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Peroxisome proliferate activator receptor-γ (PPAR-γ) gene expression and global DNA methylation as predictors for insulin resistance in healthy adults

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ABSTRACT: Finding a feasible test for screening for insulin resistance (IR) in healthy adults can significantly reduce the incidence of plethora of metabolic and cardiovascular diseases and for this reason the current study was aimed to evaluate the prognostic value of peroxisome proliferator-activated receptor γ (PPAR-γ) gene expression and global DNA methylation in prediction of IR in healthy adults in a cross-sectional study conducted on 100 euglycemic, non-diabetic apparently healthy adults of both sexes, aged ≥45 years old with HbA1C ≤6.5%, who were categorized into insulin sensitive (IS) and IR according to Homeostatic Model Assessment of IR (HOMA-IR). Global DNA methylation was estimated with enzyme linked immune-sorbent assay using a ready commercial kit. Quantitative polymerase chain reaction (qPCR) was used to estimate PPAR-γ gene expression in relation reference gene and the results revealed that out of 100 apparently healthy subjects, 30 subjects (30%) had IR. The ΔCt of PPARγ gene was significantly lower in the IR individuals (6.49±2.46) in comparison to 8.13±1.37 of the IS individuals. However, it has relative low sensitivity and specificity (56% and 53%, respectively). There was no significant difference between individual with and without IR in the intensity of global DNA methylation that may lead to conclude that PPAR-γ gene expression is significantly reduced in individuals with IR; however, it cannot be used as a screening test for IR, due to low sensitivity and specificity. Global DNA methylation seems to have no association with IR.

KEYWORDS: Global DNA methylation; homeostatic model assessment; insulin resistance; peroxisome proliferate activator receptor-γ gene; type 2 diabetes.

1. INTRODUCTION

The defining trait of type 2 diabetes (T2DM) and a number of other cardiometabolic disorders, including obesity, hypertension, and cardiovascular disease (CVD), is insulin resistance (IR). The intricate interaction between genetic and environmental variables leads to IR [1]. Prediction of IR in healthy adults can have a great impact and individual's health as it could be used as a risk alarm to change the life style in order to avoid the aforementioned pathological conditions. Although, the Homeostatic Model Assessment of IR (HOMA-IR) is currently the reliable test for IR, it has some drawbacks mainly cost, accessibility and replicability issues [2]. Several alternatives have been proposed with varying degrees of specificity and sensitivity such as Triglyceride/HDL-c ratio had a very good predictive value for IR [3].

A member of the pleiotropic nuclear receptor 1C (NR1C) family, which is sometimes referred to as the PPAR family, is peroxisome proliferator-activated receptor γ (PPARγ) [4]. This nuclear receptor family includes a class of transcription factors that are controlled by ligands and have a broad distribution across tissues, targeting a diverse range of genes and functions [3], such as growth factors, antioxidant enzymes and proinflammatory factors. Four distinct subtypes of PPAR-γ mRNA (-γ1, -γ2, -γ3, and -γ4) are produced in humans as a result of alternate promoter use and PPAR- gene - γ splicing [5]. It has been demonstrated that PPAR-γ activation by their ligands increases glucose transporter gene expression in peripheral organs, specifically in glucose transporter 1 (GLUT1) and GLUT4. They significantly change the expression of genes in fat tissue. Furthermore, such activation was found to decreased the expression of resistin and tumor

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necrosis factor-alpha (TNF-α), two factors that cause IR. Ergo, it is plausible to assume that low expression of PPAR- γ, due to whatever cause, could be associated with IR [6].

Epigenetic means a genetic control by factors other than an individual's DNA sequence. It. acts as a link between genetic makeup and external environmental factors. Several types of epigenetic modification are presents, of which DNA methylation is the most widely studied. DNA methyltransferases transfer a CH3 group covalently to the C-5 of the DNA cytosine ring as part of an inherent epigenetic process. [7]. Alteration in DAN methylation was found to be associated with different human pathologies including metabolic disorders, different cancers, neurological disorders and aging [8]. The most prevalent type of transposable elements in humans is the Alu subfamily of repeating elements, a class of Short Interspaced Nuclear Elements (SINEs) with a length of approximately 300 base pairs (bp) that are found in the human genome in more than a million copies [9]. Changes in the DNA methylation of Alu repeating sequences are hypothesized to increase genomic instability [10] and hence increase the risk of IR. On the other hand, hypomethylation in Alu sites may cause inflammation [11,12], which contributes to the onset of IR. The current research aimed to investigate the prognostic value of PPAR-γ gene expression and global DNA methylation in prediction of IR in healthy adults.

2. RESULTS

2.1. Insulin Resistance Rate (the link between IR rate & the study to classify the adult subject to 2 groups IR & IS)

Out of 100 subjects that appeared healthy, 30 subjects (30%) had IR, while the remaining 70 patients (70%), according to the HOMA-IR model, were IS (Figure 1).

2.2. Insulin Resistance-Related Parameters

Fasting plasma glucose and HbA1c, per se, were normal with a mean of 89.37±7.9 mg/dl and 5.14±0.56%, respectively. Fasting insulin was almost normal and ranged from 3.62 to 25.3 mIU/L. However, calculated HOMA-IR varied considerably with a mean of 2.51±1.17, range= 0.69-5.37 (Table 1).

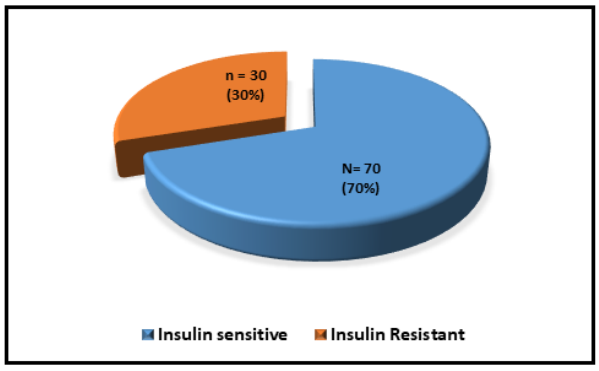

Figure 1. Insulin resistance rate in healthy subjects

Table 1. Insulin resistance-related parameters

Variables	Value
FPG, mg/dL	
Mean±SD	89.37±7.9
Range	72-109
HbA1c, %	
Mean±SD	5.14±0.56
Range	4.19-6.4
Fasting insulin, mIU/L	
Mean±SD	11.33±5.09
Median	10.3
Range	3.62-25.3
HOMA-IR	
Mean±SD	2.51±1.17
Range	0.69-5.37

2.3. Association of DNA methylation and ΔCt of PPARγ Gene with Insulin Resistance

The ΔCt of PPARγ gene was significantly lower in the IR individuals (6.49±2.46) in comparison to 8.13±1.37 of the IS individuals whereas, DNA methylation was not different in between the two groups (Table 2).

Table 2. Association of DNA methylation and ΔCt with Insulin Resistance

Variables	Insulin sensitive (70)	Insulin resistance (30)	*P*-value*
DNA methylation			
Mean±SD	2.00±1.76	1.59±1.68	0.138
Median	1.06	0.83	
Range	0.25-5.7	0.44-6.52	
ΔCt of PPARγ gene, fold			
Mean±SD	8.13±1.37	6.49±2.46	
Median	7.85	7.5	**0.013**
Range	5.7-11.8	0.8-9.3	

*Mann Whitney U test

2.4. Prognostic Value of ΔCt in Prediction Insulin Resistance in healthy individuals

Utilizing a Receiver Operating Characteristic (ROC) curve, the diagnostic value of ΔCt in the identification of IR was assessed. Area under the curve (AUC) was 0.567, 95%CI = 0.536-0.779, p = 0.013. The test's results showed 56% sensitivity and 53% specificity, 7.75 was the optimal cut off value for ΔCt. as shown in Figure 2.

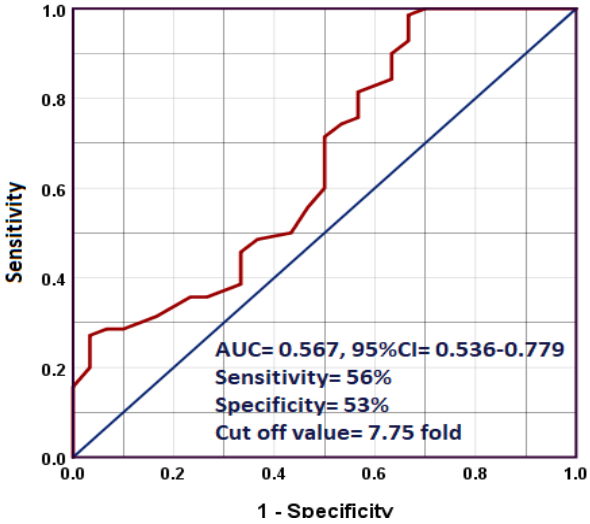

Figure 2. Receiver operating characteristic curve for ΔCt in detection of insulin resistance in healthy subjects

3. DISCUSSION

The current study's findings indicate that there was no discernible difference in global DNA methylation between IS and IR patients. This contradicts many previous studies. Zhao and colleagues [1] used 84 pairs of monozygotic American twins to investigate the relationship between global DNA methylation and IR. Using bisulfite pyrosequencing, the amount of global DNA methylation of Alu repeats in peripheral blood leukocytes was measured. The research showed that, independent of established risk factors, global DNA methylation was substantially linked to IR. After adjusting for a number of variables, an average increase in global DNA methylation of 10% was linked to an increase in HOMA of 4.55 units. In another study, Simar et al. [12] recruited 19 patients with T2DM and 25 healthy subjects. Using flow cytometry, global DNA methylation levels were determined according to cell type. Peripheral blood cells' global DNA methylation, particularly that of B and natural killer cells, was positively linked with IR. The variation in the results between these studies and ours could be attributed to several factors, the most important of which are the assay used to determine the DNA methylation, the gene involved in the measurement and participant's characteristics. In this regard, it was discovered that DNA methylation and aging are related. In skeletal muscle from aged participants compared to young subjects, two investigations reported enhanced DNA methylation in the promoter of two potential genes (NDUFB6 and COX7A1) [13, 14].

Many explanations exist, despite the fact that the chemical mechanism tying IR and global DNA methylation together is unknown up to our knowledge. Changes in DNA methylation may encourage genomic instability, which in turn may lead to IR and T2DM. On the other hand, it is likely that DNA epigenetic modifications could cause inflammation, a process linked to IR and its side effects [15]. Given that telomere shortening has been demonstrated to be epigenetically regulated and linked to both genomic instability and global DNA methylation, this suggests another plausible biological mechanism explaining the correlation between IR and global DNA methylation [16].

In the current investigation, people with IR had significantly lower levels of PPARγ expression. This outcome is consistent with another earlier research conducted globally. Ruschke et al. [18] used quantitative real-time PCR to evaluate metabolic parameters and the expression of PPARγ and PGC-1α mRNA in observational research involving 153 people. The expression of PGC-1α and PPARγ mRNA was linked to both cardiovascular risk and indicators of IR. In another research, Dubois and colleagues [19] found that obese patients with T2DM had significantly lower adipose tissue PPARγ expression than normal patients. According to Mishra et al. [20], visceral adipose tissue IR is strongly influenced by the expression of the PPARγ gene. Other studies demonstrated that PPAR-γ increases insulin-stimulated glucose uptake in insulin target cells via GLUT4 [21].

On the other hand, PPARγ agonists have been shown to significantly lower IR [22], which has led to the creation of various antidiabetic medications such as pioglitazone (Actos), rosiglitazone (Avandia), and troglitazone (Rezulin). In mice, activation of a similar receptor also has physiologic consequences that enhance insulin sensitivity [23]. Adipose tissue containing the PPARγ2 gene stimulates adipocyte differentiation, or the process of turning immature adipocytes into mature adipocytes, as well as lipid synthesis and storage in adipose tissue [24]. Maintaining insulin sensitivity requires adipocyte differentiation [25]. Adipocyte hypertrophy, adipose tissue malfunction, and a gradual build-up of triglycerides in preexisting adipocytes are the outcomes of impaired adipocyte differentiation [26]. Visceral adipose tissue is where fat accumulates when the subcutaneous adipose tissue's storage capacity is surpassed due to either overnutrition or defective adipogenesis brought on by a genetic predisposition [27]. It is thought that the ensuing lipotoxicity causes IR and T2DM. By promoting adipocyte development and increasing adipocyte flexibility, PPARγ reduces lipotoxicity [28, 29]. Moreover, it has been demonstrated that pioglitazone-induced PPARγ activation lowers postprandial triglyceride levels and delays the onset of T2DM in rats [30]. This implies that lipotoxicity, postprandial triglyceridemia, and the development of type 2 diabetes involve PPARγ.

4. CONCLUSION

Peroxisome proliferator-activated receptor γ gene expression is significantly reduced in healthy adult with IR; however, it cannot be used as a screening test for IR, due to low sensitivity and specificity. On the other hand, global DNA methylation seems to have no association with IR.

5. SUBJECTS AND METHODS

5.1. The Study Population

This study is a cross-sectional study which was conducted at Al Nahrain university/ college of medicine, Al Bayan University and Al-Mustafa University College, Baghdad, Iraq from February to August 2022. The study included 100 apparently healthy adult subjects of both sexes. Subjects enrolled in the study, are euglycemic, non-diabetic adults, ≥45 years old with HbA1C < 6.5%. the exclusion criteria of the volunteers who subjected to the current study are as the following:

- Subjects with diabetes
- Subjects with other severe chronic diseases, such as renal and cardiac diseases,
- Subjects who administered medications that may affect lipid metabolism or IR (such as anti-hyperlipidemic drugs corticosteroids and)
- pregnant and breast-feeding women,

5.2. Samples Collection and Laboratory Investigations

Eight hours of fasting were required for the participants before sample collection. Five milliliters of venous blood were drawn the following morning; two milliliters were placed in an ethylenediaminetetracetic acid (EDTA) tube, and the remaining three milliliters were placed in a gel tube. The samples were allowed to stand at room temperature for half an hour before being centrifuged for five

minutes at 3000 rpm. Before being utilized, sera have been collected in eppendorf tubes and kept at -20°C. A ready-made commercial kit was used for measuring fasting blood sugar (FBS), fasting insulin, and glycated haemoglobin (HbA1c). The HOMA-IR was computed by dividing the fasting insulin by 405 and multiplying the FBS. A prior study found that participants were classified as having IR if their HOMA-IR cut off value was more than 3.1. [3]

5.3. RNA Extraction and cDNA Creation

In order to prevent RNA degradation, RNA extraction was done on the same day as sample collection. The Direct-zol™ RNA Miniprep/Zymo/USA ready-made commercial kit was utilized to extract RNA from whole blood samples. The manufacturer's instructions were strictly adhered to. Reverse transcription of the extracted RNA into cDNA was performed using PrimeScript™ RT (Takara, Japan). This kit is a lyophilized master mix that is ready to use and includes every item needed to synthesize first strand cDNA from an RNA template..

5.4. Quantitative PCR for Measuring Gene Expression of Peroxisome Proliferate Activator Receptor-γ (PPAR-γ)

A ready commercial kit (KAPA SYBR® FAST qPCR Master Mix/ kapa/USA) was used for quantitative evaluation of PPAR-γ expression. In addition to the two primers used for the amplification of the reference gene (glyceraldehyde-3-phosphate dehydrogenase protein, or GAPDH gene), two primers were also employed for the amplification of the PPAR-γ gene in blood samples as illustrated in Table 3 and 4.

Table 3. Primers used for the amplification of the reference gene PPAR

I. Target Gene PPAR

Primer	Sequence	Tm (°C)	GC (%)
Forward	5′-CTTCTGGATTTCACTATGGAG -3′	66	60
Reverse	5 ′-AAGGTGTCCTGCTACATCAT -3′	66	58

Table 4. Primers used for the amplification of the reference gene GAPDH

II. Reference Gene GAPDH

Primer	Sequence	Tm (°C)	GC (%)
Forward	5′- TGCCCAGAACATCATCCCTG-3′	57.3	58
Reverse	5′-TCAGATCCACAACGGACACG -3′	57.1	58

Five microliters of the RNA extract, one microliter of 2.5 mM deoxyribonucleoside triphosphate (dNTPs), 0.3 microliters of the RT primer, and four microliters of RT 5x buffer (10 mM Tris pH 8.9, 1.5 mM MgCl2, 80 mM KCl) made up the reaction mixture. Pre-denaturation at 95 °C for 5 minutes is part of the PCR condition. Thereafter, there are 40 cycles of denaturation at 95 °C for 20 seconds and annealing/extension at 72 °C for 30 seconds.

The amplification and melting graphs were examined following the completion of the process. Every miRNA sample was given a threshold cycle (Ct) value, which was subsequently normalized to produce /Ct values, which were then used to illustrate the relative expression values of the reference gene.

Gene Expression was calculated according to the following equation:

Folding =2-ΔΔCT

ΔCT=CT gene - CT House Keeping gene

ΔΔCT=ΔCT Treated - ΔCT Control

5.5. Global DNA Methylation

The global DNA methylation was estimated using an enzyme linked immune-sorbent assay (ELISA) kit (5'-Methyl-2'-Deoxycytidine Quantitation/ Cell Biolabs/USA) in accordance with the manufacturer's instructions. In summary, leukocyte genomic DNA was extracted using a ready-made commercial kit (Genaid/ Taiwan). The isolated DNA was quickly cooled on ice after being incubated for five minutes at 95oC to form a single strand. Nuclease P1 and 20 mM sodium acetate were used to breakdown the

denatured DNA into nucleosides, which was then treated with alkaline phosphatase. After that, the mixture was centrifuged, and the supernatant was gathered. Each test well on the plate received 50 µL of the sample, which was then incubated for 10 minutes at room temperature. After the washing procedures, the kit's included anti-5MedCyd antibodies were added to the wells. The combination was mixed with 100 µL of the substrate solution, 100 diluted secondary anti-HRP conjugate, and stop solution. At 450 nm, the absorbance was measured.

5.6. Statistical Analysis

Version 25 of the Statistical Package for Social Sciences (SPSS) program was used for all analyses. The Shapiro-Wilk test was used to determine the normality of continuous data. Normal distribution variables were reported as mean ± standard deviation (SD) and subjected to independent t-test analysis [31]. Non-normally distributed variables were evaluated using a non-parametric Mann Whitney U-test and expressed as median and range. Chi square analysis was used to express categorical data as numbers and percentages. To evaluate the correlation between several continuous variables, Pearson's correlation was employed. In discrimination subjects with and without IR, the diagnostic value of PPAR-γ gene expression was assessed using a receiver operating characteristics (ROC) curve. Significant results were defined as a p level of ≤ 0.05 [32, 33].

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