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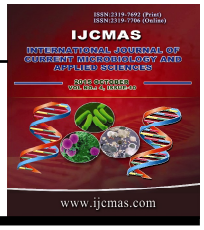

Original Research Article

The role of TNF α and Resistin Gene +299 (G□A) Polymorphism in the Development of Insulin Resistance in non obese Type 2 Diabetes Mellitus Iraqi Patients

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Keywords

Type 2 diabetes mellitus, TNF-α, RETN, Allele, Genotype, RFLP, insulin resistance

A B S T R A C T

Insulin resistance is a condition of decrease body response to natural insulin level in the serum, which is currently known as a characteristic feature of T2DM and participates in deformities in all of these tissues and organs. Thepresent study was conducted to evaluate the role of TNF-α and resistin +299(G□A)gene polymorphism in the development of insulin resistance among type two diabetic Iraqi patients. We have investigated single nucleotide polymorphisms of RETN +299(G□A) gene in 75 subjects by using. Fifty were insulin resistance diabetic patients while others were apparently healthy individuals used as control, then the serum level of TNF-α, resistin and insulin was detected by ELISA technique. The frequencies ofRETN +299(G□A) AA(32 vs 8%) genotypes and A allele (56 vs 46%)were higher in diabetic patients than control group while TNF-α level was significantly higher in patient than control groups and associated with higher mean serum concentration of TNF-α (8.28±3.21) versus (2.34±0.47), resistin (2.34±0.47) versus (1.26±0.18) and insulin level (18.79±6.45) versus (9.4±2.24),in insulin resistance diabetic patients than apparently healthy subjects. We conclude that AA genotype with A allele polymorphism are mainly expressed among insulin resistance patients and TNFα in insulin resistanceT2DMpatients was significantly higher compared with healthy group.

Introduction

In 2006, at least 171 million people worldwide suffering from diabetes and during2010 this number was increased to reach 285 million was diagnosed with DM, a prevalence of 6.4%. This is predicted to increase to 439 million, a prevalence of 7.7% by 2030. Between 2010 and 2030, the percentage of DM will increase about 20% in developed countries and 69%developing countries (Powers, 2005; Shaw *et al.*, 2010).

Insulin resistance causes decrease the ability of insulin to supply ordinary glucose and lipid homeostasis. Insulin resistance is a most pathologic condition at which the cells in the target organs unable to respond to normal serum insulin level. So that, more insulin than natural level is required so as to

adjust normoglycemyia (Eckel *et al.*, 2005). The most prevalent factor is obesity which is commonly of combined polygenetic and environmental origin. The major characteristic features of insulin resistance are uninhibited lipolysis in adipose tissue, defect in the glucose up taken in to skeletal muscles and uninhibited gluconeogenesis. Compensatory hyperinsulinemia caused by improvement β-cell releasing of insulin is consequently the most prevalent feature in insulin resistance (Granberry and Fonseca, 1999; Meier and Gressner, 2004).

TNF-alpha is an adipocytokine engaged in systemic inflammation and stimulates the acute phase reaction (Moller, 2000). TNF-α is mostly excreted by monocytes, macrophages and also by several different other cells which is include adipocytes (Giemenoand Klaman, 2005). It was thought that TNF-α contribute in the development of peripheral tissue (firstly muscle) resistance against insulin in obese persons and in patients with T2DM (Hotamisligil *et al.*, 1995). TNF-α has been shown to down-regulates many genes for "insulin receptor substrate-1 (IRS-1)", perilipin in adipocytes, adiponectin, glucose transporter 4 (GLUT4), "CCAAT/enhancer binding protein α (C/EBP-α)" and "peroxisome proliferator-activated receptor γ (PPAR-γ) "(Ruan *et al.*,2002). Also up regulate the gene expression in adipocytes which are required in several activities such as immune response, energy equilibrium and inflammation "(plasminogen activator inhibitor-1 (PAI-1), IL-1β, leptin, IL-6, resistin, vascular cell adhesion molecule-1 (VCAM-1), angiotensinogen)" (Saxena *et al.*, 1989). Adipose tissue secretes considerable quantities of TNF-α, which is partially participating in the evolution of insulin resistance in obese subjects (Ruan and Lodish, 2003). The activation of proinflammatory pathways after exposure to TNFα causes a state of insulin resistance whence of glucose capture in to muscles & adipose cells that impairs insulin signaling at the level of the insulin receptor substrate proteins (Iria Nieto-vazquez *et al.*, 2008). It was found that TNF-α have the ability to activate the "proteasome-mediated ubiquitin-dependent proteolysis" (UPS). This proteolytic system was participate in the control of receptor-associated (tyrosine-kinase activity) of the insulin receptor, it was supposed that the mechanism of TNF-α-induced insulin-resistance is mediated by the activation of the UPS-dependent proteolysis, which is contributed in the internalization of the insulin receptor, in the regulation the quantities of insulin receptor substrates 1 and 2 (IRS-1, IRS-2) and in insulin degradation (Pallares *et al.*, 2000; Wang *et al.*, 2006).

Existing studies indicates that dosage of exogenous TNF-α to the animals can generate insulin resistance, while neutralization of TNF-α can enhance the sensitive of the cells to insulin (Hotamisligil *et al.*, 1995).

Resistin is an inflammatory cytokine manufactured by adipose & immunity cells which is PMNs, macrophages and monocytes also some research find by using RT-PCR technique that resistin also produced by bone marrow (Azab *et al.*, 2012). With a molecular weight "12.5 KDa" and its length is 108 aa (Pischon *et al.*, 2007). Resistin gene is situated on chromosome 19p13.2 and spans 1369 bp with 4 exons & 3 introns. In resistin gene, non-coding SNPs was correlated with T2DM and obesity in Caucasian populations (Wang *et al.*, 2002).The effects of resistin on the development of insulin resistance and on the expression and localization of GLUT (glucose transporter) have been extensively studied (Muse *et al.*, 2004). Whose

expression is activated by "proinflammatory cytokines" and lipo-polysaccharide resulting from bacteria and suppressed by thiozolidondione drugs (TZDs) (Kaser *et al.,* 2003; Bajaj *et al.,* 2004).The responsibility of the resistin in the development of metabolic syndrome is as yet obscure. In rodents research found that RETN act as a connection between obesity, Insulin resistance and DM (Juan *et al.,* 2001; Way *et al.,* 2001). In mice, neutralization of resistin by antibody contributes in the enhancement insulin and glucose action (Pischon *et al.,* 2007).

The function of resistin linked to glucose homeostasis and insulin resistance in T2DM patients is still required to understand it. Some research can't determine any alteration in resistin levels in these diseases (Norata *et al.,* 2007; Osawa *et al.,* 2007).while other detect elevated resistin expression levels in T2DM, insulin resistance, obesity, MS, and CVD (Miyamoto *et al.,* 2009; Momiyama *et al.,* 2010). However another research show that serum resistin levels are associated in enhancing adiposity, but not effect on the degree of insulin resistance (Laudes *et al.,* 2010). These findings mention that the role of resistin in the pathogenesis of diabetes still questionable.

Materials and Methods

Patients

Fifty randomly selected 50 type 2 diabetic patients attending the diabetes mellitus center in Al-Sadder Teaching City hospital in Al-Najaf province, Iraq. The age of patients was range of 35-65years.and 25 age and sex matched healthy as controls, between December 2014 to February 2015 were included in the study. We excluded the following cases; Obese patients with BMI □25 kg/m2, Patients with renal dysfunction, Diabetic macroangiopathic complications i.e. coronary artery disease, peripheral vascular, disease thyroid diseases, heart disease and stroke, renal dysfunction, insulin therapy, Coexistent illness i.e. infections and those with Chronic inflammatory diseases: (rheumatoid arthritis, sinusitis, hay fever, psoriasis, SLE).

Methods

The following biochemical tests were done to confirm the diagnosis of diabetes mellitus, fasting blood glucose (FBG) and HbA1c, serum creatinine for check kidney state, clinical assessment including blood pressure and anthropometric variables (BMI =Weight (kg)/Height (m2).

Insulin resistance was calculated by HOMA-index according the following formula: HOMA index = [Fasting insulin(μU/ml)*FBG (mmol/l)]/22.5. Subject with HOMA more than 2.5 was considered as insulin resistance.

Serum resistin was measured by ELISA (Elabscience / china**)**.
Fasting serum insulin was calculated by ELISA. (DRG/GERMAN).
Serum concentration of TNFα was measured by using ELISA Kit (Elabscience / china). Following the manufacturer's instructions.

Genotyping

The genotypes of the RETN +299(G□A) gene were determined by PCR–restriction fragment length polymorphism (RFLP).DNA fragments extraction from EDTA blood specimens by using Accupower®Genomic DNA extraction mini kit (Whole Blood. Favorgen, Taiwan), then the RETN +299(G□A) gene was amplified by using forward primer 5'-GAGAGGATCCAGGAGGTCG-3' and the

reverse primer5'-GTGAGACCAAACGG T CCCT-3' (Bioneer, Korea). PCR amplifications were performed in Sprint Thermal-Cycler, programmed as following: initial denaturation, 95°C for 120sec, preannealing, 59°C for 60 seconds then elongation at 79°C for 120sec followed by 35cycles of denaturation at 95°C for 50 sec, annealing at 59°C for 30 secands elongation at 72°C for 90 sec, and final elongation at 72°C for 480 sec.

Then the PCR products (373bp) were visualized in an ethidium bromide-stained 1.5% agarose gel using a UV transilluminator (Figure 1). Following which they were digested with5U AluI (Thermo Fisher Scientific /USA) restriction enzymes. The digested PCR products were visualized in an ethidium bromide-stained 1.5% agarose gel using a UV transilluminator (Figure 2).

Statistical analysis

Statistical analysis was done by using SPSS (statistical package for social sciences) version 20 in which we use ANOVA (analysis of variance), independent sample T-test, chi square test, and Pearson correlation coefficient as needed. We set P value <0.05 as significant.

Results and Discussion

Demographic and biochemical profile

The demographic and biochemical profiles of both diabetic patient and control groups are shown in table 1. In which there are a significant differences between the patients and controls at all the biochemical parameters except age, and male to female ratios, creatinine, SBP and DBP. The subjects with T2DM were not obese due diet regimen after diagnosis of diabetes.

Distributions of RETN +299(G□A) genotypes and alleles in case and control groups

Distribution of RETN +299(G□A) polymorphism was detected by PCR-RFLP technique, at this locus there're three genotype; for RETN+299(G□A) AA, GA and GG (Figure 2). The frequency distribution of genotypes and alleles of RETN +299(G□A) in patient and control groups are summarized in (Table 2).

The A allele may increase a susceptibility to diabetes, whereas The G allele may be protective allele. The present study revealed statistically significant increase in AA genotype (with odd ratio 5.412, 95%cl 1.031–37.73) and combined AA+GA (with odd ratio 12.31 and 95%cl 2.62–65.71). This study was also revealed statistically significant decrease in GG genotype in T2DM in compared with control subjects. The distribution of +299 resistin allele frequencies A and G was 46% and 54% respectively in control subjects whereas in diabetic group it was 56% and 44% respectively. There was a statistically significant relation of A allele in the diabetic subjects as compared with other group ($p<0.001$). These results are harmonious with (Khalil *et al.,* 2014).This polymorphism is in an intron, +299 (G>A), which generally has not been considered to have regulatory functions. However, it has been shown that SNPs in the non-coding region, such as the 3'-untranslated gene region, can affect gene expression (Pesole *et al.,* 2001; Suriyaprom *et al.,* 2010). The potential mechanism underlying the association of the RETN +299 gene polymorphism to T2DM may be according to Suriyaprom *et al.* (2010) Who conclude that resistin gene polymorphism at +299 (G>A) probably a marker in linkage disequilibrium with other polymorphism

affecting gene expression and may contribute to increased resistin levels in Thai diabetic subjects, which may be involved in the pathogenesis of type 2 diabetes by impaired insulin action (Suriyaprom *et al.*, 2010).

Tsukada et al (2006) said that intron polymorphism of TFAP2B, a susceptibility gene to T2DM, influence adipocytokine gene expression transcriptional activity.

Table.1 Comparison of some socio-demographic data, clinical and biochemical characteristics of studied subjects

parameter		Control(25)	Cases(50)	P value
Age/years		44.72±9.08	48.14±13.74	0.264
Gender	**Male**	12(48%)	24(48%)	1
	Female	13(52%)	26(52%)	
BMI kg/m^2		22.86±1.43	23.48±1.47	0.09
Weight(kg)		81.2±14.4	74.3 ±12.4	0.03
Height(m)		1.8 ± 0.1	1.8 ± 0.1	0.78
Duration of the disease (y)		2.5 ± 2.1	----------	------
Creatinine mg/dl		0.68±0.11	0.7±0.13	0.242
FBS mg/dl		91.66±9.34	248.54±65.59	<0.001
HbA1c%		5.059±0.276	8.64±2	<0.001
Insulin Mu/ml		9.4±2.24	18.79±6.45	<0.001
Resistin ng/dl		1.26±0.18	2.34±0.47	<0.001
TNFα pg/ml		8.28±3.21	26.4±10.1	<0.001
HOMA-IR		2.1±0.5	11.59±4.97	<0.001
SBP mmHg		122.54±3.75	121.53±3.51	0.252
DBP mmHg		78.2±15.09	80.36±2.08	0.323

Table.2 Frequency and distribution of resistin genotype and allele frequencies in diabetic Patients and controls

Allele	Control(n=25)	Cases(n=50)	P value	Odd ratio	(95%CI)
AA	2(8%)	16(32%)	0.022	5.412	1.031-37.73
GA	12(48%)	31(62%)	0.248	1.768	0.602-5.231
GG	11(44%)	3(6%)	<0.001	0.081	0.015-0.381
GA±AA	14(56%)	47(94%)	<0.001	12.31	2.62-65.71
G allele	34(54%)	37(44%)	<0.001	0.276	0.126-0.601
A allele	16(46%)	63(56%)			

Table.3 Statistical analysis of biochemical parameters in association with genotype distribution in diabetic patients

Variable		AA	GA	GG	P value
BMI kg/m^2	T2DM Patient	23.94±0.69	23.22±1.73	23.93±0.72	0.260
	control	22.71±1.54	22.81±1.61	23±1.1	0.905
FBS mg/dl	T2DM Patient	328.16±29.35	241.84±31.59	125±16.39	<0.001
	control	94.82±9.48	89.34±10.29	93.72±7.25	0.443
HbA1c%	T2DM Patient	11.2±1.57	8.08±1.23	6.51±1.04	<0.001
	control	5.34±0.25	4.95±0.27	5.05±0.15	0.022
Resistin ng/dl	T2DM Patient	2.96±0.04	2.27±0.17	1.42±0.22	<0.001
	control	1.41±0.06	1.29±0.15	1.19±1.18	0.075
TNF pg/ml	T2DM Patient	39.26±4.79	24.5±6.14	10.76±1.64	<0.001
	control	7.68±1.63	7.9±1.93	5.84±2.94	0.061
Insulin Mu/ml	T2DM Patient	18.4±5.9	19.63±6.39	15.05±7.55	0.278
	control	9.82±2.02	8.73±2	8.91±3.79	0.082
HOMA-IR	T2DM Patient	14.84±4.5	11.66±4.2	4.67±2.27	<0.001
	control	2.16±0.33	2.14±0.31	2.1±0.26	0.093

Figure.1 agarose gel electrophoresis of amplified PCR product (373 bp) of RETN+299 gene: Show: DNA molecular size marker (KAPA Universal Ladder) and other lane for diabetic and control group

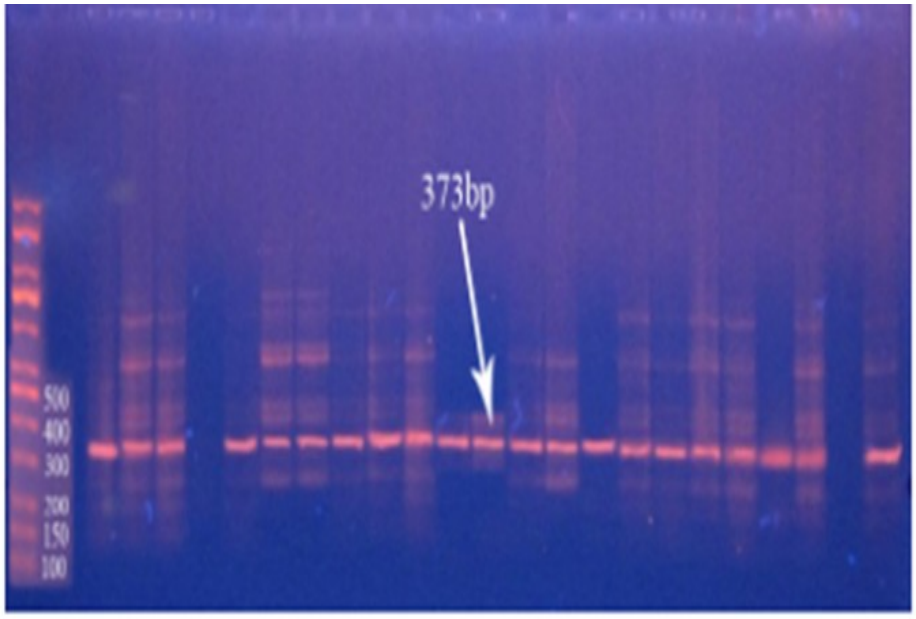
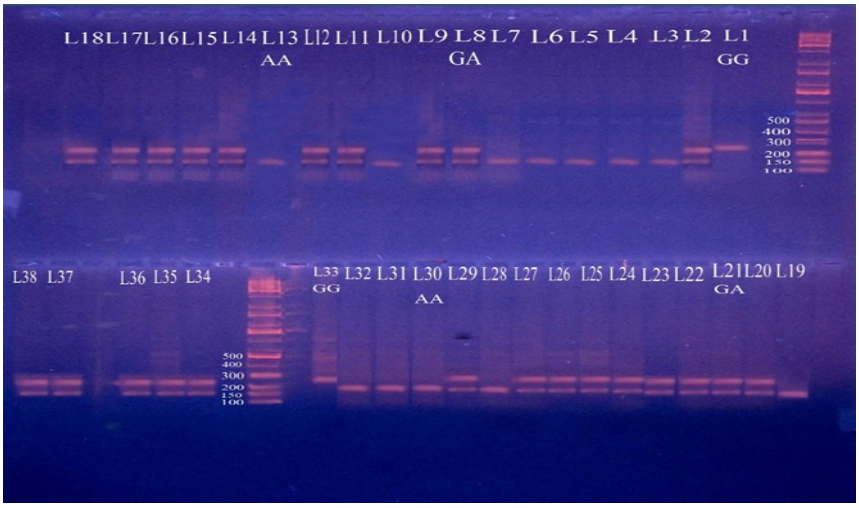


Figure.2 Agarose gel electrophoresis of RETN+299 gene after restriction digestion with Alu1 Show: DNA molecular size marker (KAPA Universal Ladder), Lane 1, 33= GG Homozygous for wild type genotype, Lane 8, 9=GA heterozygous genotype, Lane 13, 21=AA Homozygous for mutant genotype



Also Khalil *et al.* (2014) found that the risk of development T2DM was increased in the subjects who carry a polymorphic A allele in RETN (OR=3.021, p=0.0001). El-Shal *et al.* (2013) reported that the Frequency of the RETN +299 AA genotype was significantly associated with diabetic patients when compared with control group, the odds ratio (OR=3.53, P=0.005) suggested an association between the presence of the polymorphism and the prevalence of disease. They revealed that the genotypes and alleles of resistin polymorphisms +299(G>A) were significantly associated with increased risk of impaired glucose Tolerance and T2DM compared to normal glucose tolerant patients.

This results harmonious with Miyamoto et al (Miyamoto *et al.,* 2009) who found that there is a significantly increased in the AA genotype and A allele the in diabetic patients than in control individuals (p<0.0001).On the other hand GG genotype "(OR=0.06, 95% CI (2-0.11) P= 0.451)" and G allele "(OR=0.1, 95%CI (0.12-2) P=0.6)", which haven't statistical significant association with diabetic subjects. However, the risk to improve T2DM was elevated in the subject's carry the polymorphic AA and GA alleles in SNP/ RETN +299 (Suriyaprom *et al.,* 2010). Whereas resistin gene +299 (G>A) was not associated with T2DM in USA subjects and Malaysian (Coneely *et al.,* 2004; Lau and Muniandy, 2011), respectively.

We divided each of the control and T2DM into 3 subgroups according to genotype frequencies and we compared the mean value ± SD of the parameters, there were statistically significant difference as regards to mean value ±SD of FBG, serum resistin, fasting serum insulin, HOMA-IR and HbA1C % in AA subgroups of T2DM group as compared to both GG and GA subgroups. As well as there were statistically significant difference when comparing GG and GA subgroups as FBG, serum resistin, fasting serum insulin, HOMA-IR and HbA1C %. No statistically significant differences were detected as regards to the parameters when

comparing AA, GA, GG subgroupsin control group (Table 3).

This study found that the subjects with T2DM appear significantly increased resistin concentration than healthy controls and the resistin concentration in subjects with T2DM with AA and GA genotypes were significantly higher than those with the GG genotype and this results was agreement with (Asano *et al.,* 2010; Onuma *et al.,* 2010).

Tejero *et al.* (2008) mention that resistin expression may be cis-regulatory, which is denote that there were variants close to or in the RETN gene that may affect the profusion of its mRNA. The high level of resistin may be due to increase TNFα level in the serum of patients with T2DM (Saxena *et al.,* 1989). Lau and Muniandy (2011) were found that the resistin concentration was elevated in subjects with AA genotype, followed by the GA and GG. Moreover they found that A allele was strongly correlated with increased resistin levels.

Also our results found an association between RETN +299 (G□A) polymorphism and insulin resistancein T2DM group, where HOMA index wassignificantly increased in subjects with AA and GA genotype compared with subjects with GG genotype in agreement with (Khalid *et al.,* 2014). Who report that the HOMA level was significantly increase in diabetic subjects with AA and combined GA+AA genotypes when compared with control group. This study revealed a statistically significant combination between hyperglycemia& HbA1c and RETN gene +299 polymorphism. This study revealed that FBS&HbA1c level was increased in the AA and GA genotypes when compared with GG genotype in diabetic group. No statistically significant change were determined during comparing FBS& HbA1c level in AA, GA&GG subgroup in the control subjects in agreement with (Suriyaprom *et al.,* 2009). Our results were also found that insulin level was increased but not significantly ($p<0.278$) in the subject with AA&GA genotype when compared to GG genotype, this may be due to high resistin level in the subjects with AA&GA genotype because resistin effect on the insulin receptor on the target organ and causes insulin resistance which is turn increase beta cell production of insulin.

Our study found a significant association between TNF-α concentration and type two diabetes mellitus ($P <0.001$), this may be due to its main role in T2DM pathophysiology and these results was agree with (Swaroop *et al.,* 2012)**.** The elevation of TNF-α levels may be caused by the raised oxidative stress (OS) developed in the type 2 diabetes mellitus (Muhamed, 2009). OS is a serious condition which leads to the damage of various macromolcules, i.e., DNA, lipids and proteins. Cellular damage has been also reported to associate with elevated OS (Hong *et al.,* 2006).

This study revealed that the level of HOMA was elevated with increase TNF α concentration in serum of diabetic, this because the role of TNF □ in glucose metabolism resulting in high level of insulin resistance and obesity (Zou and Shao, 2008). The association between the proinflammatory activation and the insulin resistance may be supported by the evidence of Plomgaard *et al.* (2005) who have found that the infusion of TNF-α into healthy individual impaired insulin signaling and whole body glucose uptake.Many studies indicate that TNF-alphaplays a key role in the pathogenesis of obesity induced insulin resistance caused by an interaction with insulin signaling pathway. The present

results agree with Rajarajeswari et al (2011) who found that there was a correlation between serum TNFα and insulin resistance development, were considerably higher in diabetic than non-diabetic controls. However, several study have indicated that insulin resistance is independent on the change of TNF-α level (Kellerer *et al.,* 1997; Michihiro *et al.,* 2005).

In conclusion, there is significantly higher Concentration of TNF-α in insulin resistance T2DM patients in comparison to control group. This provides strong evidence that pro-inflammatory cytokine play a major role in the pathogenesis of insulin resistance. The heterogeneous GA genotype was more prevalent in most studies for different population. The presence of AA genotype and A allele in the population may predict the probability of developing insulin resistance T2DM, whereas GG genotype and G allele might serve as protective factors for the disease. Further studies with larger samples size of both obese and non-obese group for different population are required to establish an obvious idea about the prevalence of this mutation among diabetic patients and for better understanding the effect of RETN +299(G□A) polymorphism on the development of insulin resistance T2DM.

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