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Research Article

Promoter Methylation of Mitochondrial Transcription Factor A Gene and Metabolic Syndrome

Shaymaa A Abd El- Hady[1], Mohamed M El Shabrawy[1]*, Basma B Hasan[2] and Ahmed T Abdellah[3]

[1]*Clinical and Chemical Pathology, Faculty of Medicine, Suez Canal University, Egypt*

[2]*Clinical and Chemical Pathology, Faculty of Medicine, Port-Said University, Egypt*

[3]*Cardiology department, Faculty of Medicine, Suez Canal University, Egypt*

***Corresponding Author:** Mohamed M El Shabrawy, Clinical and Chemical Pathology, Faculty of Medicine, Suez Canal University, Egypt.

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Abstract

Aim: The present work is a case-control study that aimed at determining the association between the methylation of the Mitochondrial Transcription Factor A promoter and metabolic syndrome.

Subjects and Methods: The study included 100 patients with metabolic syndrome (MS) as well as 100 apparently healthy individuals as control. All study populations were subjected to interview questionnaire, physical examination, measurement of fasting blood glucose (FBG) and lipid profile (TG, total cholesterol, LDL-C, and HDL-C concentrations). DNA extraction was performed from the circulating leucocytes. DNA methylation was done by treating DNA samples with sodium bisulfate. PCR was done for detection the promoter methylation of TFAM using the specific primers.

Results: The study revealed no association between TFAM gene methylation and metabolic syndrome. However, a statistically significant association was noticed between smoking and TFAM gene methylation.

Conclusion: TFAM promoter methylation is not associated with MS.

Keywords: *TFAM; Metabolic Syndrome; DNA Methylation; PCR*

Introduction

Metabolic syndrome (MS) is a collection of an interconnected factors that increase the predisposition to obesity, insulin resistance, dyslipidemia, non-alcoholic fatty liver disease and cardiovascular diseases [1]. Metabolic syndrome is the most important medical problem in the last century with an increasing prevalence worldwide, and is becoming an important health problem [2]. According to The International Diabetes Federation 25% of the world's adult population suffer from MS [3]. In 2008, Grundy showed that the prevalence of metabolic syndrome was 15% to 63% in the European countries while the Asian countries showed a prevalence of 12.1% to 30%.

The risk of mortality was greater with the increase in waist circumference, along with increased glycaemia, triglycerides and/or blood pressure than with other associations [4]. Obesity is a contributing factor to hypertension, high cholesterol levels, low HDL cholesterol, and hyperglycemia, which are jointly associated with a high risk of cardiovascular diseases [5]. Excess fat is responsible for an increase of non-esterified fatty acids, which overloads muscles and liver, and leads to the development of insulin resistance [6].

MS results from the interaction between genetic and environmental factors [7]. A less explored but remarkable mechanism that is gaining acceptance in understanding the pathophysiology of common diseases is the epigenetic regulation of transcriptional control by DNA methylation. Interestingly, the epigenetic mechanisms can affect the regulation of several gene pathways through life and explain the gene - environment interaction, but more importantly, they are potentially modifiable [8].

Defective mitochondrial function is considered the cornerstone of metabolic syndrome. One of the main features of metabolic syndrome is defective cell metabolism that may result from imbalance between nutrient intake and utilization of nutrient for energy production [9].

Mitochondrial transcription factor A (TFAM) is essential for mitochondrial genome regulation affecting transcription initiation and replication [10]. TFAM gene is mapped on chromosome 10q21.1. TFAM is a nuclear-encoded protein of 246 amino acids. TFAM is considered a key regulator of mtDNA transcription and replication . TFAM has an vital role for the maintenance of mtDNA as it is involved in direct regulation of mitochondrial DNA (mtDNA) copy number, affecting transcription initiation and replication [7].

The role TFAM as a sequence-specific transcription factor at mitochondrial promoters has been definitely recognized. Being an abundant protein, TMFA has other additional functions. Most of TFAM is bound at different regions all over the entire length of mtDNA and so it helps in maintaining nucleoid architecture [11].

Mitochondrial and nuclear genomes encode mitochondrial electron transport enzymes and ATP synthase genes. Mitochondrial transcriptional factors include mitochondrial transcriptional factor A (TFAM), B1 (TF1BM) or B2 (TF2BM) and RNA polymerase transcript mitochondrial encoded genes. TFAM is a key regulator of mt-DNA replication and transcription. Therefore, TFAM controls mitochondrial ATP production. Although TFAM is considered as mt-DNA specific transcriptional factor, it also controls nuclear DNA transcription i.e. SERCA2a gene. Transcription of genes for mitochondrial enzymes that produce ATP and the gene for SERCA2a that consumes ATP is coordinately regulated by the same factor. The same factor, TFAM, control not only the transcription of genes for mitochondrial enzymes that produce ATP but also the transcription of genes for SERCA2a that consumes ATP [12].

Age-related alterations of oxidative stress may result in changes in TFAM activities which in turn affect mtDNA replication [13]. Methylation of TFAM promoter may lead to insulin resistance [7].

TFAM gene disruption results in mtDNA depletion and oxidative phosphorylation capacity loss [14]. Distorted mitochondrial functions, decreased ATP synthesis and increased ROS generation inducing oxidative stress result in insulin resistance and obesity/diabetes. Energy production imbalance energy utilization leading to impaired cell metabolism. High levels of glucose augment increased production of ROS that causes morphological changes in mitochondria. Impaired Mitochondrial function leads to increased production of ROS and interference with oxidation of acetyl CoA causing increased lipid and diacylglycerol which in turn results in inhibition of insulin signaling pathway. This contributes to insulin resistance and accumulation of excess glucose leading to chronic hyperglycemia [15].

So, it's important to study if methylation of mitochondrial transcription factor A promoter is associated with metabolic syndrome.

Subjects and Methods

This case-control study was carried out at the Internal Medicine clinic and Clinical Pathology department, Suez Canal University hospital. The study included 100 patients with metabolic syndrome as well as 100 apparently healthy volunteers as control (employee and attendants without MS). All participants were Egyptians and of Egyptian descendants. Ethics Committee of the Suez Canal University approved the study that was carried out in accordance with the Helsinki Declaration. An informed written consent was obtained from each individual.

According to the National Cholesterol Education Program Adult Treatment panel III (NCEP ATP III) criteria (expert panel on Detection and Treatment of High Blood Cholesterol in Adults, 2001), patients with metabolic syndrome was selected as having three or more of the following five criteria: waist circumference (cm); male ≥ 102 cm and female ≥ 88 cm, HDL-cholesterol: male < 40 mg/dl and female < 50 mg/dl, Triglyceride ≥ 150 mg/dl, Fasting glucose ≥ 100 mg/dl and/ or Blood pressure ≥ 130/85 mmHg [4].

Patients with metabolic syndrome were taking drugs for controlling their conditions. Mostly they were on oral hypoglycemic drugs, other were on insulin beside their drugs for controlling hypertension or their high lipid profile.

All study populations (patients and controls) were subjected to interview questionnaire, and measurement of body weight, height, and waist circumference in cm, systolic and diastolic blood pressure.

Blood samples were collected after 12 hours fasting and were subjected to measurement of fasting blood glucose (FBG) and lipid profile (TG, total cholesterol, LDL-C, and HDL-C) by Cobas C501 Chemistry auto-analyzer (Roche diagnostic, Germany).

DNA extraction was done from the circulating leucocytes, using the patients' whole blood, by commercially available spin column kits (QIAamp® DNA Blood Mini Kit, QIAGEN, Stanford, Valencia, CA, USA). The extracted DNA samples were stored at -20°C for the next step of methylation assay.

DNA methylation was done by treating DNA samples with sodium bisulfite, which modifies of un-methylated C (Cytosine) to U (Uracil) but the methylated C remain unmodified as they resist the conversion. Bisulfite treatment of DNA was done using EpiMark® Bisulfite Conversion kit (New England Biolabs® *Inc*, UK).

In 1980, Kuo and colleagues developed high performance liquid chromatography-ultraviolet (HPLC-UV) for quantification of deoxycytidine (dC) and methylated cytosines (5 mC) amount that is present in a hydrolysed DNA sample. It is still considered the "gold standard" assay even though significant limited use of this method because a specialized laboratory equipment is needed and a relatively large quantities (3-10 µg) of the DNA sample to be analyzed is required. In brief, first, the DNA is hydrolyzed into its constituent nucleoside bases, then the 5 mC and dC bases are separated chromatographically and the fractions measured. The 5 mC/dC ratio can be calculated and compared to the experimental and control samples [16].

Polymerase chain reaction (PCR) was done for detecting the promoter methylation of TFAM using thermocycler R-Corbett. Primers of first-stage detected a bisulfite-treated DNA but could not distinguish methylated from un-methylated alleles. Two pairs of primers were used in the second stage, one to detect un-methylated template, and the other pair for the detection of the methylated template. The forward primer of the first stage 5'-GTAAGTTGGAGGTTAGATTGAAAG -3' and the reverse one was 5'-ATAAAATCTACATTCCAACCCC-3.

The following points were considered during primer design:

- Primer length should be between 18 - 24 nucleotides.
- The melting temperature (Tm) of the primers between 65°C and 75°C, and within 5°C of each other
- The GC content should be between 40 and 60%, with the 3' of a primer ending in C or G to promote binding.
- Avoidance of runs of 4 or more of one base, or dinucleotide repeats.
- Intra- and inter-primer homology should be avoided.
- Designing primers was done using Invitrogen Vector NTI Advance Sequence Analysis Software.
- The amplicon size of the first stage is a 963 bp acting as template for the second stage.

The second stage primers were; one to amplify the un-methylated product and the other one to amplify the methylated product. Primer sequences that were used to amplify an un-methylated product were:

Sense: 5'-TAATGGGTTTTATATAGATATATGG-3'

Non-sense: 5'- CAAAAATAATAACAAAAAAAACAAA-3'

Primer sequences for the methylated reaction were:

Sense: 5'-TTAATGGGTTTTATATAGATATACGG-3'

Non-sense: 5'-AAAAATAATAACGAAAAAAACGAA-3'

The amplicon size was 102-bp.

Amplification step was carried out in a 20 ul PCR reaction mixture containing 4 ul of bisulfate-treated genomic DNA, 10 pmol of each primer (2 ul of each primer), 10 ul 5x Emorald master mix* and 2 µl PCR grade water.

The cycling conditions were as follow; initial denaturation at 95°C for 10 minutes, and denaturation for 35 cycles containing a 95°C for 30 seconds, primer annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds and 72°C for 10 minutes for final extension. The PCR products were visualized on 2% agarose gel. Gel electrophoresis was done on 2% agarose gel containing ethidium bromide to visualize PCR products using UV illumination and photograph was taken.

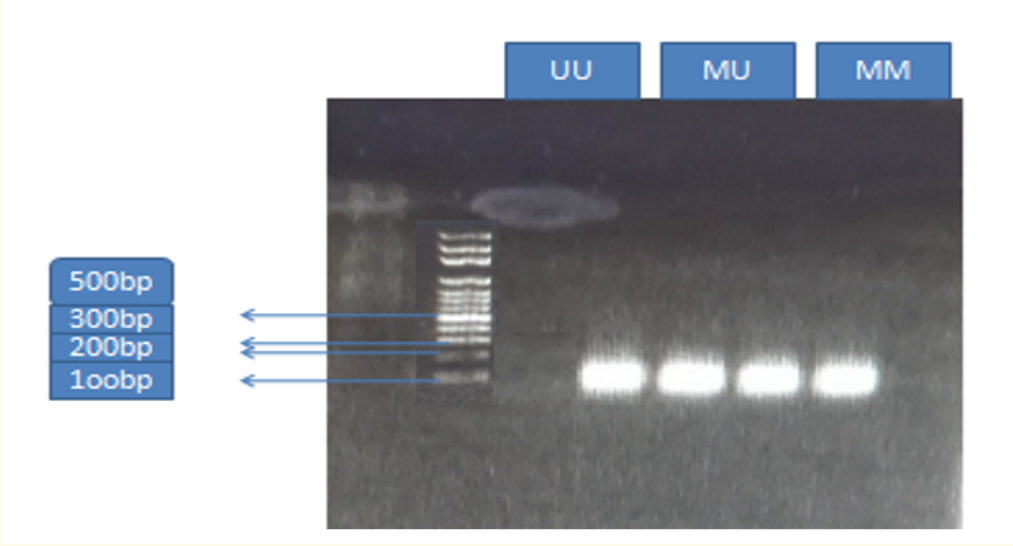

Figure 1: *Electrophoretic patterns of different TFAM 1 (methylation and un-methylation) gene.*
Lane 1: 100 bp DNA ladder
Lane 2 and 3: UU genotype
Lane 4 and 5: MU genotype
Lane 6 and 7: MM genotype

Statistical analyses

Statistical analyses were performed to investigate the association among TFAM DNA methylation, metabolic profile and metabolic syndrome. the association between metabolic syndrome with categorical variables including gender and smoking was studied by using either Pearson chi-square or Fisher exact test. The association between metabolic syndrome with categorical variables including gender and smoking was studied by using either Pearson chi-square or Fisher exact test. The association of MS with continuous variables including age, body mass index (BMI), total cholesterol, total triglycerides and glucose was tested by two sample t-test. The association between the TFAM methylation and metabolic criteria was tested by pearson correlation. The sensitivity of TFAM DNA methylation in diagnosing MS was analyzed by receiver operating characteristic (ROC) curve. The interaction of TFAM methylation and age was determined by Logistic regression. A statistically significant relation was considered if the p-value < 0.05. All the previously mentioned relations were analyzed using PASW Statistics 18.0 software (SPSS, Inc., Somers, NY, USA).

Results

Demographic and clinical criteria for both study groups are presented in table 1.

Item	MS group (n = 100)	Control group (n = 100)	p-value
Age (years)	55.9 ± 10	53.4 ± 10.1	0.240
Smokers	20	0	< 0.001
BMI (kg/m^2)	36.3 ± 5	24.3 ± 1.3	< 0.001
WC (cm)	110.7 ± 6.6	84.3 ± 8.6	< 0.001
SBP (mmHg)	146 ± 15	121 ± 10	< 0.001
DBP (mmHg)	92 ± 10	80 ± 8	< 0.001
FBS (mg/dl)	300 ± 94	92 ± 16	< 0.001
TG (mg/dl)	189 ± 39	89 ± 30	< 0.001
HDL-c (mg/dl)	39 ± 12	49 ± 14	< 0.001
T. chol (mg/dl)	205 ± 50	183 ± 36	0.027
LDL- c (mg/dl)	126 ± 45	124 ± 32	0.800

Table 1: *Demographic and clinical criteria of study groups.*

BMI: Body Mass Index; WC: Waist Circumference; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure;

FBS: Fasting Blood Sugar; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; T: Total.

FBS, TG and total cholesterol were significantly higher among MS subjects in comparison to the healthy ones. However, HDL-cholesterol was significantly lower among MS population in comparison to control ones.

MS population showed the following genotype distribution: MM genotype was 9, MU was 70 and UU was 21. The control population genotype distribution was as follow: MM genotype was 9, MU was 74 and UU was 17. There were no statistically significant differences between both groups regarding the distribution of the genotypes.

In MS group, the frequency of the M and U alleles were 88 and 112 respectively; however in the control group the frequency of the M and U alleles were 92 and 108 respectively. There were statistically insignificant differences between MS cases and healthy controls. The potential risk for metabolic syndrome in the presence of UU was 1.3 (95%CI, 0.24 - 6.6) with p-value = 0.5600, while the potential risk for metabolic syndrome in the presence of MU was 0.92 (95%CI 0.22 - 4.1) with p-value = 0.6100. The potential risk for metabolic syndrome in the presence of UU and MU was 1.0 (95%CI, 0.23 - 4.3) with p-value = 0.6400.

Item	MS group	Control group	p-value
Genotype			
MM	9	9	--------
MU	70	74	0.61
UU	21	17	0.56
Allele frequency			
M	88	92	0.76
U	112	108	0.75
Allele carrier			
M	79	83	0.865
U	91	91	--------

Table 2*: Genotype distribution, allele frequency, allele carrier of TFAM gene methylation among study groups.*

All variables examined using χ^2 test.

Multivariate backward regression analysis model shows that higher age, weight, higher BMI, higher waist circumference, fasting glucose, triglycerides, cholesterol, LDL-C, UU, MU and MM genotypes were significantly associated with the presence of metabolic syndrome with p-value of < 0.0001, 0.014, 0.025, < 0.0001, 0.0010, 0.02600, 0.02000, 0.0020, < 0.0001, 0.01100, 0.01200 and 0.01100 respectively.

Interestingly, the study found that higher BMI and fasting glucose were significantly associated with the presence of UU genotype with p-value of 0.0020 and 0.0500 respectively. Moreover, higher waist circumference (p-value = 0.0001), fasting glucose (p-value = 0.02900) and triglycerides (p-value = 0.04900) were significantly associated with the presence of MU genotype. Also, higher fasting glucose was significantly associated with the presence of MM genotype (p-value = 0.0060).

Another interesting notification, in MS group, the prevalence of UU, MU and MM among smokers were 2, 16 and 2 while among non-smokers were 19, 51 and 10 respectively. In control group, the prevalence of UU, MU and MM among non-smokers were 19, 72 and 9 respectively.

There was only significant difference regarding the prevalence of MU among smokers in comparison to non-smokers in both groups (p value = 0.0020).

Discussion and Conclusion

This study showed that the difference in the smoking status between metabolic patients and healthy controls was statistically significant. When methylation status is considered, smoking was highly associated with differential methylation in 310 genes that map to multiple biological process and cellular differentiation pathways [17].

Cigarette smoking may modulate DNA methylation, which interrogates DNA methylation at > 27,000 CpG sites located mostly in gene promoters [18].

The current study showed that there was no association between TFAM promoter methylation and MS. This was in agreement with Hashemi., *et al.* (2014) who conducted a similar study revealing that there was no association between TFAM promoter hypermethylation and MS.

Increased production of reactive oxygen species in adipocytes cause mitochondrial dysfunction predisposing to the down-regulation of GLUT4 and insulin resistance [19]. Gemma., *et al.* [20] suggested that there may be an association between TFAM promoter methylation and metabolic syndrome. Sookoian., *et al.* [21] studied the effect of epigenetic factors on the development of insulin resistance. They evaluated whether impaired insulin sensitivity might result from methylation of peroxisome proliferator-activated receptor c co-activator 1 alpha (PPARGC1A) and TFAM promoters in the liver. Their study concluded that in non-alcoholic fatty liver disease (NAFLD) patients, there was a correlation between PPARGC1A promoter methylation level, homeostatic model assessment- insulin resistance (HOMA-IR) and plasma fasting insulin levels, while there was an inverse relation between TFAM promoter methylation and fasting insulin.

In an animal experiment conducted by Holmstrom., *et al.* [22] to study how tissue-specific role of mitochondrial respiratory capacity predispose to impaired insulin sensitivity and type II diabetes. They revealed that levels TFAM were decreased together with high levels of proteins associated with mitochondrial dynamics in obese diabetic mice. The researchers concluded that mitochondrial dysfunction corresponded to insulin resistance in liver and skeletal muscle in obese diabetic mice.

van Otterdijk., *et al.* [1] showed that methylation levels were not different in patients with MS and T2D and healthy controls, while there was a trend to significant difference in methylation level regarding Paternally-expressed gene 3 protein (PEG3). Differences in individual CpG loci methylation were observed even though no difference was shown in methylation of the studied genes.

In a study of epigenetics of the human genome in diabetic patients, insulin resistant patients were similar to healthy individuals as regards the differentially methylated although some loci were nominally significant [23].

Mitochondrial transcription factor A (TFAM) affects transcription initiation and replication and therefore has an essential role in direct regulation of mitochondrial genome. In type II diabetes, alterations in mitochondrial DNA replication might occur due to age-related modifications of oxidative stress could impact by regulating the TFAM activity [24]. Impaired mitochondrial transcription is caused by decreased transport TFAM to mitochondria in addition to and decreased linkage between TFAM and mitochondrial DNA [25].

This unexpected result could be explained by some reasons. First, the interaction between TFAM with some MS components as arterial hypertension and dyslipidemia still unclear. Second, the association between promoter TFAM methylation and MS criteria is still unknown. Third, the number of the studied population was limited. Fourth, TFAM promoter methylation may be affected by other factors as ethnicity and environment.

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