Research Article

Study of Mitochondrial DNA copy Number Variation in Peripheral Blood of Type 2 Diabetes Patients: A Pilot Study

Nancy Taneja[1], Rajesh Khadgawat[2], Baibaswata Nayak[3], Shalini Mani[1]*

[1] Department of Biotechnology, Jaypee Institute of Information Technology, A-10, Sector 62 Noida, India.

[2] Department of Endocrinology, All Indian Institute of Medical Sciences, New Delhi, India.

[3.] Department of Gastroentrology and Human Nutrition Unit, All Indian Institute of Medical Sciences, New Delhi, India.

***Corresponding author's E-mail:** shalini.mani@jiit.ac.in

Received: 10-05-2017; **Revised:** 22-05-2017; **Accepted:** 14-06-2017.

ABSTRACT

Variations in content of mitochondrial DNA are known to be associated with different metabolic conditions such as diabetes, cancer, obesity and cardiac diseases. But there are few reports in case of Type 2 Diabetes patients and there is need of comprehensive studies which can correlate the variation in mitochondrial DNA content with T2DM. Our proposed study is an attempt to understand the correlation of mt DNA content with different clinical features of T2DM patients. Our current study aims to investigate the copy number variation of mt DNA in total 40 well characterized T2DM patients. Ten healthy age matched individuals were also involved in the study as a control. For the study of the relative mt DNA copy number measurements, Real time PCR using SYBR green method was performed and statistical analysis was done using SPSS 21.0.Following the real time PCR and ΔΔCt method of analysis, it was observed that in 80% of the studied patients, the content of mitochondrial DNA was found to be increased in comparison to control. The statistical studies further suggested that there was a slight positive correlation between mtDNA content and HbA1C of these patients. As increased mitochondrial content is proposed to be a marker for disease phenotype and our study strengthen this hypothesis.

Keywords: mt DNA, Diabetes, High Glucose, Copy number.

INTRODUCTION

Mitochondrion is the intracellular organelles and is crucial site for providing most of the cellular energy demands by producing ATP through the process of oxidative phosphorylation (OXPHOS)[1]. Every mammalian cell consists of 100-1000 mitochondria and this variation mainly depends on the energy requirements and type of the cell. Unlike any other cellular organelle, mitochondria contain its own DNA and copy number of Mitochondrial DNA (mtDNA) is 2-10 per mitochondria. It is 16.5 kbp in size and codes for total 13 proteins, which are integral component of different respiratory chain complexes involved in OXPHOS[2]. The maintenance of mtDNA content is important for the proper functioning of mitochondria and hence cellular function too. Several studies reported the association between quantitative defects of the mtDNA in diverse metabolic disease phenotypes, such as neurodegenerative diseases, cardiovascular diseases, cancer, diabetes and aging [3-7].

Type 2 Diabetes Mellitus (T2DM) is a multi-factorial disease and known to be caused due to various genetic and environmental factors. Most of the diabetic patients suffer through the condition of prolonged hyperglycaemia. This high glucose condition is proposed to cause different diabetes associated complications such as diabetic neuropathy, nephropathy, retinopathy and cardiomyopathy too[8]. As per various studies, these diabetes associated phenotypes are mainly due to hyperglycaemia induced overproduction of mitochondrial reactive oxygen species [9-11].This oxidative stress is known to cause mitochondrial damage which further leads to impaired mitochondrial function. Initially as a compensatory effect, there is rapid fission of mitochondria and increase in mtDNA copy number. As a result, though the mitochondrial activity increases but as a default by-product, the level of reactive oxygen species (ROS) also increases [12-14]. Once the level of ROS is too high, the rate of mitochondrial damage may be even higher than mitochondrial fission and ultimately in severe condition there is decrease in total mtDNA content. In support to these studies, the increase in copy number of mtDNA of T2DM patients has been observed by few of the studies [15,16]. These studies suggest that the increase in quantitative mtDNA content might be an early genetic marker for T2DM and possibly for insulin resistance syndrome [15].On the other side several studies have indicated the association of reduced mtDNA content with low insulin secretion and increased insulin resistance [17-19]. This group of study suggests that reduced mtDNA content may indicate the susceptibility towards the diabetic phenotype in these individuals.

Other than these few preliminary results with small number of patients, there is lack of comprehensive data which can correlate the variation in mitochondrial DNA content with disease phenotype of T2DM. Our proposed study is an attempt to understand the correlation of mtDNA content with different clinical features of T2DM patients.

MATERIALS AND METHODS

Subjects

Forty subjects with T2DM of age group 35-55years and ten healthy age matched subjects as controls were selected and recruited from All India Institute of Medical Sciences, New Delhi, India for this study. The study was ethically approved by the ethical committee of AIIMS, New Delhi and JIIT, Noida. As per ADA guidelines (2012), patients with fasting glucose ≥126mg/dl and postprandial glucose ≥200mg/dl and HbA1C ≥6.5% were classified as diabetic. Healthy subjects with no metabolic disease were considered as controls. A written informed consent and clinical details were collected from all the subjects participated in this study. Further, 2 ml of blood samples were collected in EDTA coated BD vacutainer from all the subjects and stored at 4°C till further use.

Genomic DNA Extraction

Total genomic DNA was extracted using the standard protocol of salting out method from the collected blood samples [20]. The DNA samples were stored at -80°C for further experiments. The quantity and quality of the DNA was determined using a ratio of in Nanodrop 2000 spectrophotometer and based upon it, all the DNA samples having OD 260/OD 280 in the range of 1.6-1.8 were selected for the study.

Real-time quantitative polymerase chain reaction (PCR) Analysis

To analyse the relative copy number of the mitochondrial DNA in all the 40 patients with respect to controls, a mitochondrial gene ND1 (target DNA) and a nuclear gene GAPDH (reference gene) was studied using Real time polymerase chain reaction (RT-PCR) by SYBR Green Method. The sequences of primers used for amplification of ND1 gene in mtDNA wereND1 F (5'-CATAAAACTCTTCACCAAAGAGCC-3') and ND1 R (5' GGGGTTCATAGTAGAAGAGCGA-3'). Similarly for GAPDH gene, the primer sequences were GAPDH F (5`-CCCCACACACATGCACTTACC-3`) and GAPDHR (5`-CCTAGTCCCAGGGCTTTGATT-3`). A 20µl reaction mixture, each for ND1 and GAPDH was prepared. The reaction mixture consisted 1x iTaq™ universal SYBR® Green supermix, 10 pmol of each primers and 0.125ng and 4ng of total genomic DNA for ND1 and GAPDH respectively. Real-time quantitative PCR was carried out in Agilent Mx3005P qPCR System. The real time PCR profile was 1 cycle of 95 °C for 5 min, followed by 40 cycles (95 °C for 10 s and 63 °C for 30s and 72ºC for 1 min). Melting curve analysis was performed to check the specificity of amplification and the absence of primer dimers was confirmed at the end of each run. The relative quantities of mtDNA and nuclear DNA in the blood samples were determined by calculating the average threshold cycle number (Ct) values of the nDNA and mtDNA for each case. The relative copy number of mtDNA in case of each patient was calculated using the equation $2^{-\Delta\Delta Ct}$, for which firstly ΔCt for each patient and control was calculated by (ΔCt = CtND1- Ct GAPDH) and ΔΔCt by the equation (ΔΔCt for each Patient = ΔCt (Patient)-Average of all ΔCt (control)).

Statistical Analyses

Statistical analyses were performed by using the Statistical Package for the Social Sciences, version 21.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $p < 0.05$. The correlation coefficients studies between the logarithmically transformed relative mitochondrial DNA copy number and various clinical parameters were conducted using Pearson test.

RESULTS

Clinical details

As per the clinical details of all the subjects, the average age of T2DM patients and controls selected for the study was 46.6± 9.63 years and 36.9±14.4 years respectively. The mean fasting Glucose and Post Prandial glucose of the patients was 173±51.1 mg/dl 261±77.1 mg/dl respectively, with average glycated hemoglobin (HbA1C) of 8.61±1.73 %.

Fold change in mtDNA content

As per our analysis, 80% of the T2DM patients (N=32) showed significant increase in the mtDNA copy number as compared to the control (p= 0.0192). The increased fold change in mtDNA was found to vary (1.01-63.8) among these T2DM patients. The decrease in the mtDNA copy numberwas observed in 20% of the patients (N=8) with fold change of -1.04 to -5.34 (Figure 1).

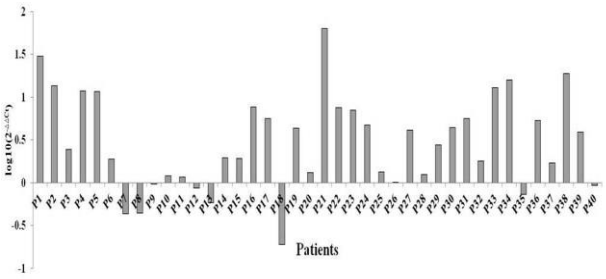

Figure 1: Bar diagram showing the relative mtDNA content (*logarithmically transformed value of relative content of mtDNA)* of 40 T2DM patients. P1-P40: Patient ID; P7, P8, P9, P12, P13, P18, P35 and P40 patients showed decrease in their relative content of mtDNA. Remaining 32 patients showed increase in mtDNA content.

Correlation of Age, Glucose levels and HbA1C levels with relative expression of mtDNA in patients

Relative mtDNA content was compared with various clinical parameters such as age, Post Prandial glucose, Fasting glucose and HbA1C (Table 1). There was a negative relation observed between mtDNA copy number variations with age of the patients (Figure 2 A). Similarly, fasting glucose and Post Prandial glucose did not show any relation with mitochondrial content (Figure 2 B, C). To

the contrary, a slight positive correlation was observed between HbA1C and relative mtDNA copy number variation in T2DM patients (r=0.164) (Figure 2 D).

Table 1: Correlation between relative mtDNA content of T2DM patients (logarithmically transformed value of relative content of mtDNA) and their clinical parameters (by Pearson coefficient method)

Parameters	r value	p-value
Age (years)	-0.14	0.931
Fasting Glucose (mg/dl)	-.031	0.847
Post Prandial glucose (mg/dl)	-.011	0.948
HbA1C (%)	0.164	0.313

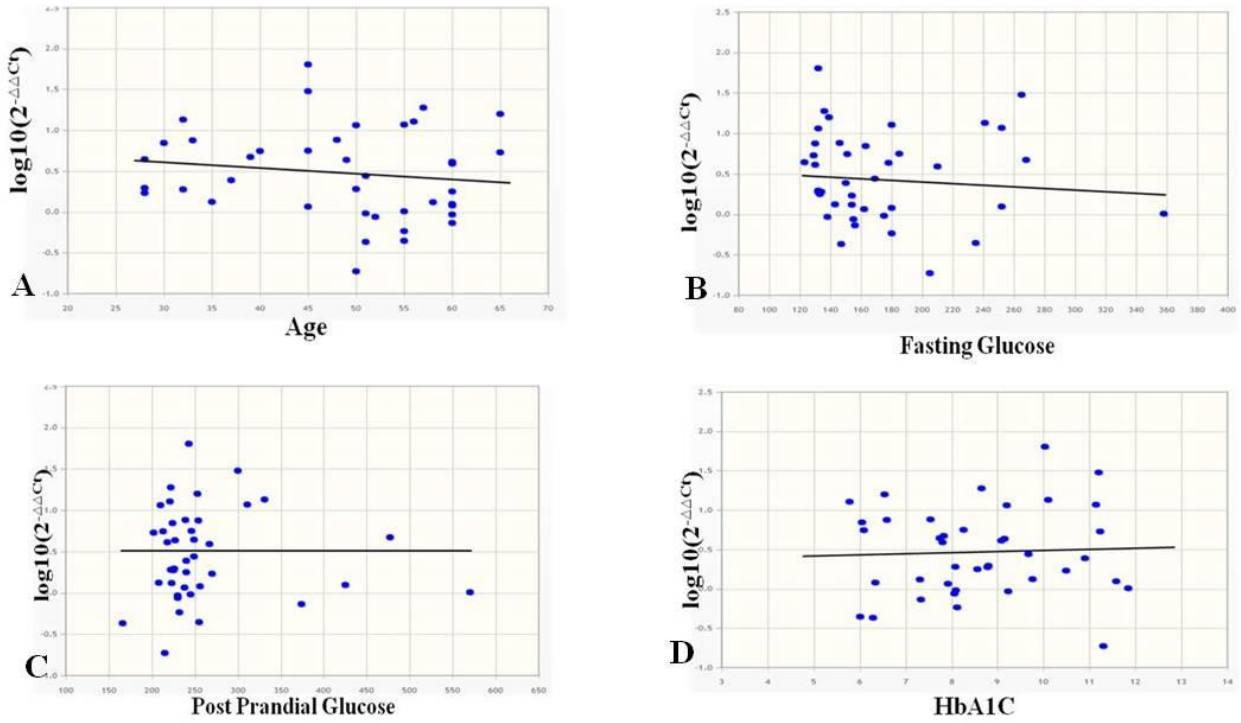

Figure 2: Correlation between relative expression of mtDNA in T2DM patients and their clinical parameters. **A)** Age (years); **B)** Fasting glucose levels (mg/dl); **C)** Post Prandial glucose levels (mg/dl); **D)** HbA1C levels (%). Only a slight positive correlation was shown between HbA1C levels and mtDNA content (r= 0.164).

DISCUSSION

Mitochondria are not only responsible for energy production, but also the major source of reactive oxygen species (ROS) generated as by-products of its metabolism. Prolonged exposure of ROS could lead to dysfunction of mitochondrial biogenesis and the alterations in mtDNA copy number, leading to pathogenesis and progressive deterioration of various metabolic diseases [21].

In current study also, we found altered mtDNA content in our T2DM patients. It was observed that there was augmented fold change of mtDNA copy number ranging from (1.01-63.8) in the T2DM patients (N=32) as compared to the control. Similar to our results, Weng *et al.*, also reported the association of increased mitochondrial DNA copy number with T2DM patients (N=125) [22]. Increased mtDNA content was further reported in T2DM patient carrying additional associated phenotype such as diabetic nephropathy [16]. Similarly, Song and colleagues also observed elevated mtDNA copy number in the offspring suffering from DM[15].

In order to understand the association between increased mtDNA content in diabetics, Lee *et al* proposed that this increase in mtDNA content could be an initial response of human cells to the oxidative stress caused by the prolonged hyperglycemic conditions of the blood to meet the energy requirement of the cells [11].

As a result of correlation studies, we did not find any relation between age of the T2DM patients and fold change of the mtDNA content. It suggests that relative change in expression of mtDNA in these patients is not due to the differences in the age but solely due to the diseased condition. In support to our observations, Miller *et al.*, 2003 also did not find any correlation between mtDNA copy number in skeletal muscle cells and different age group of these patients [23].

A slight positive correlation was observed between mitochondrial DNA content and HbA1C of our T2DM patients (r=0.164). As HbA1C is glycated hemoglobin, is reflection of the average plasma glucose levels of three months and its higher levels indicates prolonged high glucose level in the blood of these individuals. It is also well known that the prolonged hyperglycaemia induces mitochondrial ROS production and in order to cope up with this stressful condition, ultimately mitochondrial fission is initiated which lead to increase in the

mitochondrial DNA copy number in these subjects[24]. This is further supported by increased mtDNA copy number reported in the blood of diabetic nephropathy patients [16]. Thus, correlation of increased mtDNA content with diabetic phenotype seems to be highly evidenced. Hence as per our study, the content of mtDNA of T2DM patients and its correlation with higher HbA1C level, could also be suggested as marker for the diabetic phenotype.

In our study population, out of 40 patients, the decrease in the mtDNA content was also observed in 8 T2DM patients. This reduction in copy number of mtDNA was also reported in T2DM subjects by different studies [25, 26]. In 2005, Ritov and team also found the decreased mitochondrial content in skeletal muscle of patients with T2DM[27]. All these studies collectively suggest that compromised mtDNA content could further lead to low ATP production, which may ultimately affect both the insulin secretion and action in T2DM patients.

CONCLUSION

As per our study, majority of T2DM patients showed the increase in their mtDNA content which suggests that there is probably increased ROS level in these patients which may further lead to diabetic complications, if not managed. Few patients also showed the decrease in mtDNA content which highlights that these patients may be at their early stage of the disease and the condition may deteriorate if not taken care. Hence, these patients with altered mtDNA content should be advised to improve their mitochondrial metabolic activity by regular exercise, intermittent fasting, supplementing nutrients and vitamins like acetyl–carnitine, alpha lipoic acid, coenzyme Q_{10}.

Acknowledgement: Authors acknowledge the subjects who participated in the study and AIIMS New Delhi & JIIT, Noida for providing the infrastructure and financial support to conduct the study.

REFERENCES

1. Schatz G, Mitochondria: beyond oxidative phosphorylation, BBA-MolBasis Dis, 1271, 1995,123-126.
2. Falkenberg M, Larsson NG, Gustafsson CM, DNA replication and transcription in mammalian mitochondria, Annu. Rev. Biochem, 76, 2007, 679-699.
3. Chen S, Li Z, He Y, Zhang F, Li H, Liao Y, Wei Z, Wan G, Xiang X, Hu M, Xia K, Elevated mitochondrial DNA copy number in peripheral blood cells is associated with childhood autism, BMC psychiatry, 15, 2015, 50-57.
4. Cormio A, Guerra F, Cormio G, Pesce V, Fracasso F, Loizzi V, Resta L, Putignano G, Cantatore P, Selvaggi LE, Gadaleta MN, Mitochondrial DNA content and mass increase in progression from normal to hyperplastic to cancer endometrium, BMC res. notes , 5, 2012, 279-284.
5. Huang J, Tan L, Shen R, Zhang L, Zuo H, Wang DW, Decreased peripheral mitochondrial DNA copy number is associated with the risk of heart failure and long-term outcomes, Medicine , 95, 2016, 1-10.
6. Lee JE, Park H, Ju YS, Kwak M, Kim JI, Oh HY, Seo JS, Higher mitochondrial DNA copy number is associated with lower prevalence of micro albuminuria, Exp. Mol. Med, 41, 2009, 253-258.
7. Wong J, McLennan SV, Molyneaux L, Min D, Twigg SM, Yue DK, Mitochondrial DNA content in peripheral blood monocytes: relationship with age of diabetes onset and diabetic complications, Diabetologia, 52, 2009, 1953-1961.
8. Fowler MJ, Microvascular and macro vascular complications of diabetes, Clin. Diabetes, 26, 2008, 77-82.
9. Brownlee M, Biochemistry and molecular cell biology of diabetic complications, Nature, 414, 2001, 813-820.
10. Nishikawa T, Edelstein D, Du XL, Yamagishi SI, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP and Giardino I, Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage, Nature, 404, 2000, 787.
11. Lee HB, Yu MR, Yang Y, Jiang Z, Ha H, Reactive oxygen species-regulated signaling pathways in diabetic nephropathy,J. Am. Soc. Nephrol, 14,2003, S241-245.
12. Hsin-Chen LE, Pen-Hui YI, Ching-You LU, Chin-Wen CH, Yau-Huei WE, Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells, Biochemical J, 348, 2000, 425-432.
13. Lee HC, Wei YH, Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress, Int. J. Biochem. Cell Biol, 37, 2005, 822-34.
14. Lee SJ, Hwang AB, Kenyon C, Inhibition of respiration extends C. elegans life span via reactive oxygen species that increase HIF-1 activity, Curr. Biol, 20, 2010, 2131-2136.
15. Song J, Oh JY, Sung YA, Pak YK, Park KS, Lee HK, Peripheral blood mitochondrial DNA content is related to insulin sensitivity in offspring of type 2 diabetic patients, Diabetes Care, 24, 2001, 865-869.
16. Malik AN, Shahni R, Iqbal MM, Increased peripheral blood mitochondrial DNA in type 2 diabetic patients with nephropathy,Diabetes Res Clin Pract, 86, 2009, e22-24.
17. Curran JE, Johnson MP, Dyer TD, Göring HH, Kent JW, Charlesworth JC, Borg AJ, Jowett JB, Cole SA, MacCluer JW, Kissebah AH, Genetic determinants of mitochondrial content, Hum Mol Gen, 16, 2007, 1504-1514.
18. Zhou Y, Ning Z, Lee Y, Hambly BD, McLachlan CS, Shortened leukocyte telomere length in type 2 diabetes mellitus: genetic polymorphisms in mitochondrial uncoupling proteins and telomeric pathways, Clin. Transl. Med, 5, 2016, 8-14.
19. Kim JA, Wei Y, Sowers JR, Role of mitochondrial dysfunction in insulin resistance, Circ. Res, 102, 2008, 401-14.
20. Miller SA, Dykes DD, Polesky HFRN, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic acids res, 16, 1988, 1215.
21. Malik AN, Czajka A, Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction?, Mitochondrion, 13, 2013, 481-492.
22. Weng SW, Lin TK, Liou CW, Chen SD, Wei YH, Lee HC, Chen IY, Hsieh CJ, Wang PW, Peripheral blood mitochondrial DNA

content and dysregulation of glucose metabolism, Diabetes Res Clin Pract, 83, 2009, 94-99.

23. Miller FJ, Rosen feldt FL, Zhang C, Linnane AW, Nagley P, Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age, Nucleic acids res,31, 2003, e61-68.
24. Al-Kafaji G, Golbahar J, High glucose-induced oxidative stress increases the copy number of mitochondrial DNA in human mesangial cell, BioMed. Res. Int, 2013, 2013, 1-8.
25. Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, Neschen S, White MF, Bilz S, Sono S, Pypaert M, Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents, J Clin Invest, 115, 2005, 3587-3593.
26. Alán L, Špaček T, Zelenka J, Tauber J, Berková Z, Zacharovová K, Saudek F, Ježek P, Assessment of mitochondrial DNA as an indicator of islet quality: an example in Goto Kakizaki rats, InTransplant. Proc, 43, 2011, 3281-3284.
27. Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE, Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes, Diabetes, 54, 2005, 8-14.

Source of Support: Nil, **Conflict of Interest:** None.