Polymorphism of P-selectin (- 2123C/G, rs1800807 C/G) and IL-4 -1098 in a sample of Iraqi Diabetes Mellitus Type 2

تعدد الاشكال الوراثية للعامل P-selectin عند الموقع 2123 C/G والموقع 1098 لجين البين ابيضاضي 4 في عينة من العراقيات المريضات بالسكري من النوع الثاني

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Abstract

Diabetes is a major public health problem because it is world's sixth cause of death. An inflammatory disease that associated with many inflammatory markers, which is a key for complications of vascular endothelial system, literally for high risk populations. Some adhesion molecules were studied in the sera of Iraqi Arab female as P-selectin associated with inflammatory disease and Cytokines. This work tend to evaluate the association between soluble P-selectin level, polymorphism for mutation site 2123C/G (rs1800807) and the role of P-selectin and IL-4 polymorphism in the developing of Diabetes Mellitus type 2. Genetic polymorphism of P-selectin gene was investigated at the position -2123C/G, Present study illustrated three genotypes (GG, GC, CC), with the significant protective effect for G allele and Etiological effect for C allele. The Polymorphism of IL-4- gene at the position IL-4 -1098 was presented with three genotypes (TT, TC and CC), Furthermore the result of C allele frequency (RR=1.45 with EF=0.312) reflect an Etiological effect for C allele with positive association with disease. All present factors in the first view may occur as independent risk factors but after a deeply focusing on results, it seems as if there's a sequence functions among them, the result in a synergist effect interfere with Diabetes Mellitus type 2

Key word: P-selectin, IL-4, Polymorphism, IL-4 -1098, P-selectin(- 2123C/G, rs1800807 C/G) and diabetes

الملخص

السكري من المشاكل الصحية الكبرى في العالم ويعد المسبب السادس للوفيات عالميا. مرض التهابي يرتبط بالعديد من الدلائل الالتهابية والتي تعتبر بمثابة المفتاح لمضاعفات النظام البطاني الوعائي خاصة في المجتمعات التي تمتلك نسبة عالية من عوامل الخطورة. بعض عوامل الالتصاق درست تراكيزها في مصل المريضات العراقيات العربيات مثل عامل الالتصاق P كعامل التهابي يرتبط بالحركيات الخلوية. لذا فالعمل الحالي يسعى لتقييم العلاقة بين المستوى المصلي السائل للعامل وتعدد الاشكال الوراثي عند الموقع 2123 (rs 1800807) C/G -وعلاقته بتعدد الاشكال للبين ابيضاضي 4 عند الموقع 1098- وتطور مرض السكري. تعدد الاشكال الوراثي لعامل الالتصاق p-selectin والذي اختير عند الموقع2123-C/G في الدراسة الحالية له ظهرت ثلاثة تراكيب وراثية (GG, GC, CC) وظهر تاثيرا وقائيا معنويا للاليل G وتاثيرا وبائيا للاليل C. في حين تعدد الاشكال لجين البين ابيضاضي عند الموقع -1098 اظهر ثلاثة تراكيب وراثية هي (TT, TC, CC) وعكست نتائج تكرار الاليل C دوره الوبائي والارتباط الموجب مع المرض. كل العوامل الحالية للوهلة الاولى تظهر وكانها عوامل خطورة مستقلة عن بعضها البعض لكن بعد التدقيق في النتائج يتضح ان هناك ترابط وضيفي متسلسل يجعلها متضافرة وتتداخل مع مرض السكري من النوع الثاني.

الكلمات المفتاحية: عامل الالتصاق سلكتين P عند الموقع -2123 ، تعدد الاشكال ، البين ابيضاضي 4 عند الموقع -1098، السكري

Introduction

Diabetes is a major public health problem because it is world's sixth cause of death. An inflammatory disease that associated with many inflammatory markers, which is a key for complications of vascular endothelial system, literally for high risk populations [1]. The disturbance of endothelial factors and circulating levels of adhesion molecules which are elevated in the sera of patients. Some adhesion molecules were studied in the sera of Iraqi Arab female as P-selectin with ABO blood group [2], E-selectin have showed significant increasing level associated with allele A and B of ABO system [2] P selectin is one of surface adhesion molecules [3]. The elevated synthesis of P-selectin may play an important role in the delivery of protein to the cell surface[12], which associated with cardiovascular disease, cardiovascular mortality and sudden cardiac death among male dialysis patients [4].

P-selectin gene is located on chromosome 1q21-q24, spans > 50 kb and contains 17 exons in humans.[3] It is constitutively expressed in megakaryocytes (the precursor of platelets) [4] and endothelial cells [5]. In ischemic

stroke patients, plasma P-selectin concentration was reported to be highly correlated to plasminogen activator inhibitor-1 activity and tissue plasminogen activator activity [4]. Expression of present factor is induced by two distinct mechanisms. First, it is synthesized by megakaryocytes and endothelial cells, then it is sorted into the membranes of secretory granules. [6] When megakaryocytes and endothelial cells are activated by agonists such as thrombin, then it rapidly translocated to the plasma membrane from granules.[7] Secondly, increased levels of P-selectin mRNA and protein are induced by inflammatory mediators such as tumor necrosis factor-a (TNF-a), LPS, and interleukin-4 (IL-4). IL-4 increases P-selectin transcription in human [8,9,10].

Some genetic polymorphisms located in the promoter region of the P-selectin gene have been described, as well as its effect on the protein sequence. 2123 C/G, gene polymorphism in some other countries have related reports. We evaluated the association between soluble P-selectin and polymorphism for mutation site 2123C/G (rs1800807) with other risk factors in present work. Moreover cytokines play a key roles in the pathogenesis of diabetes in which the pattern of cytokine expression are changed, as well as many researchers in immune and genetics field believed that DMT2 is associated with cytokine imbalance and alter pattern of Th2 to Th1 immune response [11], as well as a previous study suggested that gene polymorphisms of IL-4 play an important role in the pathogenesis of diabetic nephropathy in Iranian patients with DMt2[12].Therefore it become so important to investigate the role of genetics polymorphism for P-selectin with IL-4 in the developing of Diabetes Mellitus type 2.

Materials and Methods

Present study included 720 Iraqi females, whose age ranged from (20–55) years, attending NDC for checking and found to be newly diagnosed as having type 2 diabetes. Two hundred forty Iraqi Arab females control subjects who were apparently normal in a term non diabetic, non-hypertension and age, gender matched with the studied group were selected to be control group. The study was done in NDC (National diabetes Center)/AL-Mustansiryia University, during the period from September 2012 to November 2013. All patients and controls were with no history of hyperlipidemia, hypertension, renal disease, heart disease, smoking, alcohol taking and were free of acute illness and infection at time of sampling. Both patients and control were divided into four sub groups according to the type of blood groups. Patient underwent full clinical examination. Central obesity (CO) was measured with the subject standing midway between the lower rib margin and iliac crest where's the hip was measured at the level of great Trochanters. Body mass index (BMI) was calculated as weight divided by height squared (Kg/m²).

A-Serological test conducted by the following

1-Soluoble P-selectin (sP-selectin) kit from eBioscience, Human P-selectin ELISA for quantitative detection, Europe. The work done in private Lab (Al-Karam Lab).

2-Interleukine-4 (IL-4) kit from PLAB-CLIA A daltis, ELISA.

B-Chemical test conducted by the following

1- Kit for plasma glucose measurement, Randox Company, united kingdom◦

2-Glycosylated Hemoglobin (HBA1c) kit, NycoCard, AXIS-SHIELD PoC AS, Norway.

3-Total cholesterol kit, Spin reacts, Spain

4-High Density Lipoprotein (HDL) – Cholesterol kit, Randox, united kingdom

5-Atherogenic index) AI) calculated according to this formula: =serum-total cholesterol /HDL-cholesterol

C-.1. Experimental methods for genotyping

Two mL fasting quiet state peripheral cubital vein blood were collected, and then placed in EDTA anticoagulant tube. Use TIANGEN Biochemical technology co. kit, LTD (Beijing) of genomic DNA extraction kit to extract genomic DNA.

2-Extracted DNA samples were stored at 20^{o} C.

The amplified PCR products were digested with the *Pst I* restrictive enzyme (New England Bio Lab, Beverly, MA, USA) overnight at 37^{o}C. The digested products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide at 70 volt for 1.30hour. PS genotypic test results Ps gene 2123 C/G polymorphism loci, PCR amplification products fragment size is 189 bp, *Pst I* restriction fragment of the situation in accordance with restriction endonucleases. There are three kinds of genotypes, CC-type (106 and 76 bp 2 bands). CG type (189, 106 and 76 bp 3 bands), GG-type (189 bp 1 band).

Table (1): Primer sequences for genotyping P-selectin polymorphisms (- 2123C/G rs1800807 C/G).

Polymorphism	Reference SNP	ID Ref SNP alleles	Primer sequences	restriction enzyme	Reference
-2123C/G	rs1800807		-CCGTTTAATTAGC CAGTAGTGATG-3 -CCGAAGTGTGGTATGTAGACTAG TAG-3	*Pst I*	Bai *et al* 2014

C-3- Cytokine CTS-PCR-SSP Tray Kit from Germany by University Clinic Heidelberg for determine the polymorphism of 13 cytokine at 22 position in the promoter region of different type of Interleukin for present study the IL-4 has been chosen. The PCR primers were prepared to identify alleles, genotypes and some haplotypes at these regions. These primers were designed by the Department of Transplantation Immunology, University Clinic Heidelberg (Germany) according to the WHO international nomenclature committee of cytokines. Each tray in the kit contained allele-specific, ready-for-use primers, which consisted of 48 PCR dried lyophilized primer mixes that were dispensed in 96 well of the tray, to carry out cytokine genotyping for two individuals (48 wells per sample). Each well of the tray 96 wells was designated by a digit-letter combination from H1 to A12). The PCR mix positions were named numerically for each cytokine that correspond to locus specificities in the tray.

The master mix (CYT) supplied with the kit and consisted of ammonium sulfate, Tris Buffer, magnesium chloride, glycerol (glycerin), cresol Red and deoxyribonucleotides (dNTPs).

Statistical Analysis

Statistical analyzed was performed using the computer programmer SPSS (Statistical Package for Social Sciences) version 14. The data were given as mean ± standard error (SE.), and differences between two samples were assessed by t -test. Allele frequencies of gene was calculated by direct gene counting method, while significant departure from Hardy-Weinberg (H-W) equilibrium was estimated using H-W calculator for two alleles, which is available free online at http://www.had2know.com/academics/Hardy-Weinberg- equilibrium- calculator -3-alleles.html. Alleles and genotypes were presented as percentage frequencies, and significant differences between their distributions in DMt2 patients and controls were assessed by two-tailed Fisher's exact probability (P). In addition, relative risk (RR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between cytokine alleles and genotypes with the disease. The RR value can range from less than one (negative association) to more than one (positive association). If the association was positive, the EF was calculated, while if it was negative, the PF was given. These estimations were calculated by using the WINPEPI computer programs for epidemiologists. The latest version of the WINPEPI package (including the programs and their manuals) is available free online at http://www.brixtonhealth.com.

Results and Discussion

Most studied parameters showed significant increase level in patients group compared to healthy control group, but HDL showed non-significant increased. Table (2). The present results showed agreement with previous information about slightly increasing level of P-selectin in the sera of patients with Diabetic Human Retina and Choroid (8). Although another published work demonstrated the critical role of P- selectin in inducing neointimal lesion formation in mutant Mice after arterial injury [9]. Soluble forms of these molecules are released from shedding or proteolysis cleavage from the endothelial cell surface and may reflect overexpression of their respective membrane-bound forms expression of endothelial cellular adhesion molecules activate the adherence and transmigration of leukocytes into the sub endothelial space, subsequently leading to endothelial and sub endothelial structural changes[10]. The level of IL-4 increase significantly in patients sera (6.35±2.04) compared to healthy individual (0.48 ± 0.30).This result goes with previous study noticed the protective role of IL-4 against progressing diabetes by inhibiting the activity of natural killer cell and T-cell against beta cell [11].

The present data has recorded a significant increasing p-selectin level of cholesterol (222.8 ± 5.2) compared to healthy control (161.1 ± 9.1), so the same for the other types of lipids except HDL which showed a non-significant increasing P≥0.05, cholesterol can cause thickening and narrowing walls of the blood vessels, by depositing on the endothelium of arteries cause (Atherogenic) to Atherosclerosis. Therefore, cholesterol is considered as the major cause of cardiovascular disease [13].

The means of FBG and HbA1c were significantly higher p-selectin in patients compared to control. This result is agreement with previous Iraqi documentation about females with DMT2 [14]. Atherogenic index in present data was significantly higher p-selectin level in patients compared to healthy control, these results agree with previous Iraqi studies which were showed the same result for lipid profile in Iraqi female diabetic patients [15]. The present data demonstrates that Type II diabetes is associated with increased serum TG and reduced HDL-cholesterol, and the precise cause of the low HDL–C in type 2 diabetes may be the consequence of insulin resistance, increased lipid production and increased activities of cholesterol ester transfer protein (CETP) and endothelial lipase [16]. As well as glycaemia leads to increase transferring excessive amount of blood sugar into lipid [13]. The present results showed increasing level of BMI, Co. with other hand glucose, glycated hemoglobin, most lipid profile and IL-4 that agree with previous study illustrated increasing level of all these parameters with IL-4 in obese and non-obese diabetic patients, which explain the protective role of IL-4 [17]. Moreover IL-4 activity as inhibitory factor for lipid accumulation in fat tissue in patients sera with hyperglycemia [18].

Table (2): Mean ± SE of some parameters in Iraqi Arab females.

Parameters	Study groups		P-value
	Control group mean ± SE	Patients group mean ± SE	
P-selectin (ng/ml)	23.79 ± 2.44	34.55 ± 1.87	0.025*
IL-4 (Pg/ml)	0.48 ± 0.30	6.35± 2.04	0.040*
FPG (mg/dl)	95.8 ± 5.48	190.97± 10.32	0.001*
Cholesterol mg/dl	158.1 ± 9.8	222.8 ± 5.2	0.001*
Triglyceride(mg/dl)	104.3 ± 5.00	156.87 ± 9. 5	0.0001*
HDL (mg/dl)	56.90 ± 2.6	60.1 ± 2.3	0.061
HbA 1C (%)	4.9 ± 3.9	9.11 ± 1.3	0.031*
Ath. Index	4.2 ± 0.19	5.2 ± 0.2	0.006*
BMI(kg/m$^{2)}$	24.2 ± 3.2	31.4 ± 5.01	0.021*
C.O	95.3 ± 9.4	112.2 ± 18.1	0.041*

S = significant differences (P<0.05)* NS= Non significant

Genetic polymorphism of P-selectin gene was investigated at the position -2123C/G, that corresponded to two alleles (G and C), which was presented with three genotypes (GG, GC and CC) in DMT2 patients and healthy control figure (1).The results showed that genotypes frequencies in control group was in a good agreement with Hardy-Weinberg equilibrium(HWE), no significant differences between the observed and expected frequencies in healthy Control, while a significant differences between the observed and expected frequencies in Patients with DMT2 that deviation from HWE may be because the small size of sample and blood relation Table (3).

Table (3): Observed numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of P-selectin – 2123C/G genotypes and alleles in study groups.

Groups			Genotype and Allele frequency					H-W X^2 P ≤
			GG	GC	CC	*G*	*C*	
Diabetes type2 (No. =110)	Observed	No.	55	32	23	142	78	0.0001*
		%	50	29.10	20.90	64.55	35.45	
	Expected	No.	45.83	50.35	13.83	Not Estimated		
		%	41.66	45.77	12.57			
Controls (No. = 50)	Observed	No.	27	16	7	70	30	0.0923
		%	54	32	14	70	30	NS
	Expected	No.	42.5	21	4.5	Not Estimated		
		%	49	42	9			

S = significant differences (P<0.05)* NS= Non significant

Comparing patients to controls revealed that GG genotype showed a significant decreased frequency in patients (50 vs. 54%; P = 0.0001). The preventive fraction (PF) of such negative association was 0.148. In term of allele

frequencies, G allele frequency was decreased in patients compared to controls (64.0 vs. 70%), while C allele frequency was increased (35.0 vs. 30%); however, G allele variations felt short of significance P < 0.0001 according to Fisher's Exact Probability, while C allele not significant 0.373. The relative risk (RR) of such two associations (negative for G allele and positive for C allele) was 0.30 and 1.28, respectively Table (4). Present study illustrated the significant P<0.05protective effect for G allele and Etiological effect for C allele. Present data showed that the wild type of genotype structure was GG in a sample of Iraqi population While in other study, the frequencies of the 2123G allele among the Xinjiang population were 0.741[19].

Table (4): Statistical analysis of associations between P. selectin$_{-2123}$ C/G genotypes or alleles and (Diabetes disease Versus Controls).

Type of Comparison	P-selectin Genotype	Statistical Evaluation				
		Relative Risk	Etiological fraction	Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Diabetes	GG	0.85		0.148	0.733	0.41-1.76
Disease	GC	0.87		0.128	0.713	0.40-1.94
Versus	CC	1.62	0.384		0.384	0.61-4.83
Controls	G	0.30		0.704	0.000*	0.15-0.57
	C	1.28	0.220		0.373	0.75-2.22

S = significant differences (P<0.05)* NS= Non significant

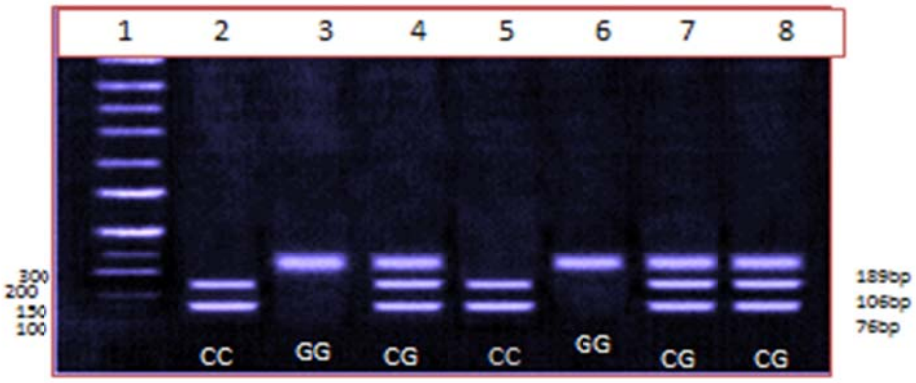

Fig. (1): The PCR-RFLP assay for analyzing the Ps gene 2123C/G polymorphism yields different fragments according to genotype after *Pst I* digestion at 75 V. for 1.30 h. (lanes 1 M :DNA leader 1000bp; 4,7,8 heterozygous CG genotype; Lanes 2,5 were mutated homozygous CC genotype; Lanes 3,6 were homozygous GG type.

Genotype of IL-4- gene at the position -1098 was presented with three genotypes (TT, TC and CC) that corresponded to two alleles (TandC). The comparing between observed and expected frequencies of these genotypes illustrated non-significant differences P<0.05, therefore they were consistent with Hardy-Weinberg equilibrium (HWE) in both groups of patients, and control. In addition, comparison between patients and control revealed non-significant differences in the distribution of IL-4 -1098 allele's frequencies.

Table (5): Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of IL-4 $_{-1098}$ genotypes in study groups.

Groups			IL-4-$_{1098}$ Genotype TT	TC	CC	*T*	*C*	H-W X^2 P ≤
Dibetes type 2 (No. = 49)	Observed	No.	21	18	10	60	38	
		%	42.85	36.73	20.41	61.22	38.78	
	Expected	No.	18.37	23.27	7.37	Estimated		
		%	37.48	47.48	15.04			
Controls (No. = 40)	Observed	No.	21	13	6	55	25	0.1 NS
		%	52.50	32.50	15.00	68.75	31.25	
	Expected	No.	18.91	17.19	3.91	Estimated		
		%	47.27	42.97	9.77			

S = significant differences (P<0.05)* NS= Non significant

Among patients, the frequencies of TT genotype (42.85 VS. 37.%; RR =0.68% and PF 0.321) and T allele (61.22 vs. 68.75%; RR =0.59) were non significantly decreased in patients compared to controls (P = 0.1 and 0.1, respectively), so the association PF=0.72 has a preventive effect . In contrast, TC genotype frequency is (36.73 vs. 32.50% and RR=1.21 with EF=0.171) show increasing ratio in patients comparing to control with etiological effect. Even there is no significant variation between patients and controls in the distribution of IL-4 -1098 for all genotypes and alleles, but the frequencies of CC genotype (20.41 vs. 15.0 %) were non significantly increased in patients compared to controls with C allele frequencies (38.74 vs. 31.25. Furthermore the result of C allele frequency (RR=1.45 with EF=0.312) reflect a Etiological effect for C allele with positive association with disease Tables (5 and 6).This result may conflict a weak action of IL-4-1098 Genotype on DMT2 this finding is supported by a previous one demonstrated that a long term exposure of rat pancreatic islets to IL-4 resulted in an inhibitory effect to some of the islet cells function [20]. This result agree with previous result about which illustrated an association between polymorphism of IL-4 genotype with diabetes type2 and obesity but in fact it was weakly association [21].

Table (6): Statistical analysis of associations between IL-4_1098 genotypes (Diabetes disease Versus Controls).

Type of Comparison	IL-4-$_{1098}$ Genotype	Statistical Evaluation Relative Risk	Etiological	Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Diabetes Disease Versus Control	TT	0.68		0.321	0.399	-1.71
	TC	1.21	0.171		0.823	-3.21
	CC	1.45	0.312		0.586	0.42 -5.36
	T	0.69		0.72	0.345	0.19 -2.36
	C	1.47	0.321		0.354	0.59 -3.71

S = significant differences (P<0.05)* NS= Non significant

Moreover, there's an association between them that in agreement with previous study mention that the increase in P-selectin expression induced by IL-4 results from increased transcriptional activation of the P-selectin gene. The postreceptor signaling pathway giving rise to the prolonged increase in P-selectin expression induced by IL-4 [22], Present result disagree with previous study illustrated that IL-4 gene genotype show no any significant differences between diabetes type 2 with control in Iranian population [23] and that may because expression of Cytokines is different from person to person and in different population [21].

Some kind of immune-genetically association among present study parameters represent by them role. IL-4 is secreted by T-helper Lymphocytes (Th2), stimulating humeral immunity with different types of immune cells such us Macrophage, basophile, eosinophil, immunoglobulin class switching and B-Lymphocyte proliferation, [17] so eosinophil has the ability to effectively use the adhesion molecules as p-selectin and others for tethering and firm adhesion to expressed on the surface of endothelial cells, at the same time IL-4 stimulated endothelial cells in order to increase adhesion under physiological stress condition.

The overall, based on present result, it may be concluded that the polymorphism of P-selectin and IL-4 -1098 can effect on Diabetes mellitus type 2 that conclusion is supported by all previous result, Although we need to emphasize that the complication with gradient progress of disease are very complex, besides are associated with many factors: - Genetics, environmental and lifestyle of patients with Cytokines that play key role in the progression of pathogenesis of diabetes. However all that need further studies to focus on their association among them.

Finally our Recommendation that researchers should done more studies with focusing on links between all present parameters and their polymorphism to understand their real effect on disease progress.

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