease, in addition to diabetic nephropathy per se (N.I., unpublished observation). Based on these findings, we screened a group of diabetic subjects who had end stage renal disease (ESRD), as well as a group of patients with nondiabetic ESRD, for the mt.3243A>G mutation, in order determine the prevalence and contribution of this mutation to the development of kidney disease in Japanese patients.

Subjects and methods

Subjects

We studied two groups of subjects. The first group consisted of 135 unrelated Japanese patients (M/F, 91/44) with diabetes mellitus (type I/type II, 11/124) and ESRD. All had been treated for diabetes and had started dialysis treatment at the Diabetes Center of Tokyo Women's Medical University between January 1991 and December 1998. The

age at diagnosis of diabetes in this group was 36.2 ± 14.1 years (mean ± SD) and the duration of diabetes was 16.8 ± 7.6 years. The age at the start of dialysis treatment was 52.7 ± 12.7 years. The second group consisted of 92 patients with ESRD, but without diabetes, who were receiving hemodialysis treatment at the hemodialysis unit of the Department of Nephrology, Tokyo Women's Medical University. The clinical features of the diabetic subjects were obtained retrospectively through their medical records. This study was approved by the Institutional Review Board of Tokyo Women's Medical University and was carried out in accordance with the principles of the Declaration of Helsinki II. Informed consent was obtained from all subjects.

Detection of mt.3243A>G mutation

Genomic DNA was prepared from peripheral blood, using a standard procedure. The presence of the mt.3243A>G mutation was identified, using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. An 854-bp fragment of mitochondrial DNA was amplified, using PCR and primers 5′-CACTGTTCCTTAAATAGGG-3′ (nt 2603–2622), and 5′-AGCGAAGGGTTGTAGTAGCC-3′ (nt 3437–3456) in the presence of [α-^{32}P]-dCTP. PCR was performed in a 25-μl volume containing 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM dNTPs, 10 pmol of each primer, 0.2 U Taq DNA Polymerase (Amersham, Little Chalfont, UK), and 200 ng of genomic DNA. The PCR consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. A 1.0-μl aliquot of the reaction was digested in a 20-μl volume with 20 U of *Apa*I at 37°C for 2 h. The PCR products were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography. The mt.3243A>G mutation created a site for the enzyme *Apa*I and, with the primers described above, the A-allele gave a fragment of 854 bp and the G-allele, fragments of 641 and 213 bp.

The tissue distribution of the mt.3243A>G mutation was measured in DNA prepared from the postmortem tissues of patient 5 (see Table 2), who died at 47 years of age. The degree of heteroplasmy was assessed using an image analyzer.

Statistical analysis

Student's *t*-test and the χ^2 test were used for statistical comparisons, and $P < 0.05$ was considered significant.

Results

The mt.3243A>G mutation was found in heteroplasmic form in 8 of the 135 patients with diabetes and ESRD, but in none of the 92 patients with ESRD alone. Each of the subjects with the mt.3243A>G mutation had initially been diagnosed with type II diabetes. The clinical features of patients with ESRD and diabetes, with and without the mt.3243A>G mutation, are summarized in Table 1. The mean ages at the time of the study, at the diagnosis of diabetes, and at the start of dialysis treatment were significantly lower in the group of patients with the mutation. There were no significant differences between the two groups with regard to the duration of diabetes or the length of time between age at diagnosis of diabetes and start of dialysis treatment. The mean time interval between the diagnosis of diabetes and the start of dialysis in the eight patients with the mutation was 17.5 ± 7.3 years (range, 4–23 years). The subjects with the mutation were, on average, shorter and weighed less than those without the mutation.

The specific clinical features of the eight patients with the mt.3243A>G mutation are shown in Table 2. Proteinuria preceded the diagnosis of diabetes in two patients. Deafness was present in all eight patients. Five patients had undergone skeletal muscle biopsy to examine the cause of muscle atrophy that became prominent during the course of follow-up. Gomori-trichrome staining

Table 1. Clinical characteristics of Japanese patients with diabetes mellitus and ESRD with and without the mt.3243A>G mutation

	mt.3243A>G+	mt.3243A>G-	*P* Value
Number (M/F)	8 (5/3)	127 (86/41)	
Age at the time of the study (years)	48.0 ± 6.9	58.3 ± 12.8	0.027*
Age at diagnosis of diabetes (years)	26.3 ± 5.8	36.8 ± 14.2	0.04*
Duration of diabetes (years)	22.4 ± 8.1	21.9 ± 7.8	0.85
Age at start of dialysis (years)	43.7 ± 5.6	53.3 ± 12.8	0.04*
Interval before dialysis (years)[a]	17.5 ± 7.3	16.8 ± 7.7	0.79
Height (cm)	152.5 ± 6.7	162.1 ± 8.4	0.002*
Weight (kg)	42.3 ± 5.1	59.4 ± 10.0	<0.0001*
Body mass index (kg/m^2)	18.2 ± 2.2	22.5 ± 3.1	0.0002*

* $P < 0.05$
Data values are means ± SDs
ESRD, End stage renal disease
[a] Time interval between diagnosis of diabetes and start of dialysis

Table 2. Clinical characteristics of the Japanese patients with diabetes mellitus, ESRD, and the mt.3243A>G mutation

Subject	1	2	3	4	5	6	7	8
Sex	F	M	M	M	F	M	F	M
Present age (years)	(49)[a]	(41)[a]	42	60	(47)[a]	(50)[a]	50	41
Height (cm)	148.0	159.6	159.2	151.0	140.0	155.6	149.5	156.6
BMI (kg/m^2)	17.1	15.1	20.1	20.1	18.9	17.5	17.0	15.9
Maximum BMI	18.3	20.8	20.9	22.4	20.9	19.8	18.8	18.6
Current treatment	Insulin	Insulin	Insulin	Insulin	Insulin	Insulin	Insulin	Insulin
Complications								
Neuropathy	+	+	+	+	+	+	+	+
Retinopathy	PDR	PDR	PDR	SDR	PDR	PDR	PDR	PDR
Detection of proteinuria (years)	27	33	[b]	39	[b]	27	27	[b]
Age at diagnosis of diabetes (years)	39	27	27	40	29	22	22	20
Age at start of dialysis (years)	43	40	37	53	47	45	47	38
Duration between diagnosis of diabetes and start of dialysis (years)	4	13	10	13	18	23	23	18
Age at diagnosis of deafness (years)	36	NA	36	NA	25	40	42	31
Muscle biopsy (RRF)	+	+	NT	NT	+	+	+	NT
Brain CT/MRI				NT				NT
Calcium deposits	+	−	−		+	−	−	
Atrophy	+	+	+		+	+	+	
Infarction	+	−	+		+	−	+	
Stroke-like episodes	+	+	−	−	+	+	+	−
MELAS	+	+			+	+	+	
Maternal diabetes	+	+	−	−	+	−	+	+
Family history of renal dysfunction	+	−	+	+	−	−	−	+

ESRD, End stage renal disease; BMI, body mass index; RRF, ragged-red fibers; CT, computed tomography; MRI, magnetic resonance imaging; MELAS, myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; NA, not available; PDR, proliferative diabetic retinopathy; NT, not tested

[a] Age at death

[b] Evident at first visit

showed the presence of ragged red fibers in the biopsy samples of all five patients. Brain computed tomography/magnetic resonance imaging (CT/MRI) scanning in six patients revealed the following findings either singly or in combination: calcium deposition in the basal ganglia, cerebral/cerebellar atrophy, or cerebral infarction. In addition, five patients had a history of stroke-like episodes, such as syncope or severe headaches, during the course of treatment. Five patients (subjects 1, 2, 5, 7, and 8) had a history of maternal diabetes or impaired glucose tolerance (IGT), and four patients (subjects 1, 3, 4, and 8) had a family history of renal dysfunction (Fig. 1).

The younger sister of subject 1 died of acute renal failure, the details of which were not available, and the mother had diabetes and deafness, but had not received hemodialysis treatment. The brother of subject 3 had deafness, diabetes, and moderate kidney dysfunction. His father also had ESRD. His mother had died of a myocardial infarction, but was not known to have had either diabetes or renal dysfunction. The mother of subject 4 had ESRD, and one of his sisters had diabetes mellitus, MELAS, and ESRD. The maternal aunt and mother of subject 8 had diabetes with deafness. His older brother, who is positive for this mutation, also has diabetes, deafness, and renal dysfunction.

We were able to carry out histological studies on kidney tissue from one of these eight patients. This female patient (subject 5) died of multiorgan failure after sustaining a bone fracture; the resulting rhabdomyolysis had led to acute-on-chronic renal failure. The kidneys showed scattered nodular diabetic glomerulosclerosis, compatible with classical diabetic nephropathy. Some tubules contained densely eosinophilic hyaline casts that were positive for myoglobin. We examined a number of tissues, including kidney, pancreas, and cerebrum, for the presence of the mt.3243A>G mutation, and all showed heteroplasmy for this mutation. The degree of heteroplasmy was greater than 50% in the kidney and cerebrum, and it was 40% in the pancreas (data not shown).

Discussion

We identified the mt.3243A>G mutation in 8 of 135 (5.9%) subjects with ESRD and diabetes mellitus (type I and type II), and in none of 92 nondiabetic subjects with ESRD alone. Each of the patients with the mt.3243A>G mutation was initially diagnosed with type II diabetes. Hearing loss was observed, but additional neurological manifestations were not evident at presentation. Five of these patients subsequently exhibited clinical characteristics of MELAS, although none of these features were evident when they first came to clinical attention. The years at which hemodialysis was started in the eight carriers of the mutation ranged from 1992 to 1998: two patients started in 1992, one started in 1993, two started in 1994, one started in 1995, one started in 1996, none started in 1997, and one started in 1998.

The mt.3243A>G mutation has been reported to have a prevalence of 0.9% (5/550) in a randomly selected sample

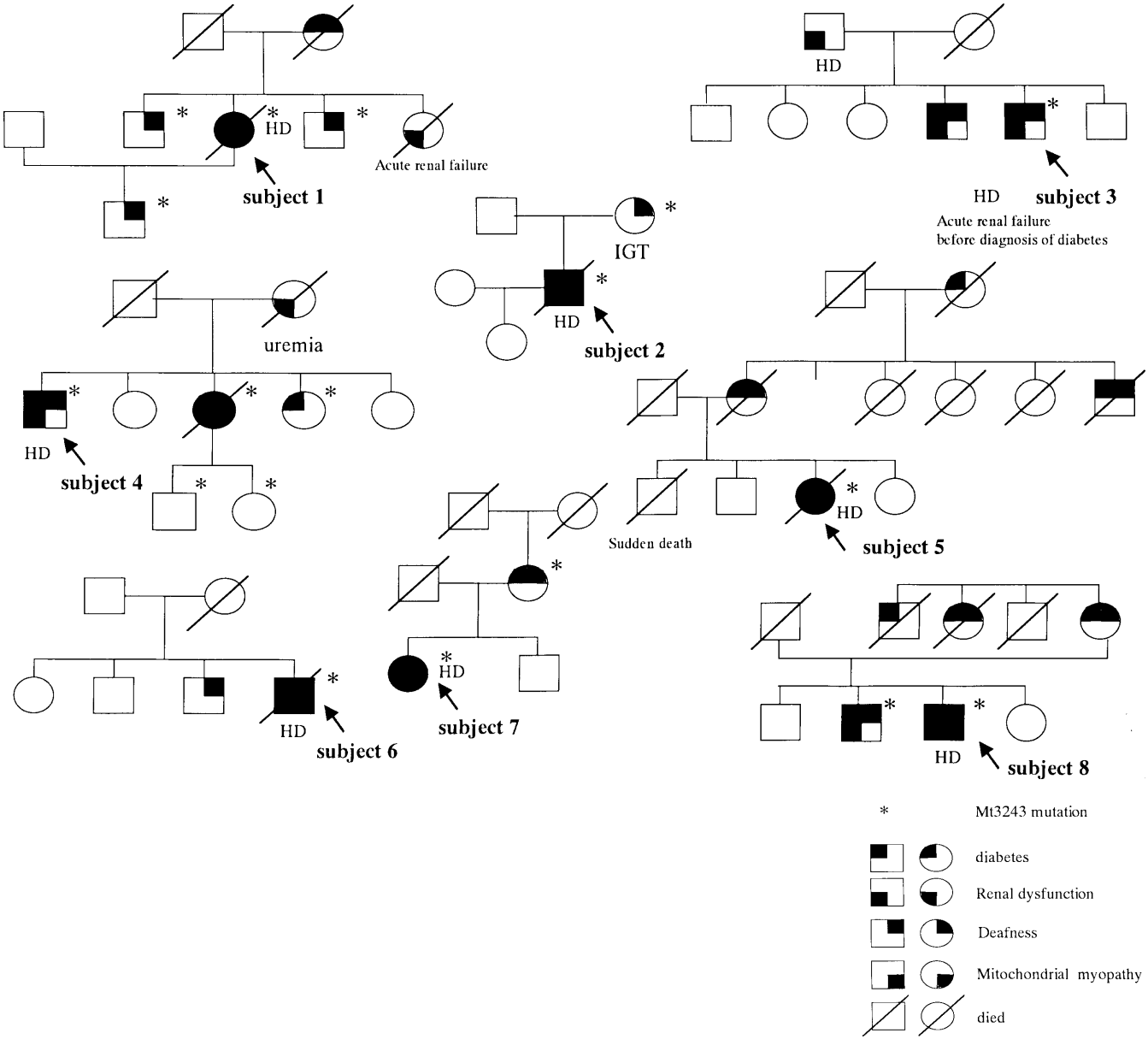

Fig. 1. Pedigrees of the eight subjects who were positive for the mt.3243A>G mutation. *HD*, Hemodialysis; *IGT*, impaired glucose tolerance

of unrelated Japanese diabetic patients (including those with type I and those with type II diabetes) (Otabe et al. 1994). Thus, the mt.3243A>G mutation has a 6.5-fold risk of ESRD (8/135 vs 5/550, respectively; $\chi^2 = 13.704$; $P = 0.0002$). Jansen et al. (1997) identified four individuals with this mutation from three families who suffered progressive kidney disease in association with diabetes and hearing loss, and emphasized the existence of a form of kidney disease that is characterized by the presence of this mutation. Our results are consistent with their observation, and support the notion that the mt.3243A>G mutation is one of the genetic factors involved in the development of kidney dysfunction in patients with diabetes. However, our results differ from those of Yamagata et al. (2000), who found the mt.3243A>G mutation in only 1 of 132 (0.8%) Japanese patients with diabetes and ESRD, a value similar to that in the general diabetic population in Japan (Otabe et al. 1994). The reason for the difference between the results of our study and that of Yamagata et al. is not known.

All eight patients that we identified with the mt.3243A>G mutation had typical features of MIDD, such as lean body and short stature with deafness. The etiology of kidney dysfunction in these eight patients was clinically diagnosed as diabetic nephropathy, because all of them had proliferative retinopathy. It was not possible to obtain the histological findings from these patients, because they had already developed ESRD. Thus, it is not clear whether the diabetic nephropathy in these patients was exacerbated in association with the mutation, or whether another form of kidney disease was involved. Five of the eight patients have been diagnosed with MELAS, but at present, the remaining three patients have not been diagnosed with this condition.

The mt.3243A>G mutation has been found in 80% of patients with MELAS and is the most common cause of this condition (Goto et al. 1990). However, there is no report in the literature regarding the proportion of diabetic patients with the mt.3243A>G mutation who have MELAS. There is also little information about kidney involvement in patients with MELAS (Brown and Wallace 1994; Ciafaloni et al. 1992; Goto et al., 1990, 1992; Koo et al. 1993). The renal dysfunction in MELAS has been reported to share features with renal tubular acidosis, Bartter's syndrome, and Fanconi syndrome, with focal glomerulosclerosis being a typical histological finding in these patients (Hsieh et al. 1996). Our results, and those of Jansen et al. (1997), indicate the need for further study of the mt.3243A>G mutation in diabetic patients with ESRD.

In conclusion, we suggest that the mt.3243A>G mutation may be a contributing genetic factor in the development of ESRD in Japanese patients with diabetes mellitus.

Acknowledgments We thank the patients for their willing participation in this study. This study was supported by grants from the Ministry of Health and Welfare of Japan (for Research on Human Genome and Gene Therapy), the Ministry of Science, Culture, and Sports of Japan (70203370, 12204102), the Uehara Memorial Foundation, and the Japan Diabetes Society Foundation.

References

Brown MD, Wallace DC (1994) Molecular basis of mitochondrial DNA disease. J Bioenergetics Biomembranes 26:273–289

Ciafaloni E, Ricci E, Shanske S, Moraes CT, Silvestri G, Hirano M, Simonetti S, Angelini C, Donati MA, Garcia C (1992) MELAS: clinical features, biochemistry, and molecular genetics. Ann Neurol 31:391–398

Goto Y, Nonaka I, Horai S (1990) A mutation in the $tRNA^{(LEU)(UUR)}$ gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348:651–653

Goto Y, Horai S, Matsuoka T, Koga Y, Nihei K, Kobayashi M, Nonaka I (1992) Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): a correlative study of the clinical features and mitochondrial DNA mutations. Neurology 42:545–550

Hsieh F, Gohh R, Dworkin L (1996) Acute renal failure and MELAS syndrome, a mitochondrial encephalomyopathy. J Am Soc Nephrol 7:647–652

Jansen JJ, Maassen JA, van der Woude FJ, Lemmink HA, van den Ouweland JM, t'Hart LM, Smeets HJ, Bruijn JA, Lemkes HH (1997) Mutation in mitochondrial $tRNA^{Leu(UUR)}$ gene associated with progressive kidney disease. J Am Soc Nephrol 8:1118–1124

Koo B, Becker LE, Chuang S, Merante F, Robinson BH, MacGregor D, Tein I, Ho VB, McGreal DA, Wherrett JR (1993) Mitochondrial encephalopathy, lactic acidosis, stroke-like episodes (MELAS): clinical, radiological, pathological, and genetic observations. Ann Neurol 34:25–32

Otabe S, Sakura H, Shimokawa K, Mori Y, Kadowaki H, Yasuda K, Nonaka K, Hagura R, Akanuma Y, Yazaki Y, Kadowaki T (1994) The high prevalence of the diabetic patients with a mutation in the mitochondrial gene in Japan. J Clin Endocrinol Metabol 79:768–771

van den Ouweland JM, Lemkes HH, Ruitenbeek W, Sandkuijl LA, de Vijlder MF, Struyvenberg PA, van de Kamp JJ, Maassen JA (1992) Mutation in mitochondrial $tRNA^{(Leu)(UUR)}$ gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. Nature Genet 1:368–371

Yamagata K, Tomida C, Umeyama K, Urakami K, Ishizu T, Hirayama K, Gotoh M, Iitsuka T, Takemura K,. Kikuchi H, Nakamura H, Kobayashi M, Koyama A (2000) Prevalence of Japanese dialysis patients with an A-to-G mutation at nucleotide 3243 of the mitochondrial $tRNA^{Leu(UUR)}$ gene. Nephrol Dial Transplant 15:385–388