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Analysis Of Cell Free DNA And Cf-Mt DNA As Molecular Markers InPatients With Type-2 Diabetes Mellitus

Alaa Ramthan Hussein and Prof. Dr. Zafir Hassan Ghali

Department of Biology, College of Education for pure sciences, University of Wasit, Iraq.

Abstract

Type 2 diabetes mellitus (T2DM) is a major cause of morbidity and mortality worldwide, and the incidence is increasing. Cell free circulating DNA (cf DNA) derivedfrom dead cells and detectable in plasma and serum, is a potential biomarker for a variety of diseases including T2DM. The calculation of gene expression fold change was made using relative quantification using RCR. The results revealed that the mean ±SE of Ct values of cf-n DNA and cf-mt DNA for each studied group. The Ct value of cf-n DNA ranged from 10.10- 29.4 with a mean±SE20.4 ± 0.38. In control group, the Ct value ranged from 18.7 - 21.2 with a mean±SE19.9 ± 0.174. The difference in Ct values between the two groups was found to be non-significant. However, The mean ±SE Ct value of plasma cell free mitochondrial DNA was 31.4 ± 0.7 in T2DM patients group. It ranged from 20- 36. In control group, the Ct value ranged from30.6– 36 with a mean±SE 32.9 ± 0.3. There was a significant difference in the Ct mean values between the two studied groups (p=0.04). Results of ΔCt for control group displayed a mean of 12.9 ±0.384 ,whereas for patients group 10.9±0.835 with a significant difference p=0.02 when compared patients to controls. Results of ΔΔCt showedsignificant difference for patients group with a mean -1.94± 0.835 .The fold of gene expression in patients group of cf-mt DNA was 18.9 time higher than that of control group .These results indicate that cf-mt DNA increases significantly in patients with type2 diabetes mellitus.

Conclusion: These results reveals that both cf-n DNA and cf-mt DNA could be significant biomarkers for type2DM .

Keywords: Cell free DNA, cf-mt DNA, type-2 diabetes mellitus, RT-PCR

Introduction

The term "Diabetes mellitus" is used to describe a set of metabolism diseases characterized by persistent hyperglycemia. It is caused by either reduced insulin production or insulin effectiveness, or, more commonly, both (Petersmann et al., 2018).There are three types of diabetes mellitus: type 1diabetes, type 2 diabetes, and gestational diabetes. Diabetes, if not properly controlled, can lead to serious health issues (Hassan et al., 2020). Relative insulin insufficiency induced by pancreatic-cell failure and insulin resistance in target organs define Type 2 diabetes. One of the world'smost prevalent and fastest-growing diseases, with 693 million individuals expected to be affected by 2045, an increase of more than fifty percent from 2017 (Cho et al., 2018).This illness is currently one of the most common sources of disease burden across the world (Safiri et al., 2019). Thus according worldwide estimates approximately 451 million individual worldwide had diabetes mellitus in 2017, with more than 5 million fatalities due to the disease (Cho et al., 2018). Cell-Free DNA (cf-DNA) is severely degraded DNA fragments that may be found in every human's peripheral blood. The hematopoietic system provides the vast bulk of cf-DNA. The composition of cf-DNA can alter under specific physiologic or pathologic conditions (Pantel and Alix-panabie,2010). With a half-life of less than an hour, cf DNA molecules are quickly removed from circulation (Fan et al., 2010). Cell free DNA is a double-stranded molecule that appears in both plasma and serum and is made up of tiny fragments (70 to 200 base pair) and bigger fragments with molecular weights of up to 21 kilobases (Jahr et al., 2001). Necrosis, Apoptosis, and active cellular secretion are three potential causes for the occurrence of c-f DNA (Bronkhorst et al., 2019). Cell-free mitochondrial DNA is acharacteristic of many age-related illnesses and has been investigated as a diagnostic for systemic inflammation (Silzer et al. 2019(. The presence of mt DNA in plasma andserum was originally discovered more than 20 years ago. Nearly 10 years later, a connection was established between higher levels of circulating, cell-free mt DNA (ccf-mt DNA) and higher rates of mortality in trauma sufferers. (Ware et al., 2020). The aimof this study is to investigate the clinical significance of cell-free DNA (cf DNA) in patients with type 2 Diabetes mellitus (T2DM).

Materials and Methods

This study included (40) patients with T2DM (20 males and 20 females), and (20) apparently individuals (controls) (10 males and 10 females) their age range was (mean ± SD=20, median = 44.2) They were selected in Al-Rifai General Hospital in Dhi Qar Governorate. The glycemic status of diabetic patients and controls was used to differentiate between them. Oral consent was taken from all participants. Age, gender,weight, body mass index, smoking, medications, and medical or family history of diabetes were measured using standardized questionnaires. Five milliliters of blood were collected from all participants,. 5 ml of whole blood samples were withdrawn from subjects vein after 8 hours of fasting and delivered quickly to EDTA-K3- containing plastic tube. Plasma was collected by blood cooling centrifugation divided in to several aliquots , and kept in – 80 o C° until final analysis.

DNA Extraction

DNA isolation from plasma was performed Quick-gDNA TM Blood Mini Prep CatalogNos . D3072& D3073 according to the manufacturer's guidelines.

Plasma DNA Quantification by RT-PCR

Baseline plasma cf- mt DNA and cf- n DNA were quantified by real time PCR using KAPA SYBR® FAST qPCR Master Mix(2X) kit . primers sequences used in RT-qPCRβ-globin gene forward 5' -GTGCACCTGATCCTGAGGAGA -3' , reverse 5'- CCTTGATACCAACCTGCCCAG-3', MT-ND1 forward 5'-AACATACCCATGGCCAACCT-3', reverse 5'-AGCGAAGGGTTGTAGCCC-3'.
The gene expression levels and fold changes were quantified by measuring the threshold cycle (Ct) employing the KAPA SYBR® FAST qPCR Master Mix (2X) Kitcomponents . Every reaction was done in a duplicate and included a non-template control (NTC), non-amplification control (NAC) and non-primer control (NPC) as negative control. The thermal profile for cf-DNA and mt-DNA expression was as follows: denaturation at 95°C for 5 min, followed by 40 cycles of 20 sec at 95 °C, 20 sec at 60 °C and 20 sec at 72°C. DNA concentrations expressed asng/µl.

Statistical Analysis

Data were analyzed with the SPSS 21.0 software (SPSS Inc ., Chicago, IL, USA).The comparison of significant (P-value) in any test was : S = Significant difference ($P < 0.05$) HS = Highly Significant difference ($P<0.01$) , and NS = Non Significant difference. Analysis of variance (ANOVA) test was used to determine the differences between the three groups and within groups.

Results and Discussion

Table 1 reveals the results of this study.

The calculation of gene expression fold change was made using relative quantification

.This depends on normalization of Ct values calculating the ΔCt. Table (1), reveals themean ±SE of Ct values of cf-n DNA and cf-mt DNA for each studied The Ct value of cf-n DNA ranged from 10.10- 29.4 with a mean±SE20.4 ± 0.38. In control group, the Ct value ranged from 18.7 -21.2 with a mean±SE19.9 ± 0.174. The difference in Ct values between the two groups was found to be non-significant. However, The mean ±SE Ct value of plasma cell free mitochondrial DNA was 31.4 ± 0.7 in T2DM patients group. It ranged from 20- 36. In control group, the Ct value ranged from30.6– 36 with a mean±SE 32.9 ± 0.3. There was a significant difference in the Ct mean values between the two studied groups (p=0.04). Results of ΔCt for control group displayed a mean of 12.9 ±0.384 ,whereas for patients group 10.9±0.835 with a significant difference p=0.02 when compared patients to controls. Reults of ΔΔCt showed significant difference for patients group with a mean -1.94± 0.835 .The fold of gene expression in patients group of cf-mt DNA was 18.9 time higherthan that of control group as shown in table (1).These results indicate that cf-mt DNA increases significantly in patients group. These results indicate that cf-mt DNA increases significantly in patients group. The two studied groups of the present study were divided into two subgroups: high expression when the fold of gene expression was above 1and low gene expression when the fold of gene expression was lower than
1. The frequency of high expressing patients was 25% and for control group was 0.00.It is well shown that that the high expression of cf-mt DNA was evident in patients withtype2DM in comparison to control group. There was a significant statistical differencebetween two groups p value ≤ 0.01 .

Prostate cancer patients had a 3-fold increase in mt DNA copy number compared to benign controls, according to Mehra et al., (2007). However, Diabetes patients had substantially greater amounts of cf-mt DNA than healthy controls, with plasma 2.33- fold higher and serum 2.08-fold higher, according to Rosa et al., (2020). (equivalent to230 percent and 208 percent of healthy controls values, respectively).

An elevation in mitochondrial copy number has been proposed as a possible compensatory impact for the oxidative damage-induced overall decrease in mitochondrial respiratory performance. The shortage of histones and other DNA- protecting proteins, as well as DNA repair mechanisms that are less effective than those found in n DNA, allows for greater degradation in mt DNA, which might be a cause of ROS production and cell death (Kumar et al., 2017). In cells experiencing oxidative stress, mt DNA damage is far more widespread and lasts much longer than nuclear DNAdamage under diabetes circumstances (Cao et al., 2019). According to several research, the reduced mt DNA content in the peripheral blood of diabetics may be linked to the

development of T2DM (Singh et al., 2007; Xu et al., 2012). Several recent investigations have established a connection between mitochondrial malfunction or abnormalities in mitochondrial biogenesis and insulin resistance or type 2 diabetes development. The tissues of mice and human individuals with insulin sensitivity or type 2 diabetes have decreased expression levels of genes encoding subunits that make up respiratory enzymes, according to these research, reduced respiratory enzyme complex activity, decreased expression of mitochondrial biogenesis genes, mutation or deletion of mitochondrial DNA, decreased bioenergetic capacity, or problems in beta oxidationof fatty acids (Wang et al., 2012).

Table (1) : Comparison between cells free nuclear DNA and mt-DNA in Ct, Δ Ct, ΔΔ Ct, and fold

Study groups	**Mean Ct of cfn DNA**	**Mean Ctof mt DNA**	**ΔCt (Ct of mt-n**	**ΔΔCt**	**Fold of gene expression**
Control	0.174±19.9	32.9 0.32	0.384±12.9	0.00	1
Patients	0.38±20.4	31.4±0.7	0.385±10.*96	0.835±*1.94	**18.9
P-value	0.02				
Significance	Significant				

* significant at p value ≤ 0.05, * significant at p value ≤ 0.01.

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