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Polymorphism variation of l-selectin the pro213ser (rs2229569) in Iraqi Arab patient with type2 diabetes mellitus (T2DM)

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Abstract: Type2 diabetes mellitus (T2DM) is a disorder of metabolism and complex disease affected by genetic, environmental factors, and associated with inflammation, occurs when the pancreas either doesn't make enough insulin or the body doesn't use the insulin as it should, lead to insulin resistance (IR) along with gradual loss of ß-cell secretory capacity . The aim of this study was to investigate the role of L-selectin gene (P213S) polymorphism in diabetes mellitus type 2 patients. Study includes seventy six Iraqi Arabs patients (male and female) having newly diagnosed type 2 diabetes mellitus (T2DM) and fifty three Iraqi Arabs healthy subjects matched in age, sex and ethnic group. Patients and healthy subjects were genotyped, by PCR-RFLP analysis. Genotype spread analysis indicates that P213S is more frequent in the T2DM group (54.0 %), compared with the control group (16.9%). Thus our results suggest L-selectin (P213S) gene play a role in the development of DMT2 in Iraqi Arabs patients.

Keywords: Polymorphism , L-Selection gene, Type2 diabetes, Mellitus.

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***Search is derived from the first researcher's letter.**

Introduction:

Diabetes mellitus or Hyperglycemia is a hereditary, chronic and endocrine metabolic disorder which causes deaths worldwide (1). It characterized by deficiency or fail in keeping normal glucose homeostasis level (increased blood glucose) (2).

Type 2 diabetes the most common form of diabetes, about (90%_95%) of people are with type 2 diabetes (3).Type 2 diabetes is a multifactorial , chronic disease and due to a combination of environmental and genetic risk factors (4). DMT2 happened when the pancreas either doesn't make enough insulin or the body doesn't use the insulin as it should, drive to insulin resistance (IR) along with gradual loss of ß-cell secretory capacity (5).

Cell adhesion molecules (CAMs) are Cell adhesion molecule located on the cell surface involved in binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion. In essence, cell adhesion molecules help cells stick to each other and to their surroundings (6). CAMs have four different groups, which are defined by different structures and functional characteristics: cadherin's, integrin's, selectins and immunoglobulin (Ig)-like proteins (7).

Selectins CD62 (cluster of differentiation 62) are cell adhesion molecules have a number of transmembrane domains, and bind themselves to the carbohydrate groups of neighboring cell surfaces (bond is heterophilic) (8). Selectins (type I transmembrane proteins) are a family of mammalian vascular adhesion molecules involved in the tethering and deceleration of cells in lymphatic &blood stream on capillary endothelium (9). They are plays a high role in the passage of leukocytes to sites of inflammation by mediating the initial attachment and rolling of leukocytes on vascular endothelium before to integrin-dependent arrest and extravasation (10). Selectins are related to DMT2, a local study showed P-selectin plays a role in Iraqi female patients with DMT2 (11), another local study showed E-selectin plays a role in Iraqi patients with DMT2 (12, 13).There are three types of selectin: P-selectin, E-selectin, and L-selectin (14).The genes for the selectins are located on chromosome number one (8).

L-selectin (SELL, CD62L) gene location on chromosome 1 (1q24.2), and the total length of the L-selectin gene 27kbp, which is consisting of 9 exons and 8 introns (15). L selectin(CD62L), a type I membrane protein, is a leukocyte adhesion molecule constitutively expressed on leukocytes(16) including lymphocytes (naive T cells and subsets of memory T cells), neutrophils, monocytes, eosinophils, haematopoietic progenitor cells, and immature thymocyte (17). SELL functions mediate both lymphocyte homing to peripheral lymph nodes and initital recruitment leukocyte accumulation at sites of inflammation (18). L-selectin plays important roles in rolling leucocytes followed by activation, marked adhesion, and regulation of immune cell`s homing to the infected tissues (19). L-selectin (CD62L) is cleaved enzymatically from the leucocyte surface membrane after cell activation and released into the blood stream, soluble L-selectin (sL-selectin) is functionally active (20).

Materials and Methods:

Seventy six Iraqi Arabs patients (male and female) having newly diagnosed type 2 diabetes mellitus (T2DM) were examined by the AL-Yarmouk Teaching Hospital. We chose patients who suffer from diabetic type II less than six years, no history of hyperlipidemia, heart disease, hypertension, renal disease, no thyroid dysfunction, and were free of acute illness and infection at time of sampling, their age range was between (26-77) years, with Fifty three Iraqi Arabs healthy subjects matched in age range was between (24-71) years, sex and ethnic group.

Laboratory test were include:

1- Anthropometric measurements included:

Weight (kg), height (m), Central Obesity (CO) (Abdominal circumference) Waist to hip bones ratio (WHR) was measured by tool and recorded in (cm) and body mass index (BMI) was calculated by dividing the body weight in Kg the square of the height in (cm) according to the following equation:

BMI = Weight (Kg) / [Height (cm)]2 .

2-Chemical parameters include:

Fasting blood glucose (FBG), Glycosylated hemoglobin (HbA1c), Uric acid (UA), and Lipid profile (Total Serum Cholesterol T-c, Serum Triglycerides TG-c, Serum High Density Lipoprotein Cholesterol LDL-c, and Very Low Density Lipoprotein-Cholesterol VLDL-c) were measured in serum by auto analyzer ARCHITECT c4000 Methods Photometric, Potentiometric, and Turbid metric. The HbA1c test from blood samples that collected in EDTA tubes from patients and controls. HbA1c was measured by Genius PA54 Specific Protein Analyzer method Nephelometry. While S-LDL, S-VLDL, and Atherogenic index of plasma (AI) detected mathematically by using the formula

-LDL-cholesterol= Total cholesterol-HDL-cholesterol- $\frac{\text{Triglycerides}}{5}$

-S.VLDL-C= TG - TC/5 in mg/dl.

-Atherogenic Index = $\frac{\text{Serum – total cholesterol}}{\text{HDL–cholesterol}}$

3-DNA genotyping

The genomic DNA was extracted from the peripheral venous blood of patients and control (2ml) in tube containing ethylene diaminetetraacetic acid (EDTA), using the protocol of Genomic gSYNC™ DNA purification Kit and then stored at -20°C. The Pro213Ser (rs2229569) L-selectin (SELL) gene polymorphism genotyping was detected by Polymerase chine reaction and Restriction fragment length polymorphism (PCR-RFLP) technique. 186 bp was amplified, using the following primers:

F 5' - TGA TTC AGT GTG AGC CTT TG – 3' and

R 5' - CTT GAC AGG TTG GTT CTG – 3'.

Table 1 shows the PCR mix (working solution):

Table (1): the mixture of working solution.

Working Solution	
Go Taq Green Master Mix	25 µl
Primer Forward	1.5 µl of each primer
Primer Reverse	1.5 µl of each primer
Free Nuclease water	17 µl
DNA template	5 µl
Final volume 50µl	

The PCR program has an initial denaturation at 94 ^{0}C for 5 minutes 1 cycle, 35 cycles at (94^{0}C Denaturation for 30 second, 58^{0}C Annealing for 45second and 72^{0}C Elongation for 45 second),and a final step at 72^{0}C final Elongation for 5 minutes 1 cycle. The quality of amplicons has been checked by means of electrophoresis (agarose 2%, TBE 1x). The genotypes spread within the genotypes were determined by digestion of (20µL) PCR product with (0.5 µL) of *Hph1* enzyme (New England Bio labs, Inc. Beverly, USA) for a 3 hour at 37°C followed inactivation at 65 °C for 10 minutes. The restriction fragments have been viewed in UV light, after electrophoresis in 4% agarose gel and ethidium bromide for staining genotype PP yieled one fragment 186bp, genotype SS yieled two fragment 141 and 45, genotype PS yieled three digestion fragments of 186,141, and 45.

Statistical Analysis:

Statistical analysis was done using SPSS version 21 computer software (statistical package for social sciences) and Microsoft Office Excel (Microsoft Office Excel for windows; 2010). Data were analyzed by using t-test (independent t-samples t-test) used to value significant difference among means. $P \leq 0.05$*, $p \leq 0.01$** was considered statistically significant, while $p > 0.05$ was considered non-significant. Frequencies of L-selectin genotype and allele were compared between T2DM and controls using Fisher's exact test when appropriate, 95% confidence intervals (CIs) was calculated to value the relative risk conferred by a particular genotype and allele. Compare the observed genotype frequencies among the subjects with the expected genotype frequencies by using Hardy-Weinberg equilibrium was tested for with a goodness of fit X^2-test with one degree of freedom.

Results:

The statistical analysis of Anthropometric parameters in study groups is shown in Table 2, the mean of BMI significantly in patients group (31.1495±0.82175) compared to control group (28.3927±0.74754) respectively. Central Obesity (CO) (Abdominal circumference) were significantly higher in patients group (43.8816 ± 0.65640) compared to control group (40.8302±0.68295) respectively.

Table (2): Statistical analysis of Anthropometric parameters in patients and control

Characteristics	Patients group Mean ± SE	Control group Mean ± SE	t- test	P –value
BMI (kg/m^2)	31.1495±0.82175	28.3927±0.74754	2.365	.020*
CO(cm)	43.8816 ± 0.65640	40.8302±0.68295	3.141	.002**

Significant differences $p \leq 0.05$*, $p \leq 0.01$, non-significant $p > 0.05$**

The results show in Table 3 the comparison between patients and control groups according to the chemical parameters. The mean of most chemical parameters (FBG, HbA1c, TG-c, VLDL-c, and UA) were significantly higher in patients group (204.5658 ±8.36986, 9.0026±0.19779, 186.2368±11.0716, 36.9842±2.17970, 4.1199±0.13420) compared to control group (90.2642±1.38155, 4.9557 ±0.08304, 141.1887 ±10.75486, 28.2340±2.15075, 4.8053±0.18320) respectively, while the level of HDL-c showed significant below ($P \leq 0.05$) reduced in patients group (38.5921±1.38110) compared to control group (43.0377 ±1.77037). (T-c, LDL-c, and AI) were non - significantly higher in patients group (197.4737±6.12740, 119.7526±5.08372, 5.4550±0.23063) compared to control group (190.6038±5.04072, 117.8792± 4.37025, 4.8079±0.24218) respectively.

Table (3): Statistical analysis of chemical parameters in patients and control

chemical parameters	Patients group Mean ± SE	Control group Mean ± SE	t-test	P-value
FBG(mg/dl)	204.5658 ±8.36986	90.2642±1.38155	13.474	.000**
HbAlc (%)	9.0026±0.19779	4.9557 ±0.08304	18.866	.000**
T-C(mg/dl)	197.4737±6.12740	190.6038±5.04072	0.812	.418NS
TG-C(mg/dl)	186.2368±11.0716	141.1887 ±10.75486	2.812	.006**
HDL-C(mg/dl)	38.5921±1.38110	43.0377 ±1.77037	2.004	.047*
LDL-C(mg/dl)	119.7526±5.08372	117.8792± 4.37025	0.279	.780NS
VLDL-C(mg/dl)	36.9842±2.17970	28.2340±2.15075	2.761	.007**
UA (mg/dl)	4.1199±0.13420	4.8053±0.18320	3.090	.002**
AI (%)	5.4550±0.23063	4.8079±0.24218	1.890	.061NS

Significant differences $p \leq 0.05$*, $p \leq 0.01$, non- significant $p > 0.05$**

PCR-RFLP analysis has showed the presence of all three genotypes of P213S polymorphism in the L-selectin gene. The presence of a restriction site for Hph I enzyme (213ser allele) and the amplicon digestion generates two fragments of 141bp and 45 bp. When the restriction sites is not created (pro213 allele), the amplicon is not digested and it maintains its size of 186 bp (Figure 1).

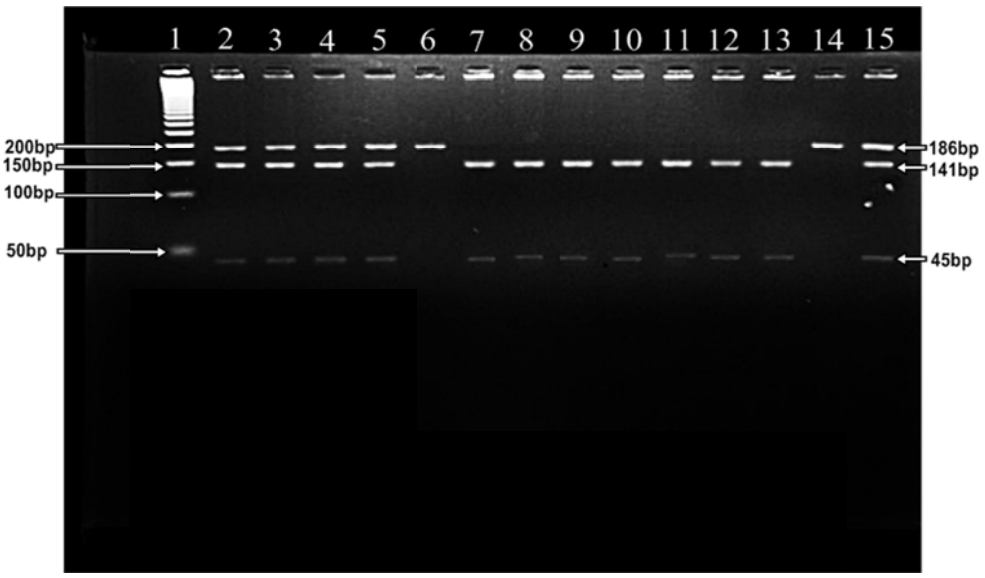

Figure (1): The electrophoresis results for Pro213Ser polymorphism (lines 1: DNA leader 50 bp; lines 2,3,4,5,15 :ProSer genotype; lines 6,14: ProPro genotype ;lines 7,8,9,10,11,12,13: SerSer genotype).

Genetic polymorphism of L-selectin gene was inspected at the position P213S (rs2229569), which was presented with three genotypes (PP, PS, and SS) that corresponded to two alleles (P and S) in T2DM patients and controls. It was observed that genotypes frequencies in both groups of subjects were in a good agreement with Hardy-Weinberg (H-W) equilibrium, and there

were high significant differences between the observed and expected frequencies (TotleX^2 =18.427). Comparing patients to controls revealed that PS genotype showed increased frequency in patients (54.0 *vs*. 16.9%).PP genotypes showed decreased frequency in patients (14.5 *vs*. 32.1%). SS genotypes showed decreased frequency in patients (31.5 *vs*. 51.0%). In term of allele frequencies, record S allele frequency (89) and percentage (58.6 %) in patients compared to P allele frequency (63) and percentage (41.4 %); while S allele frequency (63) and percentage (59.4 %) in controls compared to P allele frequency (43) and percentage (40.6 %) (Table 4).

Table (4): Observed numbers and percentage frequencies Hardy-Weinberg (HW) equilibrium of L-selectin genotypes and alleles in T2DM patients and controls.

Groups			Genotype or Allele					X^2 for alleles
			PP	PS	SS	P	S	
Diabetes Type 2 (No.=76)	Observed	No.	11	41	24	63	89	P= 3.774 *
		%	**14.5**	**54.0**	**31.5**	**41.4**	**58.6**	
	Expected	No.	16.5	29.5	30.0	Not Estimated		
		%	21.7	39.0	39.5			
Controls (No.=53)	Observed	No.	17	9	27	43	63	S= 4.447 *
		%	**32.1**	**16.9**	**51.0**	**40.6**	**59.4**	
	Expected	No.	11.5	20.5	21.0	Not Estimated		
		%	21.7	38.7	39.6			
X^2 Total comparison among genotypes			18.427**					

Significant differences p ≤ 0.05*, p ≤ 0.01, non- significant p > 0.05**

Comparing patients to controls revealed that, the frequency PP genotype showed a relative risk (0.44) .It shown as a linked genotype of the infection risk the value of preventive fraction (PF) was (0.283),it appeared significant differences were found (0.030) according to the Fisher's Exact Probability and the period of confidence was between (0.29-0.98)under 95%. The frequency PS genotype showed a relative risk (5.73) .It shown as a linked genotype of the infection risk the value of preventive fraction (PF) was (0.825),it appeared high significant differences were found (0.000) according to the Fisher's Exact Probability and the period of confidence was between (2.31-15.07) under 95%. The frequency SS genotype showed a relative risk (0.36) .It appeared as a protective genotype of the infection risk the value of preventive fraction (PF) was (0.462),it appeared significant differences were found (0.029) according to the Fisher's Exact Probability and the period of confidence was between (0.14-0.92)under 95%.

Study data showed P allele a relative risk (1.04) .P allele shown as a protective allele of the infection risk the value of preventive fraction (PF) was (0.036),it appeared non-significant differences were found (0.898) according to the Fisher's Exact Probability and the period of confidence was between (0.63-1.71) under 95%, and showed S allele a relative risk (1.73) .It shown as a linked allele of the infection risk the value of preventive

fraction (PF) was (0.009),it appeared non-significant differences were found (0.949) according to the Fisher's Exact Probability and the period of confidence was between (0.59-1.73) under 95% (Table 5).

Table (5): Statistical evaluations of associations between L-selectin genotypes or Allele's andT2DM.

Type of Comparison	L-selectin Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological Or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Diabetes Disease Versus Controls	PP	0.44	0.283	0.030 *	0.29-0.98
	PS	5.73	0.825	0.000 **	2.31-15.07
	SS	0.36	0.462	0.029 *	0.14-0.92
	P	1.04	0.036	0.898 NS	0.63-1.71
	S	1.73	0.009	0.949 NS	0.59-1.73

Significant differences $p \leq 0.05$*, $p \leq 0.01$, non- significant $p > 0.05$**

Discussion:

The comparison among study groups according to anthropometric parameters showed significant increased (BMI and CO) in patients compared to control. In obese patients, adipose tissue releases a high amount of non-esterified fatty acids (NEFA), hormones, glycerol, and pro inflammatory cytokines (such as IL-6 and IL-18). These elements are implicated in the development of insulin resistance (21).

And (BMI) associated with increased value risks of (T2DM). While the same parameters showed decreased level in Iraqi Arab patient in previous report (22, 12).Current results are consistent with a local study showed significant increased BMI and CO in patients compared to control (11).

The present data indicated that the mean of FBG and HbA1c were high significant in diabetic patient compared to control, these result agree with previous Iraqi study demonstrated that the combination of FBG and HbA1c identifies individuals who are at risk of progression to Type2 DM (22, 12). Current results are consistent with a local study showed significant increased level of FBG and HbA1c in patients compared to control (23, 24, 11). Pancreatic beta-cells induce proliferation from Short term exposure to increasing glucose concentrations; after a prolonged exposure to increased glucose concentrations the proliferative capacity is suppressed (25). Increase the glycation of common proteins such as hemoglobin, forming Hemoglobin A1c (HbA1c) when increased concentrations of glucose. Hemoglobin A1C is known to correlate with blood glucose levels over the lifetime of the RBC, which is approximately 120 days (26).

The mean of lipid profile (TG, and VLDL) in present data was significantly higher in diabetic group compared to healthy control, HDL significantly below ($P \leq 0.05$) in patients compared to control. These results agree with other study, lipid abnormalities are common in T2DM patient's .Dyslipidemias make diabetic patients apt to develop cardiac heart disease and other complications of atherosclerosis (27). Study result agree with local study showed significant increased level of (TG, HDL, and VLDL) (22) While,

result disagree with local study in Iraqi female patient with DMT2 showed non-significant level of HDL (11). Study result disagrees with local study showed non-significant increased level of (TG, HDL, and VLDL) (12). Present data shown non-significantly increasing level of T-C, LDL and AI in patients compared to control. These results disagree with other study (27), while disagree with local study showed significant increasing level of (T-C, LDL, and AI) (24) and disagree with another local study in Iraqi female patients showed significant increased level of (T-C, and AI) (11). In T2DM insulin resistance (IR) causes unrestricted lipolysis leading to high level fatty acid flux in liver and ends in higher hepatic triglyceride synthesis. Also the activity of endothelial insulin dependent lipoprotein lipase is lower resulting in decreased triglyceride clearance. Other processes involving apoprotein production and action of cholesteryl ester also obtain affected (28).

The Uric Acid (UA) in present data was significantly higher in diabetic group compared to healthy control, these result is compatible previous reports showed non-significant increased level of UA in patients compered to controls (29, 30). Serum uric acid (SUA) is the final oxidation product of purine nucleotides metabolism in circulation. Hyperuricemia (HUA), considered a risk factor for cardiovascular diseases (CVD) and metabolism diseases such as (T2DM) (31).The levels Uric acid, Creatinine ,and Urea are marked to renal function, elevated levels led to kidney dysfunction.DM patients more susceptible to kidney dysfunction (32).

Insulin resistance develops a number of alterations in lipid metabolism and lipoproteins composition, which renders LDL cholesterol and other lipoproteins more pathogenic in patients with type 2 DM. Hypertriglyceridemia results in decreased HDL, which is also a main feature of plasma lipid alterations observed in type 2 diabetic patients. Hypertriglyceridemia may be an increased hepatic secretion of very low-density lipoproteins (VLDL) and a delayed clearance of TG-rich lipoproteins, which may mainly be due to increased levels of substrates for TG production, and enhanced free fatty acids (FFA), and glucose levels. Thus, there is a strong association between type 2 diabetes and dyslipidemia (33).

In present study increase frequency of the genotype PS for polymorphism in the L-selectin gene in patients compared with control, while genotype PP and PS decrease in patients compared with control. S and P allele might be a possible risk factor for T2DM.It is disagree with another study done on Chinese population showed the frequency of PP and P allele in patient with DMT2 compared to controls (34,35), study result disagree with another study that showed the P allele was associated with risk of ischemic stroke as compared with S allele (36), while it in agreement with another study showed the SS genotype is more frequent in spinal muscular atrophy (SMA) patients than in control group , but agreement with same study which it showed the S allele might be a possible risk factor for the respiratory complications in spinal muscular atrophy (SMA) disease (37), while it agree with another study that showed the S allele may represent a risk factor

whereas the PP genotype may be protective for colorectal cancer (CRC) (38). SELL gene that may also interfere with risk for T2DM. The P213S (proline 213 serine) polymorphism a C/T transition (one nucleotide replaces another nucleotide of the gene) represents a good candidate in order to assess this risk. This genetic variant determines synthesis of protein with other amino acid (Serine); this leads to a change in the effectiveness of protein domain 1 and thus seems to be responsible for the interaction between leucocytes and endothelium (35). Present result shows the possible risk effect for both alleles P and S in developing disease that noticeable with increasing the percentage ratio of patient with PS genotypes.

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