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Association of *ND4L* gene 10609 mutation and hearing loss in a Korean with *ESRD* patients

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The kidney and cochlea have similar physiological characteristics, specifically the active transport of fluid and electrolytes, similar effects of aminoglycosides and some immunological factors. Several mitochondrial DNA (mtDNA) defects have been identified to be associated with hearing impairment either in syndromic or nonsyndromic forms. Dialysis patients had more oxidative stress than healthy subjects and this elevated oxidative stress leads to alterations of the mtDNA. To generate a more comprehensive analysis of the relationship between mitochondrial variation and hearing loss, two SNPs of 10609, 14668 position showed nominal levels of association with hearing loss. In our result, the mean PTA values in the ESRD patients were 28±13.9 (mean±SD) dB and 51.0±23.2 dB in low and high frequencies, which were significantly higher than those in the normal controls. 10609T〉C and 14668C〉T were significantly associated with hearing loss in the ESRD patients. In summary, our results suggest that the polymorphisms of the ND4L subunit gene might be association with ESRD patients and hearing loss.

Key words : ESRD; hearing loss; mitochondrial DNA; ND4L 10609 gene

INTRODUCTION

Chronic hemo- and peritoneal dialysis have been established as live-saving and live-sustaining modalities for the treatment of patients with end-stage renal disease (ESRD). Hundreds of thousands of persons are alive today as a result of receiving dialysis. Despite the widespread availability and advancements in dialysis, the expected remaining lifetime for children with ESRD remains low, between 35% and 47% of that of an age- and race-matched US population (Mitsnefes, 2002).

The kidney and cochlea have similar physiological characteristics, specifically the active transport of fluid and electrolytes by the stria vascularis and the glomerulus, accounting for similar effects of aminoglycosides and some immunological factors on the two organs (Ozturan and Lam, 1998). Development of both the inner ear and the kidney is affected by similar genetic factors in certain hereditary diseases as reported in Alport's syndrome and branchio-oto-renal syndrome (Thodi *et al.,* 2006). It has been reported in a number of variable cases that hearing impairment is associated with renal failures, including use of ototoxic medications, electrolyte imbalance, alterations in blood urea nitrogen and lipids(Johnson and Mathog, 1976), hypertension (Gartland *et al.,* 1991), and haemodialysis treatment itself (Bazzi et al. 1995; Serbetcioglu et al.

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2001). The incidence of sensorineural hearing loss among patients with chronic renal failure (CRF) is considerably higher than in the general population (Bazzi *et al.*,1995; Ozturan and Lam, 1998; Johnson *et al.*, 1976; Antonelli *et al.*, 1990)

Cochlear function requires a high rate of ATP production that is produced in mitochondria, and the human mitochondrial genome encodes for 13 subunits of the mitochondrial respiratory chain, 22 tRNAs, and two rRNAs. Genetic damages more often occur to mtDNA than to nuclear DNA, apparently due to lack of efficient DNA repair mechanisms. Further, the lack of protective histone proteins in mitochondria makes mtDNA more susceptible to DNA damaging agents (Nagy *et al.*, 2003). Several mitochondrial DNA (mtDNA) defects have been identified to be associated with hearing impairment either in syndromic or nonsyndromic forms, including A1555G in the 12S rRNA gene and an insertion of C at position 7472 and T7445C in the $tRNA^{Ser}$ gene (Estivill *et al.*, 1998; Hutchin *et al.*, 1993; Tiranti *et al.*, 1995; Reid *et al.*, 1994). Also, it is reported that mtDNA at position 4977 seems to predict survival in ESRD, but a reduced mitochondrial copy number seems to predict a poor outcome (Rao *et al.*, 2009), that there may be an association between mtDNA4977 del and SNHL(Bai and Seidman. 2001). However, It is not yet established whether haemodialysis treatment itself can cause negative or positive effects on hearing in patients with CRF.

Therefore, this study analyzed the mitochondrial DNA mutation for finding relation of hearing loss in patients with ESRD. Our data sequenced that mtDNA coding region was compared in a group of ESRD patients without hearing loss(n=51) and hearing loss(n=63).

Materials and methods

Audiological evaluation

After informed consent, 114 ESRD patients and 58 normal individuals were subjected to otological examination, such as otoscopy and tympanometry to check if there was any fluid or drainage in the ears. All of the patients and normal controls showed normal external and middle ear conditions. Further, the patients did not present any abnormal neurological conditions that could be associated with hearing loss. For hearing loss test, the patient and control groups underwent a basic audiological evaluation (pure-tone, high frequency and speech audiometry), using a clinical audiometer (Madsen Electornics). The degree of hearing loss was defined according to the pure-tone averages (PTA), which were based on the average dB values at low (0.25, 0.5, 1.0, and 2.0 kHz) or high (4.0 and 8.0 kHz). The mean ages of the ESRD patients and normal controls were 63 years and 60years, respectively.

Six SNP markers

we selected six SNP markers that showed relatively higher variation frequency (18 – 44 %) in the test with 50 ESRD patients. The six SNP markers were 6962G〉A, 10609T〉C, 14668C〉T, 14783T〉C, 15043G〉A, and 15301G〉A. All the other SNPs showed allele variation frequencies of less than 12%. All of the selected markers are present in the coding regions of the mitochondrial respiratory chain.

PCR amplification

Genomic DNA that includes the mitochondrial genome was isolated from leucocytes using the differential deproteinization method as previously reported (Lahiri and Nurberger, 1981). The entire mitochondrial genome was PCR amplified with 13 sets of primers. The sequences of oligonucleotide primers are available upon request. Amplification reactions were performed in a final volume of

25 l, containing approximately 100 ng of genomic DNA, 10 pmole of each primer, 0.2 mM of each dNTP, and *Taq* DNA polymerase (Takara). Thermocycling consisted of 30 cycles at 94 C for 1 min, at 57 − 61 C for 1 min, and at 72 C for 1 min, with a predenaturation at 94 C for 5 min and a final extension at 72 C for 7 min. Amplification was carried out in a GeneAmp PCR system 9700 thermocycler (Applied Biosystem). The amplification product was verified in 1.5% agarose gel.

Sequencing analysis of mtDNA

Association of polymorphic markers with hearing loss was performed by DNA sequencing analysis of the PCR fragments amplified from the total mtDNA. DNA sequencing was carried out with the primers used for the PCR amplifications, using the BigDye Terminator v3.1 cycle Sequencing Kit (Applied Biosystems). Thermocycling for DNA sequencing consisted of 25 cycles at 96 C for 10 sec, 55 C for 5 sec, and 60 C for 4 min. Electrophoresis of the extension products was carried out in the ABI Prism 3100 Genetic Analyzer.

Statistic analysis

Fisher's exact probability test was utilized to assess p and X^2 values. p−values less than 0.05 were considered to indicate statistical significance.

Results

PTA analysis in ESRD patients

The prevalence of hearing loss in ESRD patients and normal controls was measured by pure tone audiometry. The degree of hearing loss was defined according to the pure−tone average (PTA) value at both low (0.25, 0.5, 1.0, and 2.0 kHz) and high (4.0, and 8.0 kHz) frequencies. The PTA values less than 25 dB was defined as clinically normal, and the values equal to or more than 25 dB was defined as hearing loss as previously described (Hutchinson and Klodd, 1982). Out of 114 ESRD patients, 55% (63/114) and 87% (99/114) of the patients showed PTA values higher than 25 dB in low and high frequency thresholds, respectively (Fig. 1, Table 1). None of the normal controls showed PTA values higher than 25 in both low and high frequencies (Fig. 1, Table 1). The mean PTA values in the ESRD patients were 28 ± 13.9 (mean ± SD) dB and 51.0 ± 23.2 dB in low and high frequencies, respectively, which were significantly higher than those in the normal controls (14.0 ± 4.13 dB and 14.5 ± 4.3 dB in low and high frequencies, respectively: Fig. 1).

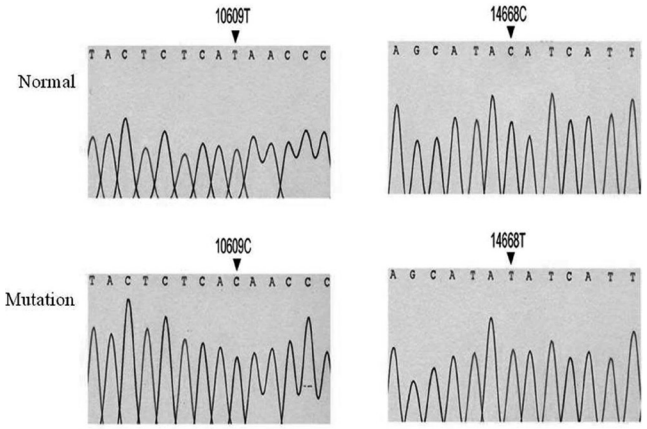

Fig. 1. Analysis of Pure−Tone Average(PTA) in normal people and ESRD patients. 58 of normal people without ESRD and 114 of ESRD patients were analyzed PTA. The mean age of normal people is approximately 60 years, mean age of patients was 63years. It was analyzed low(0.25−2 KHz) and high(4−8 KHz) frequency of PTA. (normal control average; low and high frequency, 14 dB and 15 dB ESRD patients average; low and hight frequency, 28 dB and 51dB)

Table 1. Analysis of Pure− Tone Averages in the ESRD(114 patients)

Freq(kHz)	Normal		Hearing loss	
	Right ear	Left ear	Right ear	Left ear
low frequency*	51‡	51	63	63
high frequency†	15	18	99	96

* : 0.25 − 2 KHz, †: 4 − 8 KHz, ‡: number of patients

Association of 10609T>C and 14668C>T markers with hearing loss.

For screening of mitochondrial mutations in patients with ESRD, we screened for single nucleotide polymorphic (SNP) markers in the mitochondrial genome. Using 9 sets of oligonucleotide primers that cover the entire mitochondrial coding genome, we amplified the mtDNA in 50 ESRD patients and then sequenced the PCR amplicons (Table 2). We found 104 single nucleotide polymorphisms (SNPs) in 50 ESRD patients. Among the identified SNPs, 75 SNPs were reported ones in the Mitomap database (http://www.mitomap.org), and 29 SNPs were novel ones (Table 3). Majority (83.6 %) of the 104 SNPs were transition substitution, whereas 16.3 % of the SNPs were transversion substitutions.

To the relevance of hearing loss and mitochondrial mutations in 114 ESRD patients, we divided the ESRD patients into two groups based on hearing loss and compared allele frequency of the six SNP markers between the patient groups. Allele frequency of A6962G>A, 14783T>C, 15043G>A, and 15301G>A were not significantly different between the groups with or without hearing loss. In contrast, 10609T>C and 14668C>T were significantly associated with hearting loss in the ESRD patients, showing a p-value of 0.007 and 0.045, respectively (Fig. 2, Table 4).

Discussion

Compared with normal controls, ESRD patients with he-

Table 2. Oligonucleotide primers used for PCR amplifications

Gene	Orientatio	Position	Sequence (5'-3')	Tm(°C)
*ND1**	Forward	4058-4078	5'-TCCCCTGAACTCTACACA ACA-3'	58
	Reverse	4787-4806	5'-AGTGAAAGGGGGCTATTCCT-3'	57
ND2†	Forward	4828-4846	5'-AAGGCACCCCTCTGACATC-3'	59
	Reverse	5619-5638	5'-AAAGTGGCTGATTTGCGTTC-3'	55
ND3‡	Forward	10082-10103	5'-AATCAACACCCTCCTAGCCTTA-3'	58
	Reverse	10912-10931	5'-AGGAAAAGGTTGGGGAACAG-3'	57
ND4§	Forward	10910-10929	5'-AGCTGTTCCCCAACCTTTTC-3'	57
	Reverse	11693-11713	5'-GGCGATTATGAGAATGACTGC-3'	58
ND4	Forward	11759-11778	5'-TACGAACGCACTCACAGTCG-3'	59
	Reverse	12551-12571	5'-TTAGGGAGAGCTGGGTTGTTT-3'	58
ND4	Forward	12734-12753	5'-CCGCTAACAACCTATTCCAA-3'	55
	Reverse	13571-13590	5'-CAGGGAGGTAGCGATGAGAG-3'	61
ND5‖	Forward	13573-13592	5'-CTCATC GCT ACC TCC CTG AC-3'	61
	Reverse	14479-14497	5'-TTTAGG GGG AAT GAT GGT TG-3'	55
ND6¶ *Cytb***	Forward	14478-14497	5'-CAACC ATC ATTCCCCCTAAA-3'	55
	Reverse	15336-15355	5'-CGTTTCGTGCAAGAATAGGA-3'	55
*COX1*π	Forward	6450-6469	5-CTCTTCGTCTGATCCGTCCT-3	60
	Reverse	7177-7197	5-CGAGAAAGTGTTGTGGGAAGA-3	59

*;NADH dehydrogenase 1, †;NADH dehydrogenase 2, ‡; NADH dehydrogenase 3, § ; NADH dehydrogenase 4, ‖; NADH dehydrogenase 5, ¶; NADH dehydrogenase 6, **; Cytochrome b, π; Cytochrome c oxidase (COX) subunit 1

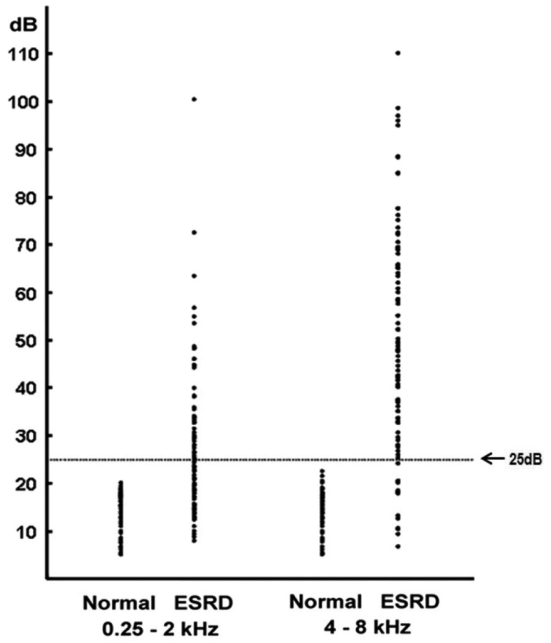

Fig. 2. Results of mtDNA sequencing. hearing health (H) show a wild-type sequence, whereas hearing loss (L) have a T to C (*) transition at base position 10609 affecting the ND4L gene, base position 14668 in the ND6 gene transition C to T(*)

modialysis treatment showed a highly significant bilateral sensorineural hearing loss at all frequencies, which was more marked in higher frequencies (Fig. 1). It was reported that many factors have been implicated for hearing impairment (Gatland *et al.,* 1991; Yassin *et al.,* 1970). There are contrasting results as to whether hemodialysis therapy may be a significant factor for hearing impairment (Rossini *et al.,* 1984; Bazzi.*et al.,* 1995; Hutchinson *et al.,* 1982). Of course, the negative factors related to hemodialysis treatment which could lead to hearing impairment are: acute hypotension, reduction in blood osmotic pressure, acute clearance of urea, increased red blood cell mass. But the direct impact of hearing impairment is not.

Dialysis patients had more oxidative stress than healthy subjects (Chen *et al.,* 2009), and this elevated oxidative stress leads to alterations of the mtDNA. Since mitochondria are a major source of ROS and are highly susceptible to oxidative damage, we evaluated the corelation of mtD-

Table 3. Novel mtDNA SNPs found in ESRD patients

Gene	Location (nt)	Amino acid change	%	Gene	Location (nt)	Amino acid change	%
*ND1**	3835C〉G	P177A	4	*CO3*	9777 G〉C	G191R	2
	3851C〉G	A182G	8		9786 G〉C	G194R	2
	3871A〉C	T189P	2		9933 C〉G	H243D	2
	3960C〉G	P218P*	8		9949 T〉G	V248G	2
CO1[†]	6192 A〉C	M97T	2		9987 T〉G	S261A	2
	6195 A〉C	N98H	2	*ND3*[§]	10073 A〉C	L5F	2
	6701 A〉G	E266E*	2	*ND4L*[‖]	10689 G〉C	G74R	2
	6818 C〉T	F305F*	2		10743 A〉C	N92H	2
	7110 T〉C	Y403H	2	*ND4*[¶]	10831 A〉G	W24W*	2
	7408 A〉C	Y502S	8		11511 A〉C	N1187T	2
CO3[‡]	7687 C〉T	I34I&	6	*ND5***	12424 A〉C	N30H	2
	7777 C〉A	V64V&	8	*ND6*[π]	14166 T〉C	N6N*	2
	7879 A〉G	K98K&	8	*CYTB*[Ⅱ]	15036 A〉C	H97P	2
	8015 C〉G	T144V	8		15037 C〉T	H97H*	8
	9303 A〉T	M33L	2				

*;NADH dehydrogenase 1, [†]; Cytochrome c oxidase (COX) subunit 1, [‡];Cytochrome c oxidase (COX) subunit 3, [§] ; NADH dehydrogenase 3 [‖]; NADH dehydrogenase 4L, [¶]; NADH dehydrogenase 4, **; NADH dehydrogenase 5, [π];NADH dehydrogenase 6, [Ⅱ]; Cytochrome b, &; synonym

Table 4. Allele frequency of 6 SNP markers in ESRD patients

Position	Gene	Amino Acid	ESRD without hearing loss (n=51)		ESRD without hearing loss (n=63)		*p* values*
6962G〉A	*COI*	353 Leu〉Leu	G 86% (44)	A 14% (7)	G 84% (53)	A 16% (10)	1.000
10609T〉C	*ND4L*	47Met〉Thr	T 96% (49)	C 4% (2)	T 76% (48)	C 24% (15)	0.007*
14668C〉T	*ND6*	2Met〉Met	C 73% (37)	T 27% (14)	C 52% (33)	T 48% (30)	0.045*
14783T〉C	*CYB*	13 Leu〉Leu	T 45% (23)	C 55% (28)	T 33% (21)	C 67% (42)	0.28
15043G〉A	*CYB*	99Gly〉Gly	G 45% (23)	A 55% (28)	G 33% (21)	A 67% (42)	0.28
15301G〉A	*CYB*	185 Leu〉Leu	G 49% (25)	A 51% (26)	G 33% (21)	A 67% (42)	0.132

*; Value was determined by χ^2 test from 2 × 2 contingency table

NA mutation in the ESRD patients with hearing loss. Mutation of mtDNA in renal failure desease reported in a wide variety of nonpathogenic polymorphisms, but no data in hearing loss. To generate a more comprehensive analysis of the relationship between mitochondrial variation and hearing loss, we have analysed chi-square. We genotyped 104 mtDNA variants in screening test and analysed data from the six SNP markers. Two SNPs of 10609, 14668 position showed nominal levels of association with hearing loss (Table 3).

The ND6 (NADH dehydrogenase 6) subunit is one of the least conserved mitochondrially encoded proteins in complex I and involved in a ubiquinone binding site. Mutations in ND6 subunit were found to decrease enzyme affinity towards decylubiquinone (DB) (Chinnery *et al.,* 2001). In our result, a similar substitution, 14668C〉T was detected in the coding region for ND6 subunit in ESRD patiant with hearing loss (Fig. 2). Although it will be need to be verified with using a large number of normal control samples, this result suggests that the 14668C〉T polymorphisms of the ND6 gene are associated with these factors in the ESRD patients. Also, the ND4L (NADH dehydrogenase 4L) subunit of mitochondrial NADH:ubiquinone oxidoreductase (complex I) is an integral membrane protein. The ND4L subunit that contains two highly conserved glutamates is essential for NADH-1 enzyme activity (Kao *et al.,* 2005; Kervinen *et al.,* 2004). We detected the presence of 10609T〉C mutation in the ND4L subunit, this position would explain the pattern of differences of amino acid (Met to Thr) (Fig. 2). The polymorphisms within the conserved site of the glutamates region might have influence on the expression level by suppressing the NADH-1 enzyme activity. Further

analysis using the transmitochondrial cell cybrid system will be necessary to establish the functional significance of the 10609T〉C mutation in the complex I gene of mtDNA.

In summary, our results suggest that the SNP of the ND4L subunit gene might be association with ESRD patients and hearing loss.

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References

1. Antonelli AR, Bonfioli F, Garrubba V, Ghisellini M, Lamoretti MP, Nicolai P, *et al*. Audiological findings in elderly patients with chronic renal failure. *Acta Otolaryngol Suppl*. 1990, 476:54–68.
2. Bai U, Seidman MD. A specific mitochondrial DNA deletion (mtDNA4977) is identified in a pedigree of a family with hearing loss. *Hearing res* 2001, 154:73–80.
3. Bazzi C, Venturini CT, Pagani C, Arrigo G, D"Amico GD. Hearing loss in short and long term haemodialysed patients. *Nephrol Dial Transplant* 1995, 10:1865–1868.
4. Chen JB, Lin TK, Liao SC, Lee LC, Liou CW, Wang PW, *et al*. Lack of association between mutations of gene-encoding mitochondrial D310 (displacement loop) mononucleotide repeat and oxidative stress in chronic dialysis patients in Taiwan. *Journal of Negative Results in BioMedicine* 2009, 8:10–16.
5. Chinnery PF, Brown DT, Andrews RM, Singh-Kler R, Riordan-Eva P, Lindley J, *et al*. The mitochondrial ND6 gene is a hot spot for mutations that cause Leber's hereditary optic neuropathy. *Brain* 2001, 124:209–218
6. Estivill XN, Govea A, Barcelo E, Perello C, Badenas E, Romero L, *et al*. Familial progressivesensorineural deafness is mainly due to the mtDNA A1555G mutation and is enhanced by treatment with aminoglycosides. *Am J Hum Genet* 1998, 62:27–35.
7. Gatland D, Tucker B, Chalstrey S, Keene M, Baker L. Hearing loss in chronic renal failure-hearing threshold changes following hemodialysis. *J R Soc Med* 1991, 4:587–589.
8. Hutchinson J, Klodd D. Electrophysiological analysis of auditory,vestibular, and brainstem function in chronic renal failure. *Laryngoscope* 1982, 92:833–843.
9. Hutchin, TI, Haworth K, Higashi N, Fischel-Ghodsian M, Stoneking N, Saha C,Arnos Cortopassi G. A molecular basis for human hypersensitivity to antibiotics. *Nucleic Acids Res* 1993, 21:4174–4179.
10. Johnson DW, Mathog RH. Hearing function and chronic renal failure. *Ann Otol Rhinol Laryngol*. 1976, 85: 43–49.
11. Johnson DW, Wathen RL, Mathog RH. Effects of hemodialysis on hearing threshold. *ORL J Oto Rhino lary* 1976. 38:129–139.
12. Kao MC, Nakamaru-Oqiso E, Matsuno-Yagi A, Yaqi T. Characterization of the membrane domain subunit NuoK (ND4L) of the NADH-quinone oxidoreductase from Escherichia coli. *Biochemistry* 2005, 12:9545–9554.
13. Kervinen, Patsi J, Finel M, Hassinen IE. A pair of membrane-embedded acidic residues in the NuoK subunit of Escherichia coli NDH-1, a counterpart of the ND4L subunit of the mitochondrial complex I, are required for high ubiquinone reductase activity. *Biochemistry* 2004, 43:773–781.
14. Lahiri DK, Nurberger JL. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFPLs studies. *Nucleic Acid Res* 1981, 19:5444.
15. Rao M, Li L, Demello C, Guo D, Jaber BL, Pereira BJ, *et al*. HEMO Study Group. Mitochondrial DNA Injury and Mortality in Hemodialysis Patients. *J Am Soc Nephrol*. 2009, 20(1):189–196.
16. Mitsnefes MM. Pediatric end-stage renal disease: Heart as a target. *J Pediatr* 2002, 141:162–164.
17. Nagy A, Wilhelm M, Kovacs G. Mutations of mtDNA in renal cell tumours arising in end-stage renal disease. *J Pathol* 2003, 199:237–242.
18. Rossini M, Stefano D, Febbo A, Paolo D, Bascini M. Brainstem auditory responses (BAERs) in patients with chronic renal failure. *Electroen Clin Neuro* 1984, 57:507–514.
19. Ozturan O, Lam S. The effect of hemodialysis on hearing using pure-tone audiometry and distortion-product otoacoustic emissions. *ORL J of Oto Rhino lary* 1998, 60:306–313.
20. Rao M, Li L, Demello C, Guo D, Jaber BL, Pereira BJ, *et al*. HEMO Study Group. Mitochondrial DNA Injury and Mortality in Hemodialysis Patients. *J Am Soc Nephrol* 2009, 20:189–196.
21. Reid FM, Vernham GA, Jacobs HT. A novel mitochondrial pointin a maternal pedigree with sensorineural deafness. *Hum Mutat* 1994, 3:243–247.
22. Serbetcioglu B, Erdogan S, Sifil A. Effects of a single session of Hemodialysis on Hearing Abilities. *Acta Otolaryngol*. 2001, 121:836–838.
23. Thodi C, Thodis E, Danielides V, Pasadakis P, Vargemezis

V. Hearing in renal failure. *Nephrol Dial Transplant*. 2006, 21:3023−3030.
24. Tiranti VP, Chariot F, Carella A, Toscano P, Soliveri P, Girlanda F, *et al*. Maternally hearing loss, ataxia and myoclonus associated with a novel pointin mitochondrial tRNASer (UCN) gene. *Hum Mol Genet* 1995, 4:1421−1427.
25. Yassin A, Badry A, Fatthi A. The relationship between electrolyte balance and cochlear disturbances in cases of renal failure. *J Laryngol Otol* 1970, 84:429−435.