

# Presence of mitochondrial tRNA<sup>Leu(UUR)</sup> A to G 3243 mutation in DNA extracted from serum and plasma of patients with type 2 diabetes mellitus

Sheng Zhong, Maggie C Y Ng, Y M Dennis Lo, Juliana C N Chan, Philip J Johnson

## Abstract

**Aims/Background**—An A to G substitution at base pair 3243 in the mitochondrial tRNA<sup>Leu(UUR)</sup> gene (mt3243) is commonly associated with maternally inherited diabetes and deafness, and other diseases. It is possible that cell free mitochondrial DNA exists in serum and plasma from these patients, and these samples might be a source of material for the detection of such mutations.

**Methods**—Sixteen patients with type 2 diabetes mellitus and 25 healthy subjects were tested for the 3243 mutation by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis. Plasma and serum from the 41 subjects were tested blind, without knowledge of the final diagnosis.

**Results**—PCR amplification of the mtRNA<sup>Leu(UUR)</sup> region in mitochondrial DNA (mtDNA) in serum samples revealed the presence of mtDNA in all samples. After *ApaI* digestion of the amplified DNA fragments, mt3243 was detected in the serum and plasma samples of the seven patients with diabetes who had previously been found to have this mutation in their leucocyte DNA. None of the serum/plasma samples from the healthy subjects or those patients negative for mt3243 in their leucocytes had this mutation ( $p < 0.001$ ). In addition, the degree of heteroplasmy of mt3243 appeared to be higher in serum and plasma samples than in leucocytes among mt3243 carriers ( $p < 0.05$ ).

**Conclusions**—Therefore, mtDNA and associated mutations are present and detectable in serum and plasma. Plasma and serum might be alternative sources for the molecular diagnosis of mt3243 associated diabetes mellitus, as well as other mitochondrial mediated diseases.

(*J Clin Pathol* 2000;53:466–469)

**Keywords:** mitochondrial tRNA<sup>Leu(UUR)</sup>; mitochondrial 3243 mutation; serum and plasma DNA; type 2 diabetes mellitus

(mt3243), has been found in patients with neural and muscular dysfunction, including a syndrome of myopathy, encephalopathy, lactic acidosis, and stroke like episodes; chronic progressive external ophthalmoplegia; myopathy; preeclampsia; and eclampsia.<sup>2–7</sup> This mutation is also associated with a subtype of diabetes known as maternally inherited diabetes and deafness.<sup>8–9</sup> The prevalence of mt3243 varies considerably among different ethnic groups and patients with diabetes with different modes of clinical presentation.<sup>10</sup>

Pathogenic mtDNA usually exists in heteroplasmic form, with the existence of both mutant and wild-type mtDNA in affected cells. The degree of heteroplasmy also varies considerably in different tissues and among different individuals. Leucocytes, which are currently used by most workers as the source of mtDNA, generally contain a lower proportion of mutant mtDNA than other cells such as those from muscle, brain, and oral mucosa.<sup>11–13</sup> Moreover, the amount of mutant mtDNA has been shown to decrease in leucocytes with aging.<sup>14</sup> Thus, analysis of mutant mtDNA in blood leucocytes might not be suitable in elderly people with a low degree of heteroplasmy.

The use of plasma and serum as sources of genomic DNA for molecular diagnosis has raised interest because of its non-invasive nature and ease of sample collection. Recent studies have demonstrated that tumour specific DNA and fetal DNA are detectable in the plasma and serum of patients with cancer<sup>15–19</sup> and pregnant women, respectively.<sup>20</sup> These reports prompted us to investigate whether mtDNA, the other human genome, is detectable in serum and plasma samples, and whether these samples might be an alternative source of material for the detection of mitochondrial mediated diseases. In our study, the presence of mtDNA and the most common mt3243 mutation in serum and plasma of patients with type 2 diabetes mellitus was investigated.

## Patients and methods

All subjects were ethnic southern Chinese living in Hong Kong. Sixteen patients with type 2 diabetes and 25 healthy subjects (age range, 20 to 40 years; recruited from hospital staff) were included in our study. Among the 16 patients with diabetes (age range, 23 to 79 years), seven were known to have the mt3243 mutation in their leucocyte DNA. Initially, we identified two patients with diabetes with this mutation.<sup>9</sup> Subsequently, we screened 917

Department of Clinical Oncology, Sir Y K Pao Centre for Cancer, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N T, Hong Kong, The People's Republic of China

S Zhong  
P J Johnson

Department of Medicine and Therapeutics, Sir Y K Pao Centre for Cancer  
M C Y Ng  
J C N Chan

Department of Chemical Pathology, Sir Y K Pao Centre for Cancer  
Y M D Lo

Correspondence to:  
Professor Johnson  
email:  
[pjohnson@cuhk.edu.hk](mailto:pjohnson@cuhk.edu.hk)

Accepted for publication  
8 December 1999

Human mitochondrial DNA (mtDNA) encodes the ribosomal and transfer RNAs (tRNAs) for ribosomal protein synthesis, and enzyme subunits of complexes in the respiratory chain.<sup>1</sup> Mutations in mtDNA have been reported to be associated with a variety of diseases.<sup>2–9</sup> One of the most common mutations, an A to G substitution at base pair (bp) 3243 in the mitochondrial tRNA<sup>Leu(UUR)</sup> gene

patients and identified a further five using identical methodology. These seven patients, together with nine further patients in whom the mutation was not present in their leucocytes, were included in this investigation. Of the seven patients positive for mt3243 in their leucocytes, six had a history of maternal diabetes (except patient M6), and three of them belonged to the same pedigree (M4, M8, and M9). Type 2 diabetes was diagnosed according to the 1985 WHO criteria.<sup>21</sup> Informed consent was obtained from all participating subjects and approval for our study was obtained from the Chinese University of Hong Kong clinical research ethics committee. A 10 ml blood sample was collected and aliquoted into one tube containing EDTA and one plain tube. DNA was then isolated from serum, plasma, and leucocytes and genotyped for mt3243.

#### SAMPLE PREPARATION

Blood samples collected in EDTA and plain tubes were centrifuged at 3000  $\times$ g for collection of plasma and serum, respectively. After plasma removal, the buffy coat and red blood cell pellet were saved for DNA extraction. The serum and plasma samples were recentrifuged at 3000  $\times$ g and collected in new tubes to avoid the carry over of blood cells. All the samples were then stored at  $-20^{\circ}\text{C}$  until further analysis.

#### DNA EXTRACTION FROM LEUCOCYTES, SERUM, AND PLASMA

Leucocyte DNA was extracted by a standard method involving proteinase K and phenol/chloroform.<sup>22</sup> Serum and plasma DNA were extracted by a similar method. In brief, 100  $\mu\text{l}$  of serum or plasma was mixed with an equal volume of digestion solution (20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), and 0.8 mg/ml proteinase K) and incubated at  $56^{\circ}\text{C}$  for four hours. DNA was then extracted using phenol/chloroform followed by chloroform. DNA was precipitated with ethanol and sodium acetate and air dried. The precipitated serum and plasma DNA were dissolved in 20  $\mu\text{l}$  of double distilled water, and stored at  $-20^{\circ}\text{C}$  for further analysis.

#### MT3243 GENOTYPING AND MEASUREMENT

We deliberately concealed the identity of the subjects and rearranged the order of the plasma and serum of the subjects. Mt3243 genotype was determined by PCR using the primer set: 5'-AGG ACA AGA GAA ATA AGG CCT-3' (nucleotides (nt)3130–3149) and 5'-AAC GTT GGG GCC TTT GCG T-3' (nt3423–3404).<sup>1</sup> Reactions were carried out in 20  $\mu\text{l}$  volumes containing 200 ng DNA from blood leucocytes or 2  $\mu\text{l}$  of serum or plasma DNA extracted from 10  $\mu\text{l}$  serum or plasma, 10 mM Tris/HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.5  $\mu\text{M}$  of each primer, and 0.5 U of Taq DNA polymerase. PCR was performed for 40 cycles with denaturation at  $94^{\circ}\text{C}$  for 45 seconds, annealing at  $55^{\circ}\text{C}$  for 45 seconds, and extension at  $72^{\circ}\text{C}$  for one minute. Next, 0.5  $\mu\text{Ci}$  of [ $\alpha^{33}\text{P}$ ]dATP was added and one more cycle was performed.

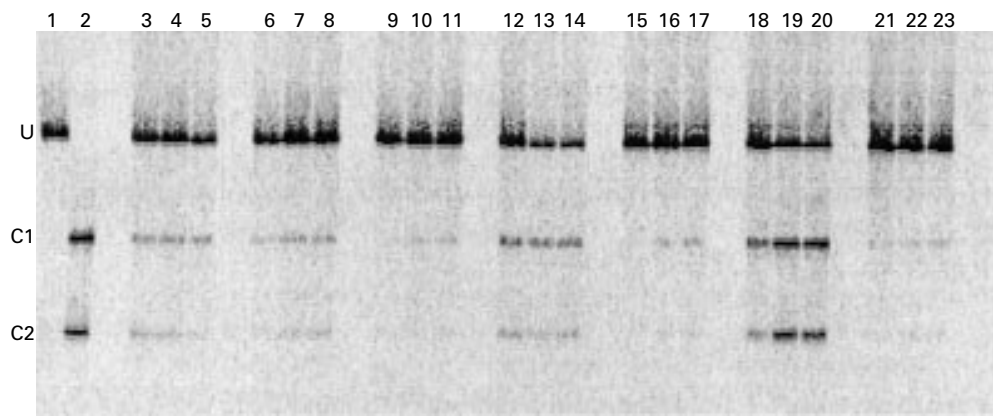
Labelling of the PCR product during the last cycle rather than the first cycle prevents underestimation of the proportion of mutant mtDNA as a consequence of heteroduplex formation during the PCR.<sup>3,9</sup> Aliquots (5  $\mu\text{l}$ ) of the PCR products were then digested with 5 U ApaI (Gibco BRL, Rockville, Maryland, USA) for two hours at  $30^{\circ}\text{C}$  and then electrophoresed using 8% denaturing polyacrylamide gels at 1000 V for two hours and visualised by electronic autoradiography. The presence of mt3243 allowed the 294 bp product to be cleaved into 180 and 114 bp fragments. Standards containing 0–100% mutant mt3243 (made by mixing a cloned DNA carrying no mt3243 mutation and another cloned DNA carrying >99% mutant mt3243 in different proportions, kindly given by Dr J van den Ouweland, Leiden University, the Netherlands) were also included in the assay. The intensity of the bands was measured by means of an Instantimager (Packard, Canberra Company, Meridan, Connecticut, USA). The proportion of mt3243 in a sample was calculated by dividing the intensity of mutant bands (114 and 180 bp) by the total intensity of both wild-type and mutant bands. Two separate analyses were carried out and the average of the two analyses was used for subsequent comparison. Differences in the degree of heteroplasmy between the leucocyte and serum fractions, and between the leucocyte and plasma fractions were analysed using the Wilcoxon signed rank test (SigmaStat 2.0). A p value < 0.05 was considered to be significant.

#### Results

The optimum amount of serum DNA for PCR amplification was assessed in healthy controls by subjecting DNA extracted from 1  $\mu\text{l}$  and 10  $\mu\text{l}$  serum to mt3243 PCR. When the PCR reaction was carried out using 1  $\mu\text{l}$  serum DNA, amplification of a 294 bp fragment was seen in 17 of 25 healthy subjects. However, all the 25 samples showed the 294 bp band when 10  $\mu\text{l}$  serum DNA was added as a template. Thus, 10  $\mu\text{l}$  serum or plasma DNA were used in subsequent experiments.

The degree of heteroplasmy of mt3243 was determined by incorporating [ $\alpha^{33}\text{P}$ ]dATP in the last cycle of PCR followed by ApaI digestion. The cleavage of the 294 bp fragment by ApaI into 180 and 114 bp fragments revealed the presence of mt3243. Labelling of the PCR product at the last cycle permitted correct measurement of the degree of heteroplasmy because heterodimers formed during PCR could not be cleaved by ApaI.<sup>23</sup> The effectiveness of the enzymatic cleavage of the PCR fragments was shown by including known percentages of mutant mt3243 (1% and 100%) (fig 1).

The mt3243 mutation was detected in the plasma and serum samples in which the mutations were detected in leucocyte DNA using the same PCR and digestion conditions. Seven plasma and serum samples from patients with type 2 diabetes were scored as positive for mt3243, whereas the other nine samples from the patients with diabetes and the 25 samples



**Figure 1** Comparison of the degrees of heteroplasmy detected in blood leucocytes, serum, and plasma in seven patients with diabetes who were positive for mutation mt3243. Lanes 1 and 2, 1% and 100% mutant mt3243 controls, respectively; lanes 3–5, 6–8, 9–11, 12–14, 15–17, 18–20, and 21–23 are degrees of heteroplasmy of mt3243 in the seven patients with diabetes (M8, M4, M9, M12, M13, M2, and M6, respectively). In each case, results are presented in the order of leucocytes, serum, and plasma samples (from left to right). U denotes the position of the uncleaved wild-type PCR product. C1 and C2 denote the positions of the restriction products of the mutant PCR product.

**Table 1** Proportions of mt3243 mutation in leucocytes, serum, and plasma of patients with type 2 diabetes mellitus

Subject	Age/Sex	Per cent mt3243 heteroplasmy		
		Leucocytes	Serum	Plasma
*M8	F/39	8.9	8.9	11.2
*M4	M/35	5.1	6.2	6.1
*M9	F/32	1.2	3.5	3.6
M12	F/37	13.0	25.2	29.3
M13	M/23	1.1	8.6	7.4
M2	F/38	13.5	35.2	36.5
M6	M/79	1.4	1.5	1.6

Each result is the average of two separate analyses.

\*Same pedigree.

from the healthy subjects showed no mutant bands. Thus, there was 100% concordance with the results obtained from leucocyte DNA ( $p < 0.001$ ).

The degrees of heteroplasmy in mt3243 were compared among serum, plasma, and blood leucocyte samples. The proportion of mt3243 in the seven affected patients varied from 1.1% to 13.5% in blood leucocytes, 1.5% to 35.2% in serum, and 1.6% to 36.5% in plasma (table 1 and fig 1). The amounts of mutant mt3243 in the serum fraction were higher than in the corresponding leucocyte fraction (Wilcoxon signed rank test,  $p = 0.031$ ). Similarly, the amounts of mutant mt3243 in the plasma fraction were higher than in the corresponding leucocyte fraction (Wilcoxon signed rank test,  $p = 0.016$ ).

## Discussion

In our study, mtDNA was detectable in plasma and serum samples from 25 healthy subjects and 16 patients with diabetes. Moreover, a mitochondrial mutation commonly found in patients with maternally inherited diabetes and deafness, mt3243, was detectable in both serum and plasma samples of patients with diabetes, and the amount of mutant mtDNA was significantly higher than that in blood leucocytes. Previous studies have shown that tumour specific DNA and fetal DNA are present in plasma and serum from patients with cancer and pregnant women.<sup>15–20</sup> This genomic DNA exists as oligonucleosomes in

which DNA is bound to histones and has a short half life.<sup>24–26</sup> In contrast, mtDNA is not bound to histones and might be more susceptible to degradation. Therefore, our observations support and extend the notion that DNA (including mtDNA) is present in the circulation and that it might be possible to use serum and plasma for molecular diagnosis of mt3243 associated diabetes.

Patients with diabetes had increased amounts of mt3243 in serum and plasma compared with leucocytes. The underlying mechanism and importance of this finding are unclear. It is possible that cell lysis from different tissues, as a result of physical and pathological damage, might lead to the release of cell free DNA into the circulation. Thus, the amount of mutant mtDNA detected in serum and plasma might reflect the average degree of heteroplasmy in different types of cells, rather than in leucocytes alone. The clinical importance of the raised mt3243 in patients' plasma or serum is worthy of investigation—for example, it might correlate with active disease staging or responses to stress or drug administration. In the latter case, serum and plasma mt3243 measurements might be potential markers of drug efficacy if drugs are developed to interfere with the relative growth of wild-type and mutation carrying mitochondria in the future.

The methodology described in our study represents an alternative approach to the examination of the genetic basis of mtDNA related disease using samples other than blood leucocytes. This might be particularly useful in elderly subjects, where mt3243 measurements are low in leucocytes.<sup>14</sup> Moreover, serum is an invaluable source in situations where the patients' tissues and blood leucocytes are not readily available—for example, in retrospective surveys involving patients who have either died or are not available, but whose serum has been saved. A similar approach may be applicable to the study of other mitochondrial mediated diseases such as the syndrome of myopathy, encephalopathy, lactic acidosis, and stroke like episodes; chronic progressive external ophthal-

moplegia; myopathy; eclampsia; and pre-eclampsia.

The authors sincerely thank Dr J van den Ouweland, Leiden University, for cloned DNAs carrying the wild-type and mutant form of mt3243. This research was supported by the Chinese University of Hong Kong direct grant 2040520, and Research Grants Council grant 4289/97M.

- 1 Anderson S, Bankier AT, Barrell BG, *et al*. Sequence and organisation of the human mitochondrial genome. *Nature* 1981;**290**:457–65.
- 2 Johns DR. The other human genome: mitochondrial DNA and disease. *Nat Med* 1996;**2**:1065–8.
- 3 Shoffner JM, Lott MT, Seibel P, *et al*. Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA<sup>Lys</sup> mutation. *Cell* 1990;**61**:931–7.
- 4 Goto Y, Nonaka I, Horai S. A mutation in the tRNA<sup>Leu(UUR)</sup> gene associated with the MELAS subgroup in mitochondrial encephalomyopathies. *Nature* 1990;**348**:651–3.
- 5 Mayr-Wohlfart U, Rodel G, Heneberg A. Mitochondrial tRNA(Gln) and tRNA(Thr) gene variants in Parkinson's disease. *Eur J Med Res* 1997;**2**:111–13.
- 6 Schnopp NM, Kosel S, Egensperger R, *et al*. Regional heterogeneity of mtDNA heteroplasmy in parkinsonian brain. *Clin Neuropathol* 1996;**15**:348–52.
- 7 Terese F, Norumm S, Torgge T, *et al*. Mutations in mitochondrial transfer ribonucleic acid gene in preeclampsia. *Am J Obstet Gynecol* 1996;**175**:1626–30.
- 8 Van den Ouweland JM, Lemkes HH, Ruitenbeek W, *et al*. Mutation in mitochondrial tRNA<sup>(Leu)(UUR)</sup> gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nat Genet* 1993;**1**:368–71.
- 9 Smith PR, Dronsfield MJ, Minovic CH, *et al*. The mitochondrial mtRNA<sup>Leu(UUR)</sup> A to G 3243 mutation is associated with insulin-dependent and non-insulin-dependent diabetes in a Chinese population. *Diabet Med* 1997;**14**:1026–31.
- 10 Rötig A, Bonnefont JP, Munnich A. Mitochondrial diabetes mellitus. *Diabet Metab* 1994;**22**:291–8.
- 11 Ciafaloni E, Ricci E, Servidei S, *et al*. Widespread tissue distribution of a mtRNA<sup>Leu(UUR)</sup> mutation in the mitochondrial DNA of a patient with MELAS syndrome. *Neurology* 1991;**41**:1663–5.
- 12 Goto Y, Horai S, Matsuoka T, *et al*. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): a correlative study of the clinical features and mitochondrial DNA mutation. *Neurology* 1992;**42**:545–50.
- 13 Matthews PM, Hopkin J, Stephenson JBP, *et al*. Comparison of the relative levels of the 3243 (A–G) mtDNA mutation in heteroplasmic adult and fetal tissues. *J Med Genet* 1994;**31**:41–4.
- 14 Hart LT, Jansen JJ, Lemkes HH, *et al*. Heteroplasmy levels of a mitochondrial gene mutation associated with diabetes mellitus decrease in leucocyte DNA upon aging. *Hum Mutat* 1996;**7**:193–7.
- 15 Mulcahy HE, Croke DT, Farthing MJG. Cancer and mutant DNA in blood plasma. *Lancet* 1996;**348**:628.
- 16 Stroun M, Anker P, Maurice P, *et al*. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* 1989;**46**:318–22.
- 17 Chen XQ, Stroun M, Magnenat J-L, *et al*. Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med* 1996;**2**:1033–5.
- 18 Nawroz H, Koch W, Anker P, *et al*. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* 1996;**2**:1035–7.
- 19 Stroun M, Anker P, Maurice P, *et al*. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* 1989;**46**:318–22.
- 20 Lo YMD, Corbetta N, Chamberlain PF, *et al*. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;**350**:485–7.
- 21 World Health Organization. *Diabetes mellitus: report of a WHO study group*. Technical Report Series 727. Geneva: WHO, 1985.
- 22 Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press, 1989.
- 23 Larsson NG, Tulinius MH, Holme E, *et al*. Segregation and manifestation of the tRNA<sup>Lys</sup> A–G (8344) mutation of myoclonus epilepsy and ragged-red fibers (MERRF) syndrome. *Am J Hum Genet* 1992;**51**:1201–12.
- 24 Bell DA, Morrison B. The spontaneous apoptotic cell death of normal human lymphocytes in vitro: the release of, and immunoproliferative response to, nucleosomes in vitro. *Clin Immunol Immunopathol* 1991;**60**:13–26.
- 25 Amoura Z, Piette JC, Chabre H, *et al*. Circulating plasma levels of nucleosomes in patients with systemic lupus erythematosus: correlation with serum antinucleosome antibody titers and absence of clear association with disease activity. *Arthritis Rheum* 1997;**40**:2217–25.
- 26 Rumore P, Muralidhar B, Lin M, *et al*. Haemodialysis as a model for studying endogenous plasma DNA: oligonucleosome-like structure and clearance. *Clin Exp Immunol* 1992;**90**:56–62.



## Presence of mitochondrial tRNA Leu(UUR) A to G 3243 mutation in DNA extracted from serum and plasma of patients with type 2 diabetes mellitus

Sheng Zhong, Maggie C Y Ng, Y M Dennis Lo, et al.

*J Clin Pathol* 2000 53: 466-469

doi: 10.1136/jcp.53.6.466

---

Updated information and services can be found at:

<http://jcp.bmj.com/content/53/6/466.full.html>

---

*These include:*

### References

This article cites 23 articles, 3 of which can be accessed free at:

<http://jcp.bmj.com/content/53/6/466.full.html#ref-list-1>

Article cited in:

<http://jcp.bmj.com/content/53/6/466.full.html#related-urls>

### Email alerting service

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

---

### Topic Collections

Articles on similar topics can be found in the following collections

[Immunology \(including allergy\)](#) (1279 articles)

[Ear, nose and throat/otolaryngology](#) (31 articles)

---

### Notes

---

To request permissions go to:

<http://group.bmj.com/group/rights-licensing/permissions>

To order reprints go to:

<http://journals.bmj.com/cgi/reprintform>

To subscribe to BMJ go to:

<http://group.bmj.com/subscribe/>