Lack of association of mitochondrial A3243G tRNA^{Leu} mutation in Iranian patients with type 2 diabetes

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Abstract
Many kinds of mutations in mitochondrial (mt) DNA have been reported to be related to the development of Diabetes Mellitus (DM), this type of diabetes has also been shown to be influenced by other genetic factors and/or environmental factors. Among them, tRNA^{Leu}(UUR) and its adjacent mtDNA NADH dehydrogenase subunit 1(ND1) region within the mt genome are linked to high susceptibility to DM. A point mutation at 3243 base pair (bp) in the mt tRNA ^{Leu}(UUR) is commonly referred to as a syndrome of mitochondrial myopathy, Encephalopathy, Lactic acidosis, and Stroke-like episodes (MELAS). In the current study, we have assessed the frequency of the A3243G in Iranian diabetic type 2 patients. DNA was obtained from peripheral leukocytes of 154 patients with diabetes Mellitus type2 (l50 with type 2 and 4 with gestational diabetes) and 40 control subjects. Insulin concentration from patients' blood was measured using Radioimmunoassay procedure. Patients showed fasting blood sugar (FBS) between 150-230 mg/dl, body mass index (BMI) between 19-32 Kg/m^2 and insulin concentration 0.9-2.35 mg/ml. PCR-RFLP, single strand conformation polymorphism (SSCP) and sequencing methods were used to detect the A3243G or other mutations in the mitochondrial tRNA^{Leu} (UUR) gene. A3243G mutation was not detected in patients. SSCP results showed a new pattern of PCR product in 6 patients. The C3316T transition mutation in the ND1 mitochondrial gene was confirmed in selected samples (n=6) by sequencing. No differences were observed between the two groups for C3316T and A3243G mutations (P=0.348). The mt C3316T mutation did not have any effect on the clinical finding of type 2 diabetes carrying this mutation. These data together with clinical characteristics of the patients may suggest that the mt C3316T mutation might be a polymorphism in the Iranian population.
Keywords: *Type 2 Diabetes; Mitochondria; A3243G mutation; C3316T mutation*

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INTRODUCTION

Diabetes mellitus (DM) affects over 150 million people world wide, with prevalence that varies markedly from population to population (Van Tilborg, 2001). Estimates predict that almost 300 million people will suffer from DM by 2025 with the vast majority being causes of diabetes mellitus type 2. Many risk factors have been identified which influence the prevalence or incidence. Factors of particular importance are a family history of diabetes mellitus, age, being overweight, increased abdominal fat, hypertension, lack of physical exercise, and ethnic background. Type 2 diabetes mellitus accounts for around 90% of all cases of diabetes mellitus. Since type 2 diabetes mellitus usually develops after the age of 40, the disease has also been called "adult onset type diabetes mellitus" (Reardon, 1992; Kadowaki and Van den Ouweland, 1994; Nakagawa, 1995).

Human mitochondrial DNA (mtDNA) is a double-stranded, 16569 base pair (bp) circular molecule that codes for 2 ribosomal and 22 transfer RNAs of mitochondrial protein synthesis and for approximately 13 polypeptides required for oxidative phosphorylation (OXPHOS) (Petras, 2003). The inheritance pattern is exclusively maternal in humans. Pathologic syndromes linked to mtDNA mutations have been shown

to result in abnormalities of OXPHOS function and have been characterized by degenerative abnormalities of the central nervous system, cranial nerves, and muscle (Shaffner, 1999; Gebrit, 1996). Therefore, mitochondrial dysfunction can affect almost all organ systems. Mitochondrial diseases show a variety of systemic manifestations. Mutations in mtDNA, including a point mutation at the nucleotide position of 3243, are known to be associated with diabetes mellitus as well as deafness. (Kobayshi and Tsukuda, 1997; Thorns, 1998). The latter has been identified in a pedigree with maternally inherited diabetes mellitus and deafness (MIDD) (Van den Ouweland, 1999; Doria and Massen, 2000; Massen, 2002, 2004).

In fact, numerous point mutations and deletions have been described in pedigrees with diabetes (Houshmand, 2003). The clinical characteristics of patients with the A3243G mutation include impaired insulin secretion, sensor neural deafness, and maternal inheritance of the disease. Once impaired insulin secretion becomes overt, most cases progressively develop a disease state of insulin dependence (Ohkubo, 2001). Our aim was to detect A3243G tRNA Leu (mtDNA) mutation in type 2 diabetes patients. The early preclinical diagnosis of type 2 diabetes might help to prevent or delay the onset of diabetes, while also allowing for selection of optimal treatment, thereby possibly avoiding diabetic complications altogether.

MATERIALS AND METHODS

In this study, 154 volunteers (87 males and 67 females) were randomly selected from patients treated at the Shariati hospital (150 patients with type 2 diabetes and 4 with gestational diabetes). The mean age was 54.5±11.3 years. Patients showed fasting blood sugar (FBS) levels of 150-230 mg/dl and body mass index (BMI) between 19-32 Kg/m^2, but they were initially treated with diet or oral hypoglycemic agents. Some patients, after several years need to be treated with insulin. 40 control subjects (23 males and 27 females) were also enrolled in our study who had BMI between 19-30 Kg/m^2 and FBS between 70-110 mg/dl. The mean age of control group was 53.8±10.2 years. The patients and control groups were selected according to ADA (American Diabetes Association) criteria. Insulin concentration from patients peripheral blood was measured using Radioimmunoassay.

mtDNA was isolated from the peripheral leukocytes of patients using a genomic DNA extraction kit (Diatom DNA extraction Kit, Genefanavaran, Tehran, Iran).

The following primers used for amplification: sense primer, (5´-CCTCCCTGTACGAAAGGACA-3´) and antisense primer (5´GCGATTAGAATGGGTACAAT3´) from nt3116-3353. PCR reactions were performed in a total volume of 25 µl containing 100 ng of total DNA; 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 20 pmol of each primer, 5 mM KCl, 200 mMTris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 1.5 unit of *Taq* DNA polymerase (Roche, Mennheim, Germany). PCR conditions were as follows: initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 60 seconds, annealing at 59°C for 60 sec, and extension at 72°C for 45 sec with a final extension at 72°C for 7 min. A 237 bp PCR fragment was separated by electrophoresis on a 8% denaturing polyacrylamide gel after cleavage by *Apa* I restriction enzyme overnight at 30°C to identify A3243G mutation. A positive control sample for A3243G was also used to ensure that experiment works well. The single strand conformation polymorphism (SSCP) method was used to detect possible mutations in this region, so the 237 bp amplified fragments were initially heated for 9 min at 99°C before loading onto the gel. Then Samples were separated on 8% polyacrylamide gel after denaturation to identify new mutation in tRNA^{Leu}(UUR) gene. Sequencing method was used to detect the mutation's position within these fragments which displayed new size pattern using the SSCP procedure (GeneFanavaran, Macrogen Korea).

Table 1. Insulin concentration, BMI and FBS in diabetic patients and control.

		BMI Kg/m^2	Insulin concentration mg/ml	FBS mg/dl
Number of Diabetic Patients	104	19-32	o.16-o.64 (normal range)	150-210
	45	25-32	≥0.64 (increased insulin)	180-230
	5	19-25	< 0.16 (reduced insulin)	150-200
Number of Non-diabetic controls	40	19-30	0.16-0.64	70-110

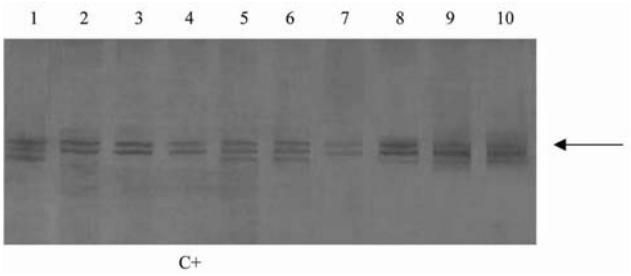

Figure 1. PCR-SSCP of the A3243G mutation. Samples were loaded on to 8% acryl amide gel, normal controls (Lane 2), and positive Control (Lane 4) and 2 patients (Lane3, 7) showed 2 bands, 6 women patients showed 3 bands (first band showed by arrow).

Statistical Analysis: Fisher's exact probability test was used to examine the association between two groups. Values of $P< 0.05$ were regarded as statistically significant.

RESULTS

Insulin measurement showed that 104 patients had normal insulin concentration (0.16-0.64 mg/ml), BMI 19-32 Kg/m^2 and FBS 150-210 mg/dl. 45 patients showed increased insulin concentration (0.64-2.35 mg/ml), BMI 25-32 Kg/m^2 and FAS 180-230 mg/dl. Five patients also showed reduced insulin concentration (0.9-0.16 mg/ml), BMI 19-25 Kg/m^2 and FAS 150-200 mg/dl (Table 1). Digestion of *Apa*I was not detected in DNA samples from the patiens. SSCP analysis showed a new band pattern in comparison to the normal healthy controls (Fig. 1). Sequencing identified a C3316T transition mutation in the ND1gene in patients (Fig. 2). This gene encodes the NADH dehydrogenase subunit 1. The C3316T mutation was detected in 6 women patients out of 154 that had higher insulin levels (0.9-1.54 mg/ml, fasting blood were between 200-230 mg/dl and their BMI were between 25-32 Kg/m^2) undergone diet therapy or drug therapy with hypoglycaemic agents. None of the controls showed C3316T mutations. No differences were observed between the two groups for the mutation of C3316T (P=0.348) and A3243G (Table 2). The mt C3316T mutation did not have any effect on the clinical finding of type 2 diabetes carrying this mutation (Table 3).

DISCUSSION

In the past years, several authors have attempted to assess the contribution of mt A3243G mutation to the genetic susceptibility of type 2 diabetes. In the Chinese population, the prevalence of mt A3243G mutation in studied population carrying type 2 diabetes varied from 0.4 - 0.8% (Xiang, 1997; Ji, 2001). Also, it was reported that the prevalence of this mutation was within a range of 1-3% in Japanese diabetic populations (Tokunaga, 1993; Katagiri, 1994; Fukui, 1997; Suzuki, 2004) and 2% in the French population. (Kishimoto *et al*., 1995). The U.K. Prospective Diabetes Study showed a low frequency of this mutation among

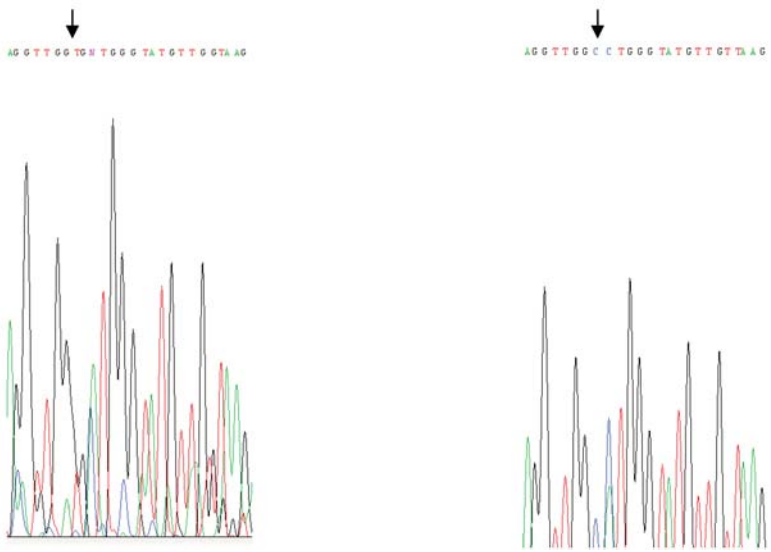

Figure 2. Sequence of the patient's and normal mtDNA using an automated sequencer (ABI 3700) showed the C3316T mutation.

Table 2. Prevalence of mitochondrial gene variation in cases with T2DM and control.

mtDNA Mutation	T2DM	Control	P-Value
C3316T	6/154=3.9%	0	0.348
A3243G	0	0	-

Table 3. Comparison between clinical profile of type 2 diabetes with C3316T mutation and individuals without mutation.

	mtDNA C3316T(+) n=6	mtDNA C3316T(-) n=148
Insulin concentration (mg/ml)	0.9-1.54	0.16-0.64
Glucose (FBS) mg/dl	200-230	70-110
BMI kg/m^2	25-32	19-30

patients with the type 2 diabetes phenotype (Saker, 1997). In a study by Abad *et al*. (1997), the A3243G mtDNA mutation was not detected in any patients examined. They concluded that this mutation may be uncommon in affected children from various ethnic and racial groups. An A-to-G mutation at nt3243 in the mitochondrial tRNA^{leu}(UUR) gene is the most common. (Lynn *et al*., 2003). However, neither the patients nor the controls had the A3243G mtDNA mutation in our study. In the present study, it was shown that the 3243 mutation is not a common mutation in Iranian diabetes type II patients. Previously, we showed that the A3243G mutation can not have any effect when its heteroplasmy status is less than 50% (Houshmand, 2003). The percentage of cells containing mtDNA with the heteroplasmic A3243G mutation varies from tissue to tissue and may be highest in affected tissues or organs (Hart *et al.,* 1994). The pancreas thus is a good source for examination of the A3243G mutation in DM patients and using peripheral blood as in our study, may result in the mutation being missed. Another reason for the results may be the small size of the sample. Therefore, a more sensitive method such as the combination of peptide nucleic acid (PNA) and allele specific PCR (Urata *et al.,* 2004) or larger sample size may be required. Recent studies have shown that many mutations in the mtDNA ND1 gene region are related to DM (Yu P, 2004 and Guo, 2005). The ND1 gene product is a NADH dehydrogenase (complex I) subunit I and may influence the function of the enzyme (Wollheim, 2000). One of ND1 gene mutations is C3316T, resulting in a transition from alanine to threonine at the protein level (Nakano, 1998). Frequency of this mutation was found to be significantly higher in Japanese type 2 diabetes patients compared to those in normal glucose tolerant controls (3.4% vs 0.9%, P=0.02), (Nakagawa, 1995; Odawara, 1996; Ji, 2001). The authors suggested that the C3316T mutation is associated with Non Insulin Dependent Diabetes Mellitus (NIDDM). C3316T mutations implicated in NIDDM were also seen at higher frequencies in patients with Gestational Diabetes Mellitus (GDM) than the controls in a Singaporean population (Chen *et al*., 2000). It was also reported that the C3316T mutation is a polymorphism unrelated to diabetes in the Chinese population (Ji, 2001). They found that the mt C3316T mutation did not exert any different effect on the major clinical characteristics (age of onset, BMI, Insulin secretary capacity) of type 2 diabetes carrying this mutation and hence is a polymorphism.

In this study C3316T mutation was detected in 6 women suffering from diabetes type II (3.9%) with insulin resistance (their insulin concentration was 0.9-1.54 mg/ml, fasting blood 200-230 mg/dl and their BMI were between 25-32 Kg/m^2) who had only undergone diet therapy or drug therapy with hypoglycaemic agents. Comparison between patients and normal control showed that there was no difference between normal group and patients (P>0.05). Also, the mtC3316T mutation did not have any effect on the clinical findings of type 2 diabetes carrying this type of mutation. Therefore, in this study of a cohort of Iranian type 2 diabetes patients and 40 normal controls, we found that the prevalence of mt A3243G mutation was zero in Iranian type 2 diabetes and mtDNA C3316T mutation seems to be a polymorphism in the Iranian population.

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References

Becker R, Laube H, Linn T, Damian MS (2002). Insulin resistance in patients with the mitochondrial tRNA(^{Leu}(UUR)) gene mutation at position 3243. *Exp Clin Endocrinol Diabetes*. 110: 291-297.

Chen Y, Liao WX, Roy AC, Loganath A, Ng SC (2000). Mitochondrial gene mutations in gestational diabetes mellitus. *Diabetes Res Clin Pract.* 48: 29-35.

Doria A, Kim H, Weremowicz S, Ercinolino T, Powers Ch Mlynarski, Bashan KA, Warram JH, Mychalekyi, Rich S S, Krolewski AS (2000). Recent advances in the genetics of maturity-onset diabetes of the young and other forms of autosomal dominant diabetes. *Curr Opin Endocrine Diabetes*. 7: 203-210.

Fukui M, Nakano K, Obayashi H, Kitagawa Y, Nakamura N, Mori H, Kajiyama S, Wada S, Fujii M, Yoshimori K, Kanaitsuka T, Shigeta H, Kondo M (1997). High prevalence of mitochondrial diabetes mellitus in Japanese patients with major risk factors. *Metabolism*, 46:793-795.

Gerbitz KD, Gempel K, Brdiczka D (1996). Mitochondria and diabetes: genetic, biochemical and clinical implications of the cellular energy circuit. *Diabetes*, 45:113-126.

Guo LJ, Oshida Y, Fuku N, Takeyasu T, Fujita Y, Kurata M, Sato Y, Ito M, Tanaka M (2005). Mitochondrial genome polymorphism associated with type 2 diabetes or obesity. *Mitochondrion*, 5: 15-33.

Hart LM, Lemkes HH, Heine RJ, Stolk RP, Feskens EJ, Jansen JJ, *et al*. (1994). Prevalence of maternally inherited diabetes and deafness in diabetic populations in The Netherlands, *Diabetologia*, 37:1169-1170.

Houshmand M (2003). Mitochondrial DNA Mutations Pathogencity and Inheritance**.** A review article, *Iranian J Biotechnol*. 1: 1-18.

Ji L, Hou X, Han X (2001). Prevalence and clinical characteristics of mitochondrial tRNA leu (UUR) mt 3243 A—>G and ND-1 gene mt 3316 G—>A mutations in Chinese patients with type 2 diabetes. *Chin Med J* (Engl). 114: 1205-7.

Kadowaki T, Kadowaki H, Mori Y, Tobe K, Sakuta R, Suzuki Y, Sakura H, Awata T, Goto Yu, Hayakawa T, Matsuka K, Kawamori R, Kamada T, Horai S, Nonaka I, Hagura R, Akanuma Y, Yazaki Y (1994). A subtype of diabetes mellitus associated with a mutation of mitochondrial DNA. *New Engl J Med*. 330: 962-968.

Katagiri H, Asano T, Ishihara H, Inukai K, Anai M, Yamanouchi T (1994). Mitochondrial diabetes mellitus: prevalence and clinical characterization of diabetes due to mitochondrial tRNA$^{Leu(UUR)}$ gene mutation in Japanese patients. *Diabetologia*. 37: 504-510.

Kishimoto M, Hashiramoto M, Araki M, Ishida Y, Kazumi T, Kanda E, *et al*. (1995) Diabetes mellitus carrying a mutation in the mitochondrial tRNA(Leu(UUR)) gene. Diabetologia 38:193-200

Kobayshi T, Nakanishi K, Nakase H, Kajio H, Okubo M, Murase T, Kosoka K (1997). Institute characterization of islet in diabetes with a mitochondrial DNA mutation at nucleotide position 3243. *Diabetes*. 46:1567-1571

Lynn S, Borthwick GM, Charnley RM, Walker M, Turnbull DM (2003) Heteroplasmic ratio of the A3243G mitochondrial DNA mutation in single pancreatic beta cells, *Diabetologia*, 46: 296-299.

Maassen JA, Hart LM, Essen E, Heine R J, Nijpels G, Jahangir Tafrechi RS, Raap AK, Janssen G, Lemkes H (2004). Mitochondrial Diabetes. *Diabetes*. 53: S103-S109**.**

Maassen JA (2002). Mitochondrial diabetes: Path physiology, clinical presentation, and genetic analysis. *Am J Medical Genetics*. 115: 66-70.

Massen JA, Van Essen E, Vanden ouweland JM, Lemekes HH (2000). Molecular and clinical aspects of mitochondrial diabetes Mellitus. *Exp Clin Endocrina Diabetes*. 109:127-134.

Nakagawa Y, Ikegami H, Yamato E, Takekawa K, Fujisawa T, HamadaY, Ueda H, Uchigata Y, Mik T, Kumahara Y, Ogihara T (1995). A new mitochondrial DNA mutation associated with non-insulin-dependent diabetes mellitus. *Biochem Biophys Res Commun*. 209: 664-668.

Nakano S, Fukuda M, Hotta F, Ito T, Ishii T, Kitazawa M, Nishizawa M, Kigoshi T, Kakinuma H, Takahashi H, UchidaK (1998).Mitochondrial DNA point mutation at nucleotide pair 3316 in a Japanese family with heterogeneous phenotypes of diabetes. *Endocr J*. 45: 625-630.

Odawara M, Sasaki K, Yamashita K (1996). A G-to-A substitution at nucleotide position 3316 in mitochondrial DNA is associated with Japanese Non-insulin- dependent diabetes mellitus. *Biochem Biophys Res Commun*. 227:147-151.

Ohkubo K, Yamano K,Nagashima M, Mori Y, Anzail K, Akehi Y, Nomiyama R, Asano T, Urae A Ono J (2001). Mitochondrial gene mutations in tRNA Leu (UUR) Region and diabetes: prevalence and clinical phenotypes in Japans. *Clin Chem*. 47: 1641-1648

Dzeja PP, Terzic A (2003). Phosphotransfer networks and cellular energetic. *J Exp Biology*. 206: 2039-2047

Reardon W, Ross RJ, Sweeney MG, Luxon LM, Pembrey ME, Harding AE, Trembath RC (1992). Diabetes mellitus associated with a pathogenic point mutation in mitochondrial DNA. *Lancet*, 340: 1376-1379

Saker PJ, Hattersley AT, Barrow B, Hammersley MS, Horton V, Gillmer MD, Turner RC (1997). low prevalence of the mitochondrial transfer RNA gene (tRNA(Leu)mutation at position 3243bp in UK Caucasian type 2 diabetic patients. *Diabet Med*. 14: 42-45.

Shoffner JM (1999) Oxidative phosphorylation diseases diagnosis. *Ann NY Acad Sci*. 893:42-60.

Suzuki S (2004). Diabetes mellitus with mitochondrial gene mutations. Japan. *Ann NY Acad Sci*.1011: 185-192.

Thorns C, Widjaja A, Boeck N, Skamira C, Zuhlke H (1998). Maternally inherited diabetes and deafness: report of two affected German families with the A3243G mitochondrial DNA mutation. *Exp Clin Endocrinal Diabetes*. 06: 384-388.

Tokunaga M, Mita S, Sakuta R, Nonaka I, Araki S (1993). Increased mitochondrial DNA in blood vessels and ragged-red fibers in mitochondrial myopathy, enchephalopathy, lactic acidosis, and stroke-like episodes (MELAS*). Ann Neurol*. 33: 275-280.

Tsukuda K, Suzuki Y, Kameoka K, Osawa N, GotoY, Katagiri H, Asano T, Yazaki Y, Oka Y (1997). Screening of patients with maternally transmitted diabetes for mitochondrial gene mutations in the tRNA$^{Leu(UUR)}$ region*. Diabet Med*. 14: 1032-1037.

Urata M, Wada Y, Kim SH, Chumpia W, Kayamori Y, Hamasaki N, *et al*. (2004) High-sensitivity detection of the A3243G mutation of mitochondrial DNA by a combination of allele specific PCR and peptide nucleic acid-directed PCR clamping, *Clin*

Chem. 50:2045-2051.

Van den Ouweland JM, Lemkes HH, Trembath RC, Ross R, Velho G, Cohen D, Froguel P , Maassen JA (1994). Maternally inherited diabetes and deafness is a distinct subtype of diabetes and associates with a single point mutation in the mitochondrial Trna (Leu)(UUR) gene. *Diabetes*, 3:746-751.

Van den Ouweland JM, Maechler P, Wollheim CB, Attardi G, Maassen JA (1999). Functional and morphological abnormalities of mitochondria harbouring the tRNA(Leu)(UUR) mutation in mitochondrial DNA derived from patients with maternally inherited diabetes and deafness (MIDD) and progressive kidney disease. *Diabetologia*, 42: 485-92.

Van Tilborg J, Van Heaften T,Pearson P, Wijmenga C (2001). Defining the genetic contribution of type 2 diabetes mellitus. *Med Genet*. 38: 569-578

Wollheim CB (2000). Beta-cell mitochondria in the regulation of insulin secretion: A new culprit in Type 2 diabetes. *Diabetologia*, 43: 265-277.

Xiang K, Wang Y, Wu S, Lu H, Zheng T, Sun D, Weng Q, Jia W, Shen W, Pu L, He J (1997). Mitochondrial tRNA(Leu)(UUR) gene mutation diabetes mellitus in chinese. *Chinese Medical J*. 110:372-378.

Yu P, Yu DM, Liu DM, Wang K, Tang XZ (2004)**.** Relationship between mutations of mitochondrial DNA ND1 gene and type 2 diabetes. *Chin Med J* (Engl). 117: 985-9.