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Association of Peripheral Blood Mitochondrial DNA Content with Type 2 Diabetic Patients

1Wahiba A. Zarouk, 2Hala M. Raslan, 2Omnia Moguib, 1Ahmed I. Abdelneam, 1Ahmed F. Abd El Razeek, 1Maged M. Mahmoud, 1Mai E. Zekrie.

1Molecular Genetic and Enzymology department and 2Internal Medicine department; National Research Center.

ABSTRACT

Aim: Mitochondrial DNA (mtDNA) content is essential for maintaining normal mitochondrial function, and the mitochondrial function is critical for the production and the release of insulin in type 2 diabetes mellitus. We investigated whether peripheral blood mtDNA content was reduced in type 2 diabetes. Methods: Fifteen Egyptian Type 2 diabetes (T2DM) and twelve normal subjects were enrolled in this study. The quantity of relative mtDNA content was measured by a real-time PCR and corrected by simultaneous measurement of the nuclear DNA. An assay based on real-time quantitative PCR was used for both nuclear DNA (nDNA) and mtDNA quantification using SYBR green as a fluorescent dye (Invitrogen, Buenos Aires, Argentina). The copy number of mtDNA and nDNA was calculated from the Ct number and by use of the standard curve Plasma glucose was analysed using automated autoanalyzer. Results: A significant difference in the mtDNA/nDNA ratio among the control subjects and patients was reported; since results from this study detected reduced mtDNA content in type 2 diabetes patients (13.36 +/- 6.13) compared to healthy individuals (109.15 +/- 49.4). Conclusion: Our results demonstrated that lower peripheral blood mtDNA content is associated with type 2 diabetes.

Key words: Mitochondrial DNA content; peripheral blood; real-time quantitative polymerase chain reaction; Type 2 diabetes mellitus.

Introduction

In human cells, mitochondria are the only organelles that contain extrachromosomal DNA. Mitochondria are essential organelles that primarily function to support aerobic respiration and to provide energy substrates, and their function is intimately related to insulin secretion and possibly insulin action (Gerbitz et al 1995, Velho et al 1996, Lee et al 2005, Ritz and Berrut 2005).

Each human cell contains hundreds of mitochondria (Approximately 300-2000 mitochondria are present in each cell) each with 2 to 10 copies of mitochondrial DNA (mtDNA), which is small, circular double-stranded DNA of 16,569 bp. Mitochondrial DNA contains 37 genes, which encode 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and 13 polypeptides. All of the encoded polypeptides are components of the respiratory chain/oxidative phosphorylation system (Anderson et al 1981, Calvo etal 2006 and Falkenberg et al 2007).

Mitochondrial genetics is unique in many ways. First, mtDNA is more susceptible to oxidative damage and has a higher mutation rate than nuclear DNA due to a lack of protective histones, lack of an efficient DNA repair system, and their function is intimately related to insulin secretion and possibly insulin action (Gerbitz et al 1995, Velho et al 1996, Lee et al 2005, Ritz and Berrut 2005).

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Second, cells contain hundreds or thousands of mitochondria; since mtDNA is present in multiple copies, pathologic mutations or variants in mtDNA often result in heteroplasmasy, the coexistence of both wild-type and mutated mtDNA. Virtually every human organ system can be affected, and many of the pathogenic mtDNA mutations are heteroplasmic. However, most mtDNA SNPs are homoplasmic and are often related to age, neurodegeneration, or metabolic disorders. Some SNPs in control regions are also associated with mitochondrial content (Brownlee, 2001 Wong 2007; Lu et al 2010;). Mitochondria are not only the power house responsible for ATP production via the Krebs cycle and oxidative phosphorylation to keep cell alive but also responsible for production of about 85% intracellular ROS during the electron transport to promote cell differentiation or induce apoptosis. The dual roles of mitochondria are responsible for life and death of the cell (Frisard and Ravussin 2006). The number of mitochondria represents
not only the capacity of ATP production but also reflects the oxidative stress to which the cell is exposed. Metabolic regulation is largely dependent on mitochondria (Yakes and Van Houten 1997 and Lee et al. 2007).

Approximately 0.5–1.5% of all diabetic patients exhibit pathogenic mtDNA mutations such as duplications, point mutations, and large-scale deletions (Kadowaki et al. 1994, Silva et al. 2000 and Maassen et al. 2007). For many years researchers have argued that type 2 diabetes is a problem of insulin sensitivity, focusing on the insulin receptors in the peripheral tissue. Other researchers have focused on the dysfunction of the beta-cells, similar to type 1 diabetes, as the primary dysfunction in type 2. Today it is recognized that both factors contribute to the disease. Beta-cell malfunction can be traced to various levels of qualitative and quantitative mitochondrial dysfunction (Ehses et al. 2007, Patti and Corvera 2010). Little attention has been paid to the quantitative aspects of mtDNA in diabetes and there is as yet no convincing evidence whether the reduction of mtDNA copy number causes enough disturbance in the glucose metabolism in peripheral tissues such as liver cells to participate in the development of diabetes. Although the depletion of mtDNA could impair mitochondrial and pancreatic beta-cell function, mitochondrial function can be measured only in the tissues of patients or animals and cannot be measured easily in noninvasive samples or in nondiseased subjects. It has previously been shown in humans that the mtDNA content in blood cells is partially heritable (Curran et al. 2007 and Xing et al. 2008). It is believed, therefore, that the peripheral blood mitochondrial DNA (pb-mtDNA) content could provide an alternative index mitochondrial dysfunction. The aim of the current study was to elucidate the association of mtDNA content with type 2 diabetes in Egyptian cases.

Methods and Procedures:

Subjects and methods:

Fifteen type 2 diabetes patients more than 25 years were recruited from outpatient clinic of Medical Services Unit at National Research Center and twelve healthy subjects; age and sex matched. Written informed consent was obtained from all the subjects who participated in this study. All patients and controls were subjected to detailed history and thorough physical examination. We excluded patients with secondary diabetes, liver or kidney disease, major organ failure, auto-immune diseases, or malignancy anywhere in the body. None of the patients had diabetic complications. Controls did not have any abnormalities regarding their physical examination and with negative family history of diabetes.

Biochemical measurement:

Serum glucose was detected for all subjects using automated autoanalyzer Cx5 (Beckman, USA).

Quantification of mitochondrial DNA using quantitative real-time polymerase chain reaction:

Nucleic acids were extracted from white blood cells from a blood sample using a standard method as described previously (Miller et al. 1988 and Kawasaki 1990). An assay based on real-time quantitative PCR was used for both nuclear DNA (nDNA) and mtDNA quantification using SYBR green as a fluorescent dye (Invitrogen, Buenos Aires, Argentina).

The primer sequences for mtDNA, mtF3212 (5’-CACCCAAGAACAGGGTTTGT-3’) and mtR3319 (5’-TGGCCATGGGTATGTT-GTTAA-3’) and those for nDNA for loading normalization, 18S rRNA gene 18S1546F (5’-TAGAGGGACAAGTGCGGTTC-3’) and 18S1650R (5’-CGCTGAGCCAGTCA-GTGT3’) were reported (Bai et al. 2004).

The PCR profile was 1 cycle of 95 °C for 2 min, followed by 35 cycles (95 °C 15 s and 60 °C 1 min). Real-time quantitative PCR was carried out in a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA). The calculation of DNA copy number involved extrapolation from the fluorescence readings in the mode of background subtracted form the Bio-Rad iCycler (Bio-Rad Laboratories) according to Rutledge. Specificity of amplification and the absence of primer dimers was confirmed by melting curve analysis at the end of each run.

The two-target amplicon sequence (mtDNA and nDNA) was visualized in agarose 2% and purified using Qiagen Qiaex II, Gel extraction Kit and dilutions of purified amplicons were used as the standard curve. The copy number of mtDNA and nDNA was calculated from the C_1 number and by use of the standard curve according to WONG et al. (Rutledge 2004).

Results:

An assay based on real-time quantitative PCR was used for both nuclear DNA (nDNA) and mtDNA quantification using SYBR green as a fluorescent dye (Invitrogen, Buenos Aires, Argentina). The copy number of mtDNA and nDNA was calculated from the C_1 number and by use of the standard curve according to Wong.
et al 2004. Amplification occur successfully in patients and controls; where diagram appear show number of cycle which occur in amplification. Also the apparatus calculate melting curve to ensure the amplification and absence of primer dimmers (melting curve appear in all cases). A significant differences in the mtDNA/nDNA ratio among the control subjects and patients was reported; since results from this study detected reduced mtDNA content in type 2 diabetes patients (13.36 +/- 6.13) compared to healthy individuals (109.15 +/- 49.4) (p<0.001).

Discussion:

Metabolic regulation is largely dependent on mitochondria, which play an important role in energy homeostasis by metabolizing nutrients and producing ATP and heat. The number and size of mitochondria are correlated with mitochondrial oxidative capacity. Decreased mitochondrial activity might be caused, at least partly, by reduced content of mtDNA. Expression of nuclear-encoded genes involved in oxidative phosphorylation is downregulated in insulin-resistant skeletal muscle (Mootha et al 2003). Thus, some investigators have speculated that mtDNA copy number might be a surrogate marker of mitochondrial function (Cho et al 2007). There is as yet no convincing evidence whether the reduction of mtDNA copy number causes enough disturbance in the glucose metabolism in peripheral tissues such as liver cells to participate in the development of diabetes.

In this study, comparing mtDNA/nDNA ratio in control subjects and patients; revealed a significant decrease of mtDNA content in type 2 diabetes patients compared to healthy individuals. The association of mtDNA content with type 2 diabetes is not confirmed in all studies and showed conflicting results; evidence is accumulating that mtDNA content is associated with type 2 diabetes. However, there is debate about whether mitochondrial dysfunction is primary or secondary to type 2 diabetes. Several lines of evidence support the idea that the alterations of mtDNA quantity may cause type 2 diabetes as one of the mitochondrial diseases. First, pb-mtDNA content decreased in the offspring of type 2 diabetic patients and, furthermore, that the pb-mtDNA content is correlated with insulin sensitivity. These findings suggest that the quantitative mtDNA status might be an early genetic marker for type 2 diabetes and possibly for insulin resistance syndrome (Song et al 2001). Elegant studies by Petersen et al (2004) have found that impaired mitochondrial function in skeletal muscle of offspring of patients with type 2 diabetes is coupled with an increase in intramyocellular lipid and insulin resistance. Thus PBMC mtDNA depletion may indicate a greater depletion of islet or muscle mtDNA content and a greater bioenergetic defect affecting islet function and insulin action, thereby leading to earlier onset of overt diabetes. Second, early studies with the Goto-Kakizaki rat, a genetic animal model of type 2 diabetes with impaired insulin secretion, found that the mitochondria of beta-cells were decreased in volume, while the islet tissue had an increased number of mitochondria per unit area, but a decrease in mtDNA copies (Serradas et al 1995). The decrease in mtDNA (approximately 50 percent per mitochondrion) was not found to be associated with any major deletions or mutations. The decrease in mtDNA was observed in the adult rat tissue (four-month old) but not in fetal tissue. These results suggest a connection between glucose-stimulated insulin secretion (GSIS) and the somatic progression of mtDNA depletion. This study shows a correlation between mitochondrial function and type 2 diabetes. The data seem to suggest that metabolic dysfunction inside the mitochondria produces increased ROS, leading to decreased mtDNA, resulting in decreased insulin secretion (Alán et al 2011). Third, another example of exogenously induced oxidative damage to mtDNA can be illustrated with streptozocin, one of the diabetogenic agents to create diabetes in test animals; reduced the levels of mtDNA content and its transcripts in pancreatic islets. Streptozocin increases ROS causing damage to mtDNA and suppression of mitochondrial transcription. Hydroxyl radicals have been shown to attack nucleosides of DNA, producing oxidized products such as 8-hydroxy-2'-deoxyguanosine (8-OHdG). This ROS attack, although known to damage nuclear DNA, can attack mtDNA at rates 3-23 fold higher (Pettepher et al 1991, Moreira et al 1991, Mecocci et al 1994 and Hamilton et al 2001). Fourth, mtDNA depletion inhibited glucose-stimulated increases of the intracellular free Ca²⁺ concentration and insulin secretion in mouse insulinoma cells, which led to glucose intolerance and hyperglycemia (Soejima et al 1996). Finally, a more direct cause-and-effect relationship between mitochondrial dysfunction and human diseases was demonstrated in the report that antiviral agents used to treat patients with AIDS could damage mitochondrial function by inhibiting the mitochondrial DNA (mtDNA) polymerase γ, resulting in hyperlactatemia, lipodystrophy (ie, the accumulation of visceral fat, breast adiposity, and cervical fat pads), and insulin resistance (Davis et al 2002).

In a follow-up study, Morino et al., 2005 proposed that patients with type 2 DM have decreased copies of mtDNA in insulin-resistant target tissues, such as skeletal muscle and adipose tissue; contributed to the decreased mitochondrial activity. Other studies argue against previous observations where low mtDNA content in blood was associated with type 2 diabetes, triglyceride storage, glucose homeostasis, insulin sensitivity, and insulin secretion (Lee et al 1998, Park et al 1999, Park et al 2001, Song et al 2001 and Weng et al 2009); Singh et al., 2007 indicated that reduced mitochondrial DNA content in peripheral blood is not a risk factor for the
development of type 2 diabetes in the offspring of patients with early onset type 2 diabetes. Reiling and his colleagues, 2010; confirmed that mtDNA content has a heritability of 35% in Dutch twins, but could not find evidence for an association of mtDNA content in blood with prevalent or incident T2D and related traits. Furthermore they were the first to show that the decline in mtDNA content might be male specific. The observed gender effect on mtDNA content, also observed by Xing et al. (2008), is probably caused by this gender-specific correlation between mtDNA content and aging. One might speculate that overall mitochondrial fitness is better retained in females, which might explain the observed difference in life span between males and females. However, this hypothesis needs further investigation. A major difference between studies is the difference in ethnicity (Reiling et al 2010). The centerpiece of the pathophysiologic mechanism of metabolic syndrome is insulin resistance; it is becoming evident that mitochondrial dysfunction is closely related to insulin resistance and metabolic syndrome. The underlying mechanism of mitochondrial dysfunction is very complex. The mechanisms of disease progression and complications in diabetic patients are probably multifactorial; one factor may be hyperglycemia-induced overproduction of reactive oxygen species (ROS) by mitochondria. Increased oxidative stress is known to cause mitochondrial damage, dysregulation of mitochondrial function, and altered mitochondrial biogenesis (Lee et al., 2000, 2002 and 2010).

It is possible that the decreased glucose uptake reduces the glucose supply for enzymes involved in glucose metabolic pathways. These enzymes may be inactivated or down regulated. Park et al 2001 suggested that the depletion of mtDNA decreased glucose utilization by suppressing glucose metabolism in addition to reducing glucose uptake. Furthermore, the attenuated activities of glycolytic enzymes could consequently reduce ATP production, which may aggravate ATP depletion in a vicious cycle. Also, depletion of mtDNA significantly attenuated the expression of all types of glucose transporters that are encoded by nuclear DNA.

The mechanisms that control mtDNA content are yet to be fully elucidated. There is evidence that PBMC mtDNA content has a large genetic determinant (Curran et al 2007) and also evidence suggesting a role for mitochondria and mtDNA content in both insulin secretion and insulin action. Thus rodent models of diabetes show reduced mtDNA content in islet cells (Serradas et al 1995) and those with genetic disruption of mitochondrial transcription factor A in pancreatic beta cells exhibit depleted mtDNA and early diabetes (Silva et al 2000). Additionally, there is increasing evidence that the monocyte–macrophage lineage plays an independent and pathogenic role in the development of type 2 diabetes. Furthermore, increased monocyte-derived macrophage infiltration of islet cells is seen in type 2 diabetes and thought to play a role in causing islet pathology in that condition. Such evidence supports a direct role of monocytes in the development of glucose intolerance. In this context, rather than being simply a surrogate for determining mitochondrial action or number in alternative tissues, reduced mtDNA in monocytes could imply a direct impact on the development and timing of type 2 diabetes (Ehser et al 2007). The mtDNA molecule is particularly susceptible to damage and defective replication by virtue of continued exposure to reactive oxygen species (ROS) and a lack of DNA repair mechanisms. Furthermore, it has been shown that mtDNA harbouring deleterious mutations are preferentially clonally amplified as a compensatory response to energy deficiency by making more mitochondria and mtDNA (Wallace 2005). However, with time, as defective mitochondria accumulate, bioenergetic and replicative function declines. Therefore, in addition to the possibility of lower mtDNA content predisposing to early-onset disease, it is equally possible that patients with early-onset disease, exposed to the ravages of longer diabetes duration, accumulate defects in the mitochondrial genome with resultant defective replication, further depleting mtDNA content (Wong et al 2009).

Although our study sample was relatively small as compared with other epidemiological and association studies, the result of this study supports the hypothesis that the mtDNA content in blood is associated with type 2 diabetes, and confirm previous observations, where low mtDNA content in blood was associated with type 2 diabetes.

References


