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SCREENING FOR SOME MUTATIONS IN MITOCHONDRIAL *ND1* GENE ASSOCIATED WITH T2DM IN IRAQI POPULATION

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ABSTRACT

This study was conducted to investigate the role of mitochondrial *ND1* gene in the incidence of type 2 diabetes mellitus (T2DM). Fifty T2DM patients and thirty apparently healthy individuals (control) were used in this study. Blood samples were collected at National Center for Diabetes treatment and Research- College of Medicine ,University of Al-Mustansiryah from both gender (42 male and 38 female), aged between 50-70 years. Statistical analysis of the questioner form showed that most of the T2DM patients are overweight with body mass index (BMI) value ≥ 25kg. Conventional polymerase chain reaction was used for identification and amplifying the specific DNA fragment and then the PCR product incubate with *Hae III* restriction enzyme, then loaded by agarose gel electrophoresis. The G3316A mutation in the NADH dehydrogenase (ND1) gene was confirmed by sequencing. Results revealed that G3316A mutation in the *ND1* gene was not associated with type 2diabetes mellitus risk in Iraqi patients. In addition, the sequence analysis indicated the presence of m.3480A>G in 4 control individuals; m.3505A>G in one T2DM patient; m. 3429C>T in 2 T2DM patients and m.3713delT in 7 control individuals and 10 T2DM patients. There was no correlation between mitochondrial *ND1* gene mutations studied herein and type 2 diabetes mellitus in Iraqi patients.

KEYWORDS: mitochondrial *ND1* gene, mutation, T2DM.

INTRODUCTION

Mitochondrion is a structure within cells that convert the energy from food into a form that cells can use. Mitochondrial DNA (mtDNA) comprises typically less than 1% of a metazoan

cell's DNA population, it is organized as a circular, double-stranded DNA molecule (Clayton, 1982), mtDNA is maternally inherited (Sutovsky, 2003). The systems of mtDNA reparation act less efficiently than those of nuclear DNA, this results in mtDNA mutation rate 10–20 times higher than nuclear DNA (Boesch *et al*., 2011). The importance of the mitochondrial genetic factors in its pathogenesis of type 2 diabetes has long been suggested, several mutations in mtDNA are indeed expressed as DM, but of more than 70 mtDNA mutations that have been suggested to be associated with DM, only one, an A3243G substitution in the $tRNA^{leu}$ gene, is in fact firmly established to be causal for DM (Pranoto, 2007). The *MT-ND1* is one of seven mitochondrial DNA encoded subunit. MT-ND1 gene provides instructions for making a protein called NADH dehydrogenase 1 (ND1),a large enzyme complex known as complex I, which is active in mitochondria (Attardi *et al* .,1986) . During oxidative phosphorylation, mitochondrial enzyme complexes carry out chemical reactions that drive the production of ATP (Galloway and Yoon, 2013).

Recent studies have indicated that many mutations in mitochondrial DNA ND1 gene region are related to the pathogenesis of many diseases ,the ND1 subunit gene is a mutational hot spot for LHON (Valentino *et al* .,2004). Also mutations in mtDNA ND1 gene at nt3316 (G-A), nt 3394(T-C) and 3426(A-G)may contribute to the pathogenesis of DM (Yu *et al*., 2004). Elango *et al*. (2014) described, in T2DM patients, novel mutations in *Cyt b*, *ATPase 8*, *ND1* and *ND5* genes excreted synergistic activity as plausible factors for the secondary complications of a patient with chronic T2DM. Iraq considered as having a medium prevalence of diabetes, in Basrah population, DM prevalence found to be within the wide range of diabetes in Middle East (Mansour *et al*., 2008). However, Mansour *et al*. (2014) found that one in five individuals had diabetes and revealed that the peak age of diabetes in both sexes was in the fourth to sixth decades of life. Diabetes was also found to affect 21.9% of Iraqis living in Sweden (Bennet *et al*., 2011). No previous studies on the correlation between the mtDNA mutations and T2DM incidence in Iraqi patients. However, in other world places, many studies indicate that there is a lot of mutations in the mtDNA cause T2DM. The present study was designed to study the correlation between mtDNA mutations in*16S rRNA*, $tRNA^{Leu(UUR)}$ and *ND1* genes with T2DM incidence.

MATERIALS AND METHODS

Subjects: Subjects included in this study were eighty person, distributed into, type 2 diabetes mellitus patients (n=50) and apparently healthy individuals(n=30) from both males and

females, with average age between 45-70 years. T2DM patients already diagnosed by specialist doctors in the National Center for Diabetes treatment and Research –University of Al-Mustansiryah – College of Medicine, with random blood glucose more than 200mg/dl distributed according to gender into twenty six females and twenty four males. Thirty apparently healthy individuals from the clinic visitors in Shalchia health center, Baghdad, Iraq, 18 males and 12 females, with random blood glucose less than 300mg/dl.

A questionnaire forma was prepared to obtain information about patients, including name, age, family history, years of diagnosis, height, weight, smoking, gender, hypertension, retinopathy and nephropathy.

DNA extraction

Total DNA was extracted from blood samples using Genaid kit. A900 bp fragment of the mtDNA from nt 2826 to nt 3726 was amplified using forward primer GAGCAGAACCC AACCTCCGAGCAG and reverse primer GATTGTTTGGGCTACTGCTCGC. PCR was carried out for 30 cycles, first cycle (initial denaturation) was at 95°C for 5 min, denaturation at 95°C for 1min annealing at 56°C for 90 sec and elongation at 72°C for 2 min 30 sec the program was applied according to Pranoto (2005). All the amplified samples were digested with *Hae III* restriction enzyme using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method to detect the presence of G3316A. The DNA fragments were separated by electrophoresis on 3.5% agarose gel for 2 hours. The presence of the substitution mutation G3316A in the *ND1* gene was confirmed by sequence analysis with AB13730XL APPLIED BIOSYSTEMS machine in NICM/USA Company.

RESULT AND DISCUSSION

In the present study, DNA was extracted from fresh blood samples of type 2 diabetes patients and apparently healthy individual , using Geneaid kit, the procedure was very efficient and showed sharp band with a good DNA concentration (30-90µg/µl),as shown in figure (1).

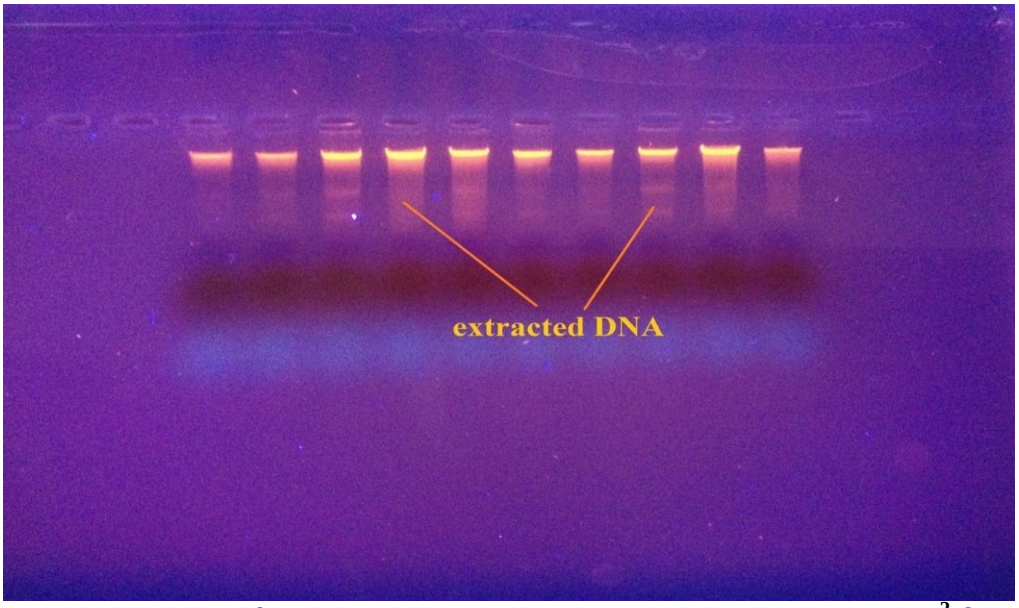


Figure 1: Genomic DNA from diabetic patients.1% agarose gel at 5 volt/cm^2 for 30 min. then visualized under U.V after staining with Ethidium bromide.

PCR: PCR was used to amplify a specific region in the mitochondrial DNA. One set of primer used according to Pranoto,(2005) was used to identify the mtDNA fragment with a molecular weight of 900 bp in the region of *16S rRNA*, *tRNA*$^{Leu(UUR)}$ and *ND1* genes. The identified fragment was appear as sharp band sized 900 base pair on agarose gel electrophoreisis (Figure 2)

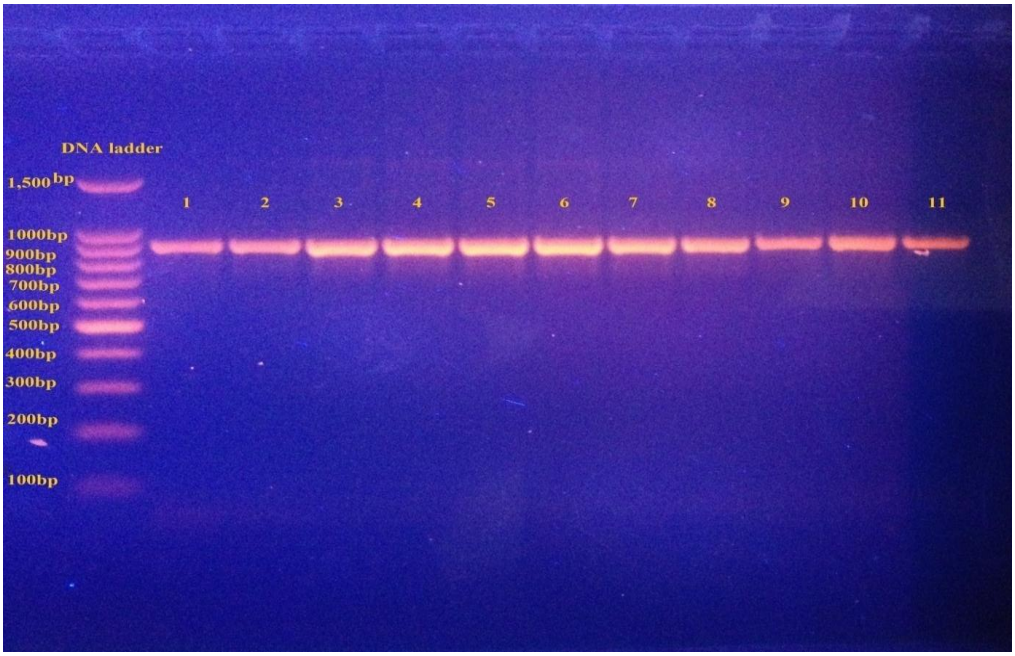


Figure 2: PCR product (900bp). The electrophoresis was on 2% agarose gel at 5 volt/cm^2 for 2 hours. Line 1 DNA ladder (100 bp), line (2-12) PCR product (900bp). visulized under U.V light after staining with Ethidium bromide.

The position of 3316 in the mitochondrial gene are located in the coding region of NADH dehydrogenase subunit (ND1). The m.3316G>A is a missence mutation which induce an exchange of amino acid Alanine for Threonine.The presence of mitochondrial G3316A mutation was determined by polymerase chain reaction amplification and restriction fragment length polymorphism (PCR-RFLP) as shown in Figure 3. Finally, mutant mitochondrial DNA was confirmed by DNA sequencing (Figure 4). The mitochondrial DNA mutation at position 3316 was found in 2 out of 30(6.7%) control individuals and this mutation was not found in patients with type 2 diabetes (n=50) ($p \leq 0.05$, OR=0.539, X^2=4.291) . This result suggest that the prevalence of the mitochondrial *ND1* gene at positions 3316 (G/A) mutation may be a polymorphism unrelated to diabetes in Iraqi patients. The G3316A mutation is a homoplasmic, which are generally considered to reflect neutral variation (Crispim *et al.*, 2008). Also, This mutation was reported as being associated with type 2 DM in Japanese diabetic subjects (Fukuda *et al.*, 1999), Chinese Han population (Liu *et al.*, 2007) and Chinese population (Tang *et al.*, 2006). However, some authers did not observe any significant differences between the frequency of this mutation among diabetic patients and healthy subjects, suggesting that this mutation is only neutral polymorphisms (Lam *et al.*, 2001; Ohkubo *et al.*, 2001; Crispim *et al.*, 2002). The G3316A mutation was found in 3.4% of patients with type 2 diabetes mellitus in Japan (Odawara *et al*, 1996).

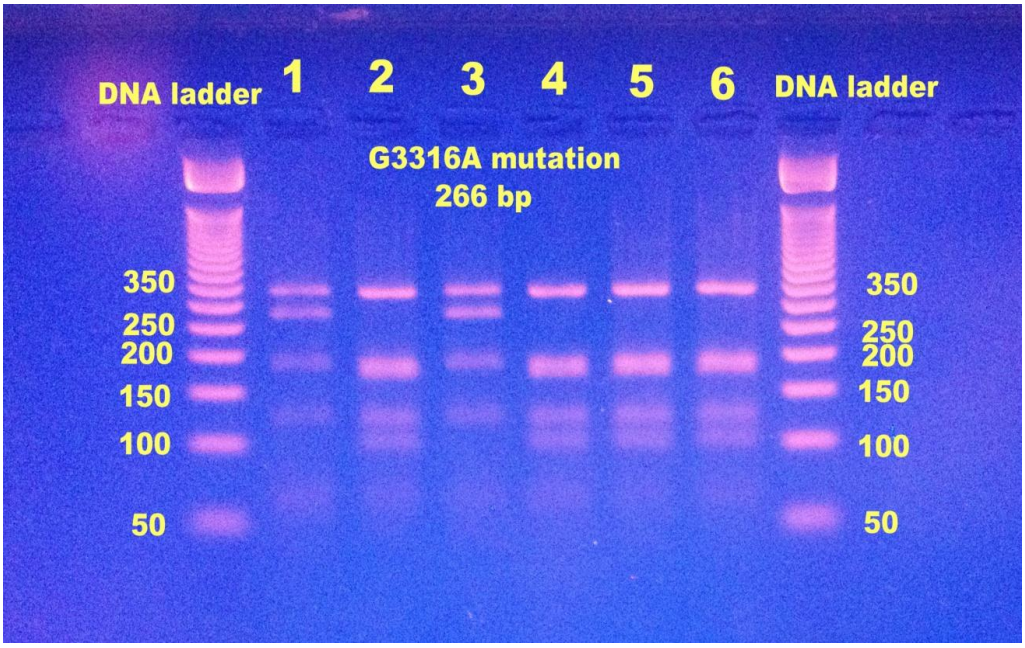
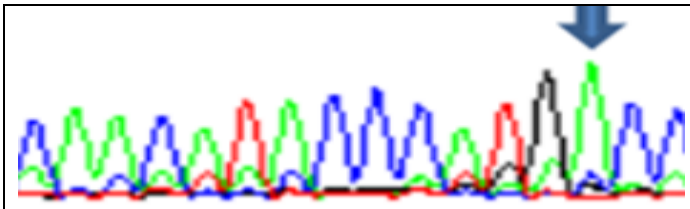


Figure 3: PCR-RFLP analysis of the *HaeIII* digest of the PCR product that contains position 3316 of the mitochondrial *ND1* gene separated on a 3.5 % agarose gel. DNA ladder=50 bp; normal cases contain fragments of 322, 180, 169, 120, 97 and 15 bp (Lanes 2,4, 5 and 6); mutant cases contain fragments of 322, 266, 180, 120 and 15 bp (Lanes1 and 3).



Query 451 TACCCATGACCAACCTC 467
Sbjct 3308 TACCCATGGCCAACCTC 3324

m.3316G>A

Figure 4.Electropherogram depicting the 3316G>A position and its flanks.

The m.3480A>G mutation was identified in 4 out of 20 apparently healthy control whereas not found in type 2 diabetes mellitus patients ($p \leq 0.01$; OR= 1.277; X^2= 7.831).The m.3505A>G mutation is missense that change the amino acid threonine to alanine. This mutation was detected in one type 2 diabetes mellitus patient whereas not identified in apparently healthy control. The incidence percentage of m.3429C>T silent mutation was higher in type 2 diabetes mellitus patients (10%) than in apparently healthy control(0%)($p \leq 0.05$; OR= 0.539; X^2= 4.291). Also, the incidence percentage of m.3713delT mutation that cause frame shift and amino acid change from valine for glutamine was 50% in type 2 diabetes mellitus patients compared with 35% in apparently healthy controls (p≤0.05; OR= 0.869; X2= 5.027). (Table 1)

Table 1: Mutations of mitochondrial *ND1* gene that identified in Iraqi apparently healthy individuals and type 2 diabetes mellitus patients in the present study.

Mutation	Group	%(n)	Type	Effect	Originality	OR	X^2
m.3348A>G	Control	20%(4)	Sub	Silent	Rs28358584	1.277	7.831**
	T2DM	0%(0)					
m.3505A>G	Control	0%(0)	Sub	Missense	RS28358585	0.0267	0.750 NS
	T2DM	5%(1)					
m.3429C>T	Control	0%(0)	Sub	Silent	Novel	0.539	4.291*
	T2DM	10%(2)					
m.3713delT	Control	35%(7)	Del	Frameshift	Novel	0.869	5.027*
	T2DM	50%(10)					

OR=odd ratio; X^2= chi square; NS=no significant; *=significant at 0.05 level; **=significant at 0.01 level.

There was no correlation between mitochondrial DNA mutations studied herein and type 2 diabetes mellitus incidence in Iraqi T2DM patients.

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