Original Article

Associations of Serum and Saliva Resistin Levels with *RETN* Gene Polymorphism –420 C/G (rs1862513) among Iraqis' Type II Diabetes

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Abstract

Background: In all societies in the world, type II diabetes (T2DM) has become a health challenge. Resistin is one of the adipokines associated with weight gain and T2DM. **Objectives:** The aim of the study is to find the correlation of resistin levels in the serum and saliva of the T2DM group and the non-diabetic (ND) control group. **Materials and Methods:** The two groups involved in our study were from Baghdad, Iraq. Resistin concentration was measured by enzyme-linked immunosorbent assay. Genotype analysis was carried out using the restriction fragments length polymorphism technique. **Results:** The results of our study showed that both glucose and resistin were elevated in the serum and saliva of T2DM compared to ND. In addition, the results of the correlation analysis showed a strong positive relationship for resistin levels between serum and saliva, and a moderate positive relationship for glucose levels in the ND group. The genotype (CC) is the most frequent in the T2DM group, which indicates that it is a risk factor for susceptibility to T2DM. No significant differences were shown for resistin concentrations among the three genotypes. No correlation was shown between minor allele (C) and resistin concentration in the T2DM group. **Conclusions:** Our findings supported the possibility of adopting saliva as substitute for serum in the detection of T2DM biomarkers in healthy group, with the need for more studies related to the possibility of replacing blood with saliva in the case of T2DM patients. In addition, our results for single nucleotide polymorphism (SNP) rs1862513 showed the need for further studies that might show the relationship of this SNP with the expression of resistin.

Keywords: Resistin, saliva, SNP, T2DM

Introduction

Type II diabetes (T2DM) is a metabolic disease with clinical importance in recent decades, it has become an epidemic all over the world that burdens health care.[1] Cell resistance to insulin, especially liver, muscle, and fat cells, and insufficient secretion of insulin is its most important characteristic. Many factors, such as age, obesity, and lack of physical activity, contribute to its development.[2,3] Overweight is the most important factor related to T2DM. adipose tissues play an essential role as energy stores, in addition to that they secrete adipokines (biologically active molecules) that have effects on fat metabolism, regulation of satiety and appetite. Insulin sensitivity and metabolism of glucose, and have important effects on biological processes such as angiogenesis, cell adhesion, bone morphogenesis, adipogenesis, and hypertension.[4,5] Resistin is one of the proinflammatory adipokines, located on chromosome 19P13.2, rich in cysteine, and has a role in glucose homeostasis.[5,6] In human, resistin is secreted by white blood cells entering adipose tissue due to an obesity-induced inflammatory response.[7] High levels of human resistin stimulate insulin resistance and have a significant effect on the occurrence of inflammation in T2DM.[8] Moreover, resistin plays an

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important role in many diseases, for instance metabolic illnesses, autoimmune diseases, and inflammation.[9] High levels of resistin have been found in both, hereditary and diet-induced obesity.[10] Elevated levels of resistin also cause inhibition of the insulin signaling pathway by activating inhibitory cytokine signaling (SOCS-3) which causes degradation of the insulin receptor substrate 1/2, thereby causing insulin resistance.[11] The involvement of resistin in obesity and type 2 diabetes has prompted multiple genetic investigations in different populations in the world.[12,13] Variations in promoter sequence of resistin—*RETN* gene including (rs1862513) has been studied in several societies, due to its effect on resistin gene expression and blood concentration.[14] Perhaps through its effect on transcription factors that bind to the resistin gene promoter.[15] It is considered as one of the strong determinants for resistin concentration in circulation.[16] Additionally, it appears to be a risk factor that increases the chances of developing T2DM.[17] Blood is frequently used as a source for measurements of biomarkers. Saliva can be used as a substitute for blood in the measurement of several biomarkers because many of the elements in saliva come from blood.[8] Biomarkers are used as indicators of normal or pathological processes. Many molecules found in vital fluids such as proteins, metabolites, lipids and microbes can act as biomarkers. Saliva is one of the biological fluids that plays an important role as a diagnostic tool because not only it reflects the pathological condition of the individual but also it contains a little amount of biomarkers that are derived from plasma, that facilitate continued monitoring of general health status and oral health as well as allow rapid, easily collectible and inexpensive detection of biomarkers.[18] Because of its many advantages over other fluids in the body, saliva as a vital fluid has aroused great interest among researchers.[19] The current study aims to evaluate the levels of fasting resistin and fasting blood glucose (FG) in the serum and saliva of the Iraqis T2DM and non-diabetic (ND) groups, and to find the correlation of the resistin and FG levels between the serum and saliva for the two study groups, in addition to determine the minor allele and genotype of single nucleotide polymorphism (SNP) (rs1862513) associated with T2DM and increased resistin level in Iraqi T2DM patients.

Materials and Methods

Study groups

Samples and data for the T2DM group and the ND group were collected from individuals attending the consulting clinic laboratory of Baghdad Teaching Hospital in Iraq from December 2021 to February 2022. The total number of samples is (88): 54 T2DM group and 34 ND group. Their ages ranged from 40 to 55 for both genders. For both groups, subjects with systemic or oral disease were excluded T2DM subjects who are partially or completely dependent on insulin were excluded, body mass index more than 27 were excluded for ND group.

Collecting of blood samples

Blood samples were collected for both groups from 8 AM to 11 AM after fasting for at least 8 h. 3 mL were collected in a gel tube to obtain the serum (it was left for an hour to clot at room temperature, then centrifuged for 15 min at 1000×*g*), 2 mL were collected in an anticoagulant tube to extract DNA.

Collection of saliva

Drooling method was used to collect the whole un stimulated saliva.[20] Before collecting saliva, the person should be asked to rinse her/his mouth with 10 mL of distilled water for 30 s to get rid of any remnants of cells and to moisten his mouth, and after resting for 10 min. About 4 mL of saliva was collected in the early morning from 8 AM to 11 AM The subjects were fasting at least 8 h.[21] After the collection was completed, saliva is immediately centrifuged for 15 min by 3000 rpm. The supernatant was kept at –20°C in Eppendorf tubes until the required test was performed.

Measurements

An estimation of the serum FG concentration for both groups was obtained from the records of the consulting clinic lab in Baghdad Teaching Hospital, Iraq in conjunction with taking serum and saliva from both groups. Fasting salivary glucose concentration for both groups was estimated according to Jouda *et al.*.[22] Which adopted the principle of oxidase - peroxidase method, or glucose (GLUC-PAP) concentration method by using Randox assay kit. Resistin concentrations were measured in serum and saliva samples for both T2DM and ND group according to sandwich enzyme-linked immunosorbent assay (enzyme-linked immunosorbent assay) by ELISA kit (Elabscience, USA).

Genotyping of resistin gene polymorphism

The *RETN* –420C>G (rs1862513) polymorphisms were genotyped by PCR-restriction fragments length polymorphism (RFLP) technique. Frozen blood samples of both groups were left at room temperature to thaw, for DNA extraction using Promega extraction kit (Relia Prep™ Blood g DNA Mini-prep System-Promega). Genotypes were determined according to what was mentioned in the study of Hashim and Al-Shuhaib.[23] PCR primers were designed according to the study of Hashem *et al.*.[24] DNA fragment of the SNPs –420 was amplified by PCR with the following primers. The following primers were used for the amplification of the target DNA sequence (5′–3′):

forward—5′-TGTCATGTTTGCATCAGCCAC-3′

reverse complement—5′-TTGGCTAATAAGTCCCTGGGC-3′.

Table 1: Comparison of fasting glucose and resistin concentrations between T2DM and ND groups

Variables	ND	T2DM	*P* value
Serum FG (mg/dL)	90.235 ± 0.759	223.096 ± 12.374	0.000
Saliva FG (mg/dL)	6.827 ± 0.197	18.815 ± 0.535	0.000
Serum resistin (pg/mL)	37.53 ± 1.479	103.041 ± 14.545	0.004
Saliva resistin (pg/mL)	9.982 ± 8.2798	91.696 ± 19.645	0.000
Number of samples	34	54	
Total number of samples	88		

T2DM: type 2 diabetics millets, ND: non-diabetics (control) group, FG: fasting blood glucose
Data are presented as the mean ± SE
Significant difference at $P \leq 0.05$—Mann–Whitney *U* test

Reaction conditions such as thermal cycles and contents of the PCR mixture were optimized to obtain high quality PCR products. Twenty microliters (20 µL) PCR reaction was conducted, 0.2 µL of Bstv2 restriction endonuclease was used to digest the PCR products overnight. The digestion products were separated using 2% agarose gel. Ethidium bromide was used for staining. Agarose gel (2%) was prepared according to the method of Samboork and Russell,[25] and 100 bp DNA ladder was used to identify the size of the resulting RFLP products. A horizontal electrophoresis device was used at 100 volts and 50 mA for 1 h. The agarose gel template was then photographed and analyzed in order to obtain results.

Statistical analysis

All statistical calculations were performed by SPSS software, Version 21.0 (IBM Corp., Armonk, NY, USA, Released 2012) and Microsoft Excel (Microsoft Corp., USA, 2010). All the results were expressed as median and 95% confidence interval (CI) were calculated. Mann–Whitney *U* test has been used for non-normally distributed data. Correlation was done using Spearman correlation. To find out the differences between the resistin concentrations of the three genotypes, Kruskal–Wallis analysis was used. Assess the categorically association variables and genetic association Performed using Hardy–Weinberg equilibrium, according to Solé *et al.*.[26]

Ethical considerations

After reviewing the research plan, research subject information, and approval form, the Ethics Committee in the Department of Life Sciences, College of Science, University of Babylon, Iraq approved according to document number 220101 on January 17, 2022. Oral consent was taken from the contributors (two study groups) before sampling was taken from them.

Results

Biochemical parameters were expressed as the mean ± SE between the two study groups and presented in Table 1. The mean of FG and resistin in serum and saliva of T2DM, were significantly higher ($P \leq 0.05$) compared

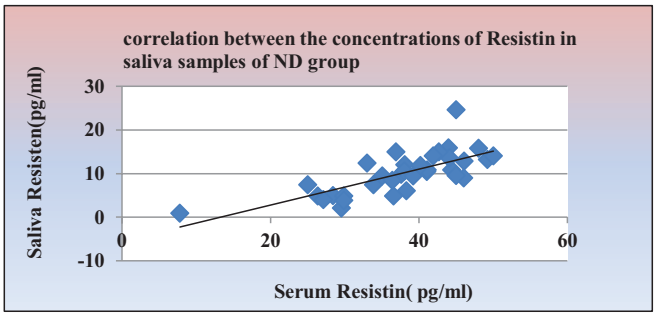

Figure 1: Correlation between the concentrations of resistin in saliva and serum samples of ND group ($P = 0.00$)

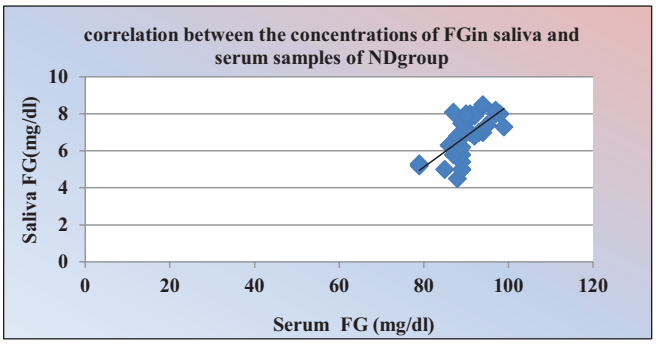

Figure 2: Correlation between the concentrations of FG in saliva and serum samples of ND group ($P = 0.00$)

to ND group. Correlation analysis [Figures 1 and 2] showed a significant positive correlation ($P < 0.01$) for ND salivary and serum resistin, *r* (0.735) *p* (0.00), salivary and serum FG, *r*(0.642) *P*(0.00) respectively. Moreover, no correlation was shown in Table 2 between serum resistin and serum FG, *r*(0.072) *P*(0.664) nor, in saliva resistin and saliva FG, *r*(0.172) *P*(0.331) in the same group. On the other hand, no correlation was seen in the T2DM group between salivary resistin and serum resistin, *r*(–0.043) *P*(0.759), salivary FG and serum FG, *r*(0.105) *P*(0.451) and also, no significant correlation was shown between serum resistin and serum FG, *r*(–0.73) *P*(0.60), salivary resistin and salivary FG, *r*(–0.236) *P*(0.086). In Table 3, our results showed that the genotype (CC) was increased by eight times in the T2DM group, compared to the ND group, OR = 8.00 (1.74–36.71) $P = 0.014$. Moreover, the

Table 2: Correlation of serum and saliva resistin with serum and saliva FG in T2DM and ND groups ND ($n = 34$)

		Serum resistin	Saliva resistin
Serum FG	*r*	0.072	
	P	0.664	
Saliva FG	*r*		0.172
	P		0.0.331
T2DM ($n = 54$)			
		Serum resistin	**Serum FG**	**Saliva FG**
Saliva resistin	*r*	–0.043		–0.236
	P	0.759		0.080
Saliva FG	*r*		0.105	
	p		0.451	
Serum FG	*r*	–0.73		
	P	0.60		

T2DM: type 2 diabetics millets, ND: non-diabetics, *r*: correlation coefficient, *P*: *P* value
Correlation by Spearman's rank (2-tailed)

Table 3: Odds ratio analysis for the distribution of alleles and genotypes of SNP (rs1862513) in resistin gene

Model	Genotype	ND *n* (%)	T2DM *n* (%)	OR (95% CI)	*P* value
Codominant	G/G	12 (35.3%)	8 (14.8%)	1.00	0.014
	C/G	19 (55.9%)	30 (55.6%)	2.37 (0.82–6.86)	
	C/C	3 (8.8%)	16 (29.6%)	8.00 (1.74–36.71)	
Dominant	G/G	12 (35.3%)	8 (14.8%)	1.00	0.027
	C/G-C/C	22 (64.7%)	46 (85.2%)	3.14 (1.12–8.77)	
Recessive	G/G-C/G	31 (91.2%)	38 (70.4%)	1.00	0.015
	C/C	3 (8.8%)	16 (29.6%)	4.35 (1.16–16.31)	
Allele					
G		43 (0.63)	46 (0.43)	**0.43 (0.23–0.8)**	0.008
C		25 (0.37)	62 (0.57)	2.32 (1.24–4.32)	

SNP (rs1862513-resistin gene) exact test for Hardy–Weinberg equilibrium ($n = 88$)

	GG	CG	CC	G	C	*P* value
All subjects	20	49	19	89	87	0.39
ND (control)	12	19	3	43	25	0.46
T2DM	8	30	16	46	62	0.41

T2DM: type 2 diabetics millets group, ND: non-diabetics (control group), OR: odds ratio, SNP: single nucleotide polymorphism

Table 4: Comparison of serum resistin and serum FG between rs1862513 genotypes

rs1862513	*N*	Serum resistin (pg/mL)	Mean rank	**P* value	Serum FG (mg/dL)	Mean rank	**P* value
GG	8	59.87 ± 25.735	20.0	0.186	181.175 ± 17.456	22.63	0.535
CG	30	121.324 ± 21.334	30.68		241.367 ± 18.99	29.35	
CC	16	90.347 ± 24.57	25.28		209.8 ± 18.312	26.47	
Total	54						

n: number, FG: fasting glucose
Data are expressed as mean ± SE, and mean Rank by Kruskal–Wallis test, significant difference at * $P \leq 0.05$

Table 5: The correlation between the C allele and T2DM serum resistin

Allele	Serum resistin (pg/mL)	
	R	*P* value
C	–0.168	0.263

Spearman correlation, *r*: correlation coefficient

frequency of the allele (C) is twice as high as the allele (G) OR = 2.32 (1.24–4.32), $P = 0.008$, which indicates that it is a risk factor for Iraqis to be susceptible to T2DM, whereas the allele (G) represents a protective factor against T2DM 0.43 (0.23–0.8), $P = 0.008$. In Table 4, our results did not show any significant difference for resistin concentration as well as fasting blood sugar between the three genotypes

in the T2DM group. In Table 5, our results showed an inverse, non-significant correlation of the allele (C) with the concentration of serum resistin in the T2DM group.

Discussion

Type 2 diabetes represents a health challenge in the world as well as in Iraq. According to estimates by the World Health Organization, the proportion of people with T2D in Iraq ranges from 8.5% to 13.9% of the total population, approximately 1.4 million type 2 diabetics.[27] Therefore, it is necessary to investigate factors that increase the risk of developing T2D. These may include variants at the level of the resistin gene.[17] Our results, regarding higher levels of both fasting blood glucose and fasting serum and saliva T2DM than in the ND group, are in agreement with the findings of Rathwa's *et al*.[17] Given that hyperglycemia is a common feature of diabetes, the expression of resistin increases with the increase in hyperglycemia.[28] Moreover, the level of serum resistin is always high in obese type II diabetics,[29] as in the type 2 diabetic group in our study. Also, our results are in agreement with other studies regarding an increase in the level of resistin in the saliva of patients with type 2 diabetes than in the control group.[30] Therefore, it is likely that the clinical applications of saliva components as potential biomarkers indicating biomolecules that are associated with the pathogenicity of T2DM will be accepted.[31] A strong positive correlation was found for the level of resistin between the serum and saliva of the ND group, which agrees with study of Mamali's *et al*.,[32] which confirms that the existence of common properties of proteins between serum and saliva gives importance for applications that investigate biomarkers in saliva.[33] The non-correlation of resistin and fasting blood glucose between serum and saliva of T2D group in our results may be due to the increased permeability of the basal membrane of salivary gland cells that is, common with diabetes, which allows for increased passage of serum proteins into saliva.[34] It is worth noting that our results may contribute to encouraging the adoption of saliva as an alternative to blood to detect the level of resistin in cases related to type 2 diabetes, such as obesity and insulin resistance,[30] and in healthy subjects.[32] It may be necessary, in the case of diabetic patients, to rely on other methods of saliva collection, such as direct collection from the parotid gland instead of obtaining whole saliva to obtain a more accurate picture of resistin level and other biomarkers when managing and monitoring T2D.[34] It has been suggested that genetic factors regulate circulating resistin level.[35] Our results indicated that there was no difference in the level of T2DM serum resistin and serum FG among the three SNP genotypes (rs1862513), these results are consistent with the studies of Menzaghi *et al*. and Hivert *et al*., respectively.[35] Also, a non-significant negative correlation was found between the (C) allele and resistin concentration, which may or may not indicate that it is the (G) allele that is, associated with an increase in the level of resistin in patients with T2D in Iraq, as in studies on the association of the (G/G) genotype for (rs1862513) with increased levels of resistin, fasting glucose, and HbA1c, in Japan,[13] in Iranian women,[36] in Thais,[37] and in Indians.[17] Contrary to the results found in Italians and Caucasians.[35] Moreover, other studies indicated that the (G) allele is a risk factor for developing type 2 diabetes,[13,37] and that resistin levels are higher in carriers of the (G/G) genotype.[34] The difference in the results between studies related to (rs1862513) may be due to genetic differences between races.[35] Or to the conditions of the study itself, for example, the number of participants in the study is too small to reflect a clear picture of the distribution of genotypes among the population.[38] Therefore, our current results indicate the need for additional studies that may explain the difference and may support our findings, perhaps by including a larger number of participants, and from all governorates of Iraq.

Conclusion

We have concluded through the results of our study, the possibility of replacing saliva with serum when measuring fasting blood glucose and resistin in healthy people in the future, with the need for more studies that reinforce this approach. In the case of T2DM patients, further modifications may be required in saliva collection and management methods. Our genetic study of the resistin gene also indicates the need for further studies, including more governorates of Iraq, to identify the allele responsible for the increase in resistin level and susceptibility to T2D.

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Conflicts of interest

There are no conflicts of interest.

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