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Methylation of *TFAM* gene promoter in peripheral white blood cells is associated with insulin resistance in adolescents

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ABSTRACT

Purpose: To explore whether DNA methylation of the mitochondrial transcription factor A (*TFAM*) promoter is associated with insulin resistance in a sample of adolescents with features of metabolic syndrome.
Methods: The data and blood samples were collected from 122 adolescents out of a cross-sectional study of 934 high-school students. The population was divided into two groups: noninsulin resistance (NIR) and insulin resistance (IR). After bisulfite treatment of genomic DNA from peripheral leukocytes, we used methylation-specific polymerase chain reaction (PCR) to assess DNA methylation of three putative methylation target sites (CpG) in the *TFAM* promoter.
Results: The ratio of the promoter methylated DNA/unmethylated DNA was 0.012 ± 0.0009 (1.2% of alleles), and inversely correlated with the biochemical features of insulin resistance (plasma fasting insulin R: −0.26, $p < 0.004$ and homeostasis model assessment (HOMA) index R: −0.27, $p < 0.002$), and obesity (R: −0.27, $p < 0.002$). Multiple regression analysis showed that the log-transformed HOMA index correlated with the status of promoter methylation of *TFAM*, independently of body mass index (BMI) Z score (β: −0.33 ± 0.094, $p = 0.00094$). Finally, the *TFAM* promoter methylated DNA/unmethylated DNA ratio was found to be significantly associated with insulin resistance as dichotomous variable (NIR $n = 45$, 0.014 ± 0.002 and IR $n = 77$, 0.011 ± 0.001, respectively, $p < 0.016$).
Conclusion: Our findings suggest a potential role of promoter *TFAM* methylation in the pathogenesis of insulin resistance in adolescents.

Introduction

According to the recent report by the National Health and Nutrition Examination Survey of the United States, the prevalence of insulin resistance in obese children of the western countries is observed to be up to 52% [1].

Despite significant effort, the understanding of the molecular processes of insulin resistance in the young population remains a major challenge, partly owing to the complex interplay between genes and environment, as well as the even more complex epigenetic mechanisms of transcriptional control, such as DNA methylation and all the processes probably necessary for the development of this phenotype. In fact, results from different animal models of nutrient perturbations, recently reviewed by Devaskar et al. showed that along with an altered *in utero* environment, tissue-specific epigenetic perturbations that permanently alter the expression of critical genes may contribute to the worldwide epidemic of Type-2 diabetes [2,3].

In addition, there has been plenty of evidence showing that mitochondrial dysfunction plays a central role in the pathogenetic processes resulting in insulin resistance and their associated complications [4,5]. Insulin resistance is associated with decreased mitochondrial number, abnormal morphology, lower levels of mitochondrial oxidative enzymes, and lower ATP synthesis in human muscle biopsies [6].

Interestingly, the molecular processes associated with mitochondrial dysfunction are subject to strong genetic and epigenetic control [7].

Mitochondrial transcription factor A (*TFAM*)[1] plays an important role in direct regulation of mitochondrial DNA (mtDNA) copy num-

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[1] *Abbreviations:* mitochondrial transcription factor A gene, *TFAM*; arterial blood pressure, ABP; methylation-specific polymerase chain reaction, MS-PCR; methylated primers, M primers; unmethylated primers, U primers; insulin resistance, IR; noninsulin resistance, NIR.

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ber, affecting transcription initiation and replication, which indicates that *TFAM* is essential for the maintenance of mtDNA [8].

In a recent study, we observed that reduced mtDNA content in the peripheral leukocytes is associated with insulin resistance in adolescents [9]. Given the above-mentioned evidence and the previous observations suggesting that mitochondrial dysfunction may be a major regulator of insulin resistance and their associated complications, we hypothesized that promoter DNA methylation of *TFAM* may be associated with the clinical features and anthropometric and laboratory variables of insulin resistance in adolescents.

Patients and methods

Data and blood samples were collected from 122 adolescents out of a cross-sectional, population-based study of 934 high-school students of self-reported European ancestry, to whom we had previously addressed the prevalence of obesity and hypertension [10,11].

Briefly, under parental supervision, the subjects responded to a questionnaire on medical history, medication, and personal habits. Anthropometrical assessments included measurement of height, weight, and waist and hip circumferences. The waist circumference was assessed in the standing position, midway between the highest point of the iliac crest and the lowest point of the costal margin in the mid-axillary line. Body height and weight were recorded in light clothing, and body mass index (BMI) was computed as weight in kilograms divided by height in meters squared. Resting arterial blood pressure (ABP) was measured after the subjects had been sitting for at least 30 min. Blood pressure was measured thrice by two investigators, at the right arm employing a mercury sphygmomanometer, using cuffs with the appropriate length and width for the upper arm. We normalized the BMI according to age and sex as established by the National Health and Nutrition Examination Survey of the US and Centers for Disease Control and Prevention website (www.cdc.gov/nchs/about/major/nhanes/growthcharts/datafiles.htm). The resting blood pressure was normalized as a *Z* score according to sex and age, using the US Task Force tables (1996).

All the investigations performed in this study were conducted in accordance with the Guidelines of the Declaration of Helsinki. Written consent from the participants and their parents were obtained, in accordance with the procedures approved by the ethical committee of our institution.

Biochemical measurements

Blood was drawn from fasting subjects who were in a supine resting position for at least 30 min. Plasma glucose, insulin, uric acid, total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol, and triglycerides were measured by standard clinical laboratory techniques. We used the homeostasis model assessment (HOMA) index (fasting insulin in micro units per milliliter, multiplied by fasting glucose in millimoles per liter, divided by 22.5) as an estimator of insulin resistance. As HOMA cut-off point to identify insulin resistance in children or adolescents is not well defined, we divided our population into two groups (noninsulin resistance (NIR) and insulin resistance (IR)) based on the median value of homeostasis model assessment of insulin resistance (HOMA-IR) of the whole sample (2.2) [9,12].

Bisulfite treatment of DNA and methylation-specific polymerase chain reaction

Nucleic acids were extracted from the white blood cells from a blood sample by a standard method as previously described [13].

We have used a previously published method [14]. Briefly, this technique is based on bisulfite treatment of genomic DNA, thereby converting all the unmethylated cytosines to uracils, while conserving the methylated cytosines. Then, genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation Kit, according to the manufacturer's protocol (Zymo Research Corporation, Orange, CA, USA).

The chemically modified DNA was subsequently used as a template for a methylation-specific polymerase chain reaction (MS-PCR) to determine the promoter methylation status of the selected CpG dinucleotides in the *TFAM* promoter.

An assay based on real-time quantitative PCR on an iCycler thermocycler (BioRad Hercules, CA, USA) was used for DNA methylation quantification, using SYBR Green (Invitrogen, Buenos Aires, Argentina) as a fluorescent dye.

For the MS-PCR experiment, two pairs of primers were used: one pair was specific for modified methylated DNA (M primers) and the other pair was specific for modified unmethylated DNA (U primers). Thus, for each sample studied, two PCRs were performed simultaneously using the M primer pair and U primer pair. Successful amplification from the M primers and U primers indicated methylation and unmethylation, respectively. A sequence starting 2,000 bp upstream from the transcriptional start site (TSS) of *TFAM* was used in the MethPrimer program (http://www.urogene.org//methprimer/index1.html) [15] to search for regions with potentially methylated CpG sites and PCR designs. The sequence was retrieved from the Database of Transcriptional Start Sites (DBTSS) at http://dbtss.hgc.jp/, with the following ID number: NM_003201, chromosome: NCBI36: 10: 59815182.59825903, TSS: 59815182.

For maximal discrimination between methylated and unmethylated alleles, M and U primers were designed to contain at least one CpG site at the 3′ end [15]. Primer sequences were as follows: For the methylated DNA template, forward primer: 5′-TTAATGGGTTTTATATAGATATACGG-3′ and reverse primer: 5′-AAAAATAATAACGAAAAAAACGAA-3′, and for the unmethylated DNA template, forward primer: 5′-TAATGGGTTTTATATAGATATATGG-3′ and reverse primer: 5′-CAAAAATAATAACAAAAAAAACAAA-3′. The resulting PCR product was 102 bp. The general PCR conditions can be obtained from the authors upon request.

The level of methylated DNA was expressed as the ratio of the estimated amount for methylated DNA to the unmethylated DNA levels, calculated for each sample using the fluorescence threshold cycle (Ct) values for a previously estimated efficiency of 2, according to a previous report [16]. We estimated the efficiency for each single sample tube using the slope of the exponential phase, as described by Ramakers et al. [17]. From the linearized curves for methylated and unmethylated reactions (correlation coefficients and mean ± SE: 0.998 ± 0.002 and 0.997 ± 0.003, respectively), we found that the PCR efficiencies were close to 2 and not significantly different for both the reactions (1.91 ± 0.02 and 1.90 ± 0.02, $n = 39$ independent samples, $p < 0.250$). Furthermore, as expected, controls for unmethylated (a purified native amplicon) and fully methylated DNA (the same amplicon treated with the DNA methylase M.SssI, New England Biolabs, Ipswich, MA, USA) gave 0 and 100% DNA methylation patterns.

In some instances, we expressed DNA methylation as a percentage (methylated DNA/(methylated DNA + unmethylated DNA) × 100). All the experiments were carried out in triplicate. The CV% was observed to be less than 5%.

The specificity of amplification and the absence of primer dimers were confirmed by melting curve analysis at the end of each run and agarose electrophoresis.

The web-based AliBaba2 program, available at http://www.gene-regulation.com/pub/programs/alibaba2, was used for *in silico* prediction of transcription factor-binding sites in the studied DNA sequences [18], a posteriori of the sequence and primers selection.

To ensure the specificity of the method and to avoid variability in the results because of the presence of two CpG dinucleotides in

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the reverse primer, we designed a degenerative reverse primer that introduced a mismatch in the second CG site, and observed the absence of amplification, regardless of the target DNA (data not shown), indicating that primers recognize the status of both the CpG dinucleotides simultaneously.

Statistical analysis

The quantitative data were expressed as mean ± SE unless otherwise indicated. As a significant variance difference was observed between the groups for most of the variables and as the distribution was significantly skewed in most cases, we chose to be conservative and assessed the differences between the groups by the Mann–Whitney *U*-test or ANOVA and Newman–Keuls test.

For multiple regression analysis, as the methylated DNA/unmethylated DNA ratio was not normally distributed, we used log-transformation of this variable. Correlation between the two variables was performed by Spearman's rank correlation test or Pearson's correlation test, after log-transformation of the variables. To perform these analyses, we used the CSS/Statistica program package, V 6.0 (StatSoft, Tulsa, OK, USA).

Results

In the analyzed sample, 45 adolescents were classified as non-insulin resistant and 77 as insulin resistant. Characteristics of the study population according to the insulin resistance status are shown in Table 1. Briefly, the IR group had significantly higher fasting plasma glucose, insulin, triglycerides, HOMA index, systolic arterial blood pressure (SABP) and diastolic arterial blood pressure (DABP) *Z* score, waist circumference, and BMI *Z* score, than the NIR group.

We used MS-PCR to assess DNA methylation of three putative methylation target sites (CpG) in the promoter of the *TFAM,* located at positions relative to TSS: −433, −442, and −499. In *silico* prediction of the transcription factor-binding sites in the studied *TFAM* sequence showed that one selected CpG dinucleotide site resides 100 bp from a putative hepatocyte nuclear factor-1-binding site. In addition, there were six predicted sites of specificity protein-1 (Sp1)-binding motif, one including the −442 CpG dinucleotide. The CpG dinucleotides and putative transcription factor-binding sites in the promoter region evaluated for DNA methylation are shown in Fig. 1.

In the whole sample, the methylated DNA/unmethylated DNA ratio of the *TFAM* promoter was 0.012 ± 0.0009 (1.2% of alleles).

TFAM promoter methylated DNA/unmethylated DNA ratio was inversely correlated with the biochemical features associated with insulin resistance (plasma fasting insulin, R: −0.26, $p < 0.004$; HOMA index, R: −0.27, $p < 0.002$; Fig. 2), the anthropometrical characteristics were associated with obesity (waist/hip ratio, R: −0.22, $p < 0.02$), which was defined as the body weight higher than the 95th percentile (R: −0.27, $p < 0.002$).

Multiple regression analysis showed that log-transformed HOMA index correlated (R^2: 0.23, $p = 0.000009$) with the status of the promoter methylation of *TFAM* (β: −0.33 ± 0.10, B: −0.50 ± 0.15, $p = 0.00094$), independent of BMI *Z* score (β: 0.33 ± 0.10, B: 0.14 ± 0.04, $p = 0.00088$).

Likewise, multiple regression analysis showed that the status of the promoter methylation of *TFAM* correlated (R^2: 0.10, $p = 0.0035$) with fasting insulin (β: −0.321 ± 0.094, B: −0.018 ± 0.005, $p = 0.0009$) and glucose levels (β: −0.216 ± 0.090, B: −0.010 ± 0.004, $p = 0.017$), independent of BMI *Z* score (β: 0.07 ± 0.09, B: 0.02 ± 0.03, $p = 0.447$).

In addition, *TFAM* promoter methylated DNA/unmethylated DNA ratio was significantly associated with insulin resistance as a dichotomous variable (NIR: $n = 45$, 0.014 ± 0.002; IR: $n = 77$, 0.011 ± 0.001, respectively; $p < 0.016$, Mann–Whitney's *U*-test).

Discussion

The possibilities that patterns of DNA methylation are variable between individuals and epigenotypes contribute to the susceptibility of complex disease have both drawn considerable attention in recent years [19].

To our knowledge, this study is the first to explore the DNA methylation status of *TFAM* promoter in adolescents with features of metabolic syndrome. *TFAM* promoter showed that 1.2% of the alleles were methylated at the particular CpGs analyzed. This low rate of methylation is expected, because they are located at a CG-rich promoter region (*TFAM* promoter was observed to have a large CpG island, showing around 450 bp in length near the transcription initiation start, with a C/G ratio of 0.87 and a GC content of 64.6%).

In addition, we observed that peripheral blood *TFAM* promoter DNA methylation at these sites was inversely correlated with insulin resistance status, considering either metabolic quantitative traits (fasting insulin, fasting glucose, and HOMA index) or insulin resistance as a dichotomous condition. Despite the fact that in our population, insulin resistance was mostly observed in obese adolescents and that DNA methylation was also negatively correlated

Table 1
Clinical characteristics of the study population according to the absence or presence of insulin resistance.

Features	Noninsulin resistance	Insulin resistance	*p* level
Number of subjects	45	77	
Age (years)	15.86 ± 1.35	15.09 ± 1.63	0.01
Sex (F/M)	28/17	46/31	NS
SABP *Z* score	0.07 ± 1.51	0.94 ± 1.58	0.004
DABP *Z* score	−0.31 ± 0.80	0.02 ± 0.85	0.03
Total cholesterol (mmol/L)	4.01 ± 0.70	4.15 ± 0.68	NS
Triglycerides (mmol/L)	0.77 ± 0.32	1.07 ± 0.52	0.0003
HDL cholesterol (mmol/L)	1.31 ± 0.26	1.23 ± 0.22	NS
LDL cholesterol (mmol/L)	2.33 ± 0.53	2.43 ± 0.61	NS
Uric acid (mmol/L)	225.1 ± 47.9	250.0 ± 65.8	NS
Glucose (mmol/L)	4.80 ± 0.35	4.99 ± 0.40	0.03
Insulin (pmol/L)	48.8 ± 12.1	102.4 ± 40.0	1×10^{-17}
HOMA	1.49 ± 0.37	3.31 ± 1.50	1×10^{-17}
BMI *Z* score	−0.09 (1.37	0.65 (0.91	0.006
Waist–Hip ratio	0.79 ± 0.05	0.81 ± 0.18	NS
Waist circumference (cm)	71.0 ± 6.6	76.3 ± 9.4	0.002

SABP: systolic arterial blood pressure; DABP: diastolic arterial blood pressure; BMI: body mass index; HOMA: homeostasis model assessment of insulin resistance. Results are expressed as mean ± SD. *p* stands for the level of statistical significance using Mann–Whitney *U*-test. NS: not significant. All measurements are in SI units.

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TFAM

-637-5'

AGACTGCAGTAGATGGTAGAATAAACAGTCTAAAGCTATATTGCTTTAAATGT

HNF-1/HNF3

GGTCTCAAGACCACCGACATTAGAACTTGTTAAATTCTGGGGAACTCGCTAAA

ATGAAGA**TTAATGGGCCCCACATAGACACACGG**AATTAAGCTCTGCGGTAA

Sp1 -499 Sp1

GGCCTTGGAATCTTGCATTTTTAGGTTTGCGAATC**CCCGCCTCTCCCGTTACTA**

Sp1 Sp1 -442 -433

TTTCTGAACTCCGAGCTCCAGCCCTGG-3'. **Transcriptional start site**

Sp1 Sp1

Fig. 1. The DNA sequence of the *TFAM* showing the methylation target sites in the promoter assessed in this study (−433, −442, and −499 in *TFAM*), and also the putative transcription factor-binding sites. Prediction of transcription factor-binding sites was made by constructing matrices on the fly from TRANSFAC 4.0 sites. Regions corresponding to forward and reverse primers are shown in bold. Transcription factor-binding sites are underlined. *TFAM:* mitochondrial transcription factor A. HNF: hepatic nuclear factor. Sp1: specificity protein-1.

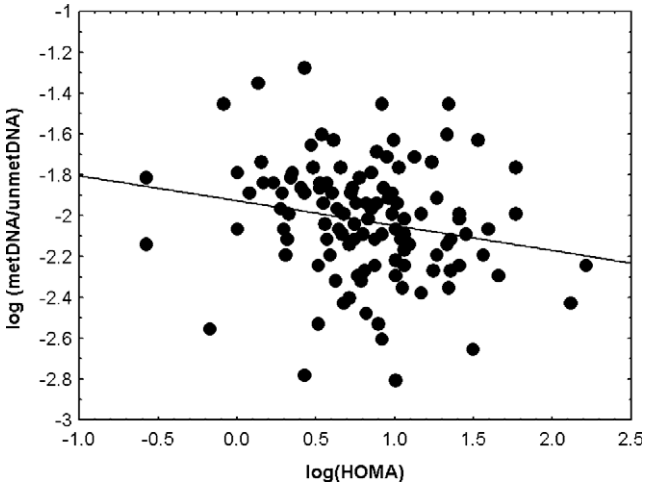

Fig. 2. Correlation between log-transformed *TFAM* promoter methylated DNA/unmethylated DNA ratio from leukocytes and log-transformed HOMA index in adolescents (R: −0.27, n = 122, $p < 0.002$).

with obesity, the observed effect of *TFAM* promoter DNA methylation on the HOMA index was independent of the BMI *Z* score.

Although association does not necessarily indicate a causal relation in this case, several lines of evidence, based on the biological plausibility of this relationship, support the association between epigenetic modifications as a potential mechanism to explain the occurrence of metabolic syndrome-related phenotypes. For instance, a recent study demonstrated the effect of epigenetic factors associated with the reduction in the expression of a gene of the respiratory complex 1 (*Ndufb6*) on age-dependent susceptibility to insulin resistance [7]. In addition, methylation of specific CpG sites in *Glut4* promoter, a gene involved in glucose transport in adipose tissue and skeletal and cardiac muscles, was observed to contribute to *Glut4* regulation [20].

Epigenetic changes may also be a factor in the metabolic programming of pancreatic β cells after fetal growth restriction, which induces diabetes later in life [3]. Finally, recent evidence from a human study demonstrated that expression of *PPARGC1A* was reduced and the level of DNA methylation of the *PPARGC1A* promoter was increased in the islets in patients with Type-2 diabetes, showing that epigenetic mechanisms are involved in impaired insulin secretion in diabetic islets [21]. Interestingly, we have observed that DNA methylation of the *PPARGC1A* promoter was positively correlated with the mother BMI in neonates [14].

Among the methylated CpG sites in the *TFAM* promoter that we evaluated in this study, there were six potential sites of binding to Sp1 transcription factor, one of them residing in the −422 CpG dinucleotide. It is worth mentioning that Sp1 has been implicated in the regulation of insulin [22].

When DNA methylation occurs at the promoter, gene expression is usually reduced, as it may inhibit the promoter activity through chromatin condensation. Thus, DNA methylation is considered to be a major regulator of transcriptional activity. However, one limitation to our study is that we were unable to measure the expression level of *TFAM* owing to the cross-sectional population-based design of our study. As a result, we could not demonstrate whether the methylation level of the *TFAM* promoter is related to the altered gene expression. However, it is important to note that the switch-like gene behavior – silent (greatly methylated) and potentially active (primarily unmethylated) – needs to be completely elucidated, as promoter hypomethylation might be required, for instance, for efficient gene expression [23]. Thus, it can be postulated that tissue-specific methylation patterns within the regulatory regions are a consequence, rather than a cause, of transcriptional activation, as it was shown that the binding and the consequent activity of some transcription factors are insensitive to cytosine methylation, and that binding of transcription factor can induce demethylation of local CpG sites in a replication-dependent manner [24]. However, weak CpG island promoters are distinct, as they are preferential targets for de novo methylation in somatic cells. Consequently, promoter sequence and gene function are observed to be the major predictors of promoter methylation states [25]. In fact, Choi et al. reported that *in vitro* methylation of the *TFAM* promoter (2378 bp) suppresses transactivation of the reporter activity [26].

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Lastly, it is worth mentioning that in this study, we explored whether *TFAM* promoter methylation could vary between individuals, which might be one of the factors that contribute to the inter-individual susceptibility to develop insulin resistance during childhood.

In summary, epigenetic gene regulation is now being considered to play a role in the pathogenesis of many complex disorders, including metabolic syndrome, sometimes, along with its acknowledged role in reprogramming during embryogenesis. Till date, little is known about the variation in DNA methylation patterns between healthy individuals. Thus, it also remains unclear regarding the extent to which intra- and inter-individual variation in DNA methylation patterns contribute to disease susceptibility.

We hope that our study acts as a pioneer, because further research is needed to confirm and extend the current findings to larger populations, to reveal the intimate mechanism by which DNA methylation variation may lead to insulin resistance in young people.

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Conflicts of financial interest

The authors have no conflict of interest to declare.

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