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RESEARCH ARTICLE

Investigation of the E-Selectin rs5368 (C468T) Single Nucleotide Polymorphism Role INA Group of Type Two Diabetes Mellitus Iraqi Patients

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Abstract

Introduction: E-select in his cell glycoprotein that is expressed on the surface of endothelial cells in response to proinflammatory cytokines and it supports the rolling of leukocytes at the sites of inflammation and tissue injury. Diabetes mellitus is a global health problem represented by metabolic disorder in which the genetic predisposition and unhealthy lifestyle interact with environmental factors to unmask the disease which might be a result of inflammation reflecting response. Aim: Detection of the E- Selectin rs5368 (C468T) Single Nucleotide Polymorphism role in a group of Type 2 Diabetes Mellitus Iraqi patients. Material and methods: The E- Selectin rs5368 (C468T) Single Nucleotide Polymorphism was characterized using Restriction Enzyme Fragment Length Polymorphism (RFLP-PCR) technique by designing new set of specific primers, then the PCR product was visualized by agarose gel electrophoresis. Results: There was no significant association found between any of the E-Selectin rs5368 (C468T) genotypes and the incidence of T2DM. Also there was no significant difference for the allele's distribution between the patients and control groups. Conclusions: This study showed a success in the primers design and the PCR- RFLP protocol and techniques for the E-Selectin rs5368 (C468T), the sequence of E-Selectin rs5368 (C468T) polymorphism is found to be in concordance with the global registered NCBI sequence. The genotyping results showed that the E-Selectin rs5368 (C468T) polymorphism is not associated with the incidence of T2DM. Also there was no significant difference for the allele's distribution between the patients and control groups.

Key words: *Type 2 Diabetes Mellitus, E- Selectin rs5368 (C468T) Single Nucleotide Polymorphism.*

Introduction

Chronic low-grade inflammation was considered as a major component in the pathogenesis of insulin resistance and Type 2 Diabetes Mellitus (T2DM) [1]. Insulin resistance (a reduced insulin-stimulated glucose intake) is associated with obesity, ageing and inactivity, genetics, environmental effect, and other cases related to chronic inflammation, begins prior to the onset of T2DM [2].

Increased levels of glucose along with free fatty acids, will strain the pancreas and insulin sensitive tissues as adipose tissue, liver and muscles, leading to the local production and release of cytokines and chemokines; also, T2DM is found to be associated with endothelial activation (and the development of the inflammatory processes) [3,4,5].

Several of those inflammatory markers were found to have elevated levels in T2DM Iraqi patients including IL-18, E-Selectin, Intercellular Cell Adhesion Molecules (ICAMs) and Vascular Cell Adhesion Molecule (VCAMs), and others which indicate an association with the incidence and the development of T2DM [6,7,8,9,10].

Studies indicated that different genetic variants (Single Nucleotide Polymorphisms, SNPs) of the inflammatory markers were correlated to different diseases including T2DM and others. E-selectin and the ICAM-1 polymorphisms are found to be involved in predisposition to atherosclerosis [11]. E-select in is an 11kDa cell glycoprotein (also known as endothelial leukocyte adhesion molecule-1(ELAM-1), CD62 antigen-like family member E (CD62E) and SELE) that is

expressed on the surface of endothelial cells in response to proinflammatory cytokines like IL-1, tumor necrosis factor and bacterial lipopolysaccharide, it supports the rolling of leukocytes at the sites of inflammation and tissue injury [12,13].The E-Selectin protein is encoded by SELE gene that is composed of 14 exons located at position 1q24.2 of Chromosome 1 and it helps in the accumulation of blood leukocytes at the inflammation scene by interposing the adhesion of cells to the vascular lining [14, 15].

Researchers found a relationship between T2DM and SNP's on the 1q21 locus of Chromosome 1, another linkage was found with the locus 1q24, and these regions contain the genes of E-Selectin [16]. Due to the lack of information on the effect of genetic variability of the previous inflammatory markers on the occurrence and development of T2DM and their impact on the clinical characteristics of Iraqi patients, this case-control study was designed to investigate the role of E-Selectin rs5368 (C468T) polymorphisms in the incidence and development of T2DM Iraqi patients.

Materials and Methods

Our study was conducted on 68 Iraqi patients (42males and 26females) that were diagnosed with Type Two Diabetes Mellitus, they were periodic patients at the Specialized Center of Endocrinology and Diabetes (Baghdad/ AL-Russafa Health Directorate) during the period between first of March to the end of June 2016.The patients were told about the purpose of the study and interested volunteers were enrolled after obtaining their consent. Patients were selected on the basis of criteria for diabetes used according to the American Diabetes Association (ADA) 2016guideline: Fasting Blood glucose (FBG) ≥126 mg/dL (7.0 mmol/L), or HBA1C ≥ 6.5% (48 mmol/mol) [17]. Sixty-one healthy subjects (31males and 30 female) were included along with patients group as a control group. This control group consisted of non-diabetic healthy individuals according to the laboratory finding of FBG (value <90mg/dL). Diabetic patients younger than 18 years old, those with less than 6 months of follow-up or pregnant women, were excluded.

Also subjects with the history of Hypertension, Coronary Artery Disease (CAD), Endocrinopathy or those taking any lipid altering medication were excluded from the study. Both patients and the control groups were characterized according to age, family history of diabetes, duration of disease, sex, weight (kg). The genotypes were characterized using Restriction Enzyme Fragment Length Polymorphism (RFLP-PCR) technique. Genomic DNA was extracted using g SYNC™ DNA Extraction Kit (Gene aid Biotech-Taiwan). The E-Selectin rs5368 (C468T) polymorphism was detected by new primer set that were designed in this study by the researchers.DNA sequence analysis was made using the Geneious 11.1.2 software (for the data sent by Macrogen's sequencing service/Korea), direct sequencing analysis using forward and reverse primers for each selected sample was performed.

NCBI BLAST was used to detect SNPs and any other alteration within the studied genomic regions. First, the PCR reaction was performed for final 50 µL reaction volume by using 25µL of 2X Go Taq® green master mix, 1.5 µL of 10M of each primer (forward and reverse), 3µL of genomic DNA and the volume was completed to 50 µL with nuclease free water, and the PCR program was done as mentioned by the reference shown in Table 1:

Table 1: Primers and PCR protocol for E-Selectin rs5368 (C468T) polymorphism

Primers			
Forward primer*	5'- GAACTATTGAAGAGCTTGGG-3'		
Reverse primer*	5'- GGGCAATCTAGGTTCAGA-3'		
Steps	Temperature (°C)	Time	Cycle number
Initial denaturation	95	5 min.	1
Denaturation	95	1 min.	35
Annealing	54	45 sec	
Extension	72	1 min.	
Final extension	72	10 min.	1
Hold	4	∞	∞

* Primers designed by the researcher

For the detection of the primary DNA fragment (353bp),15µL of the PCR product was first visualized by 1% agarose gel electrophoresis, then PCR product was digested with CviAII (5 U/ µL) restriction enzyme. Incubation time was one hour at 25°c resulting three genotypes (353bp =TT; 219 bp + 134bp = CC; 353bp + 219bp + 134bp

= CT), the enzyme recognizes the C allele (common allele) at the recognition site of the DNA fragments, agarose gel electrophoresis (2%) stained with ethidium bromide was used to separate these fragments for each sample.

The Statistical Analysis System- SAS (2012) program was used to test the effect of clinical factors on the study parameters [18]. Chi-square test was used to significant compare between percentage and least significant difference –LSD test (ANOVA) or t-test was used to significant compare between means. Estimate of correlation coefficient between variables in this study. WINPEPI computer program (version 11.63) was used to estimate the statistical significance of the p values that was calculated with Fisher's exact test as well as the Odd Ratio that was assessed by a special $\chi 2$ formula. Hardy-Weinberg equilibrium was tested by chi-squared test that was done using OEGE - Online Encyclopedia for Genetic Epidemiology studies [19].

Results

E-Selectin rs5368 (C468T) Genotyping Results

E-Selectin rs5368 (C468T) PCR-RFLP Results

The E-Selectin rs5368 (C468T) SNP was amplified using novel set of primers designed by the researcher especially for this locus in order to obtain the required DNA sequence around the SNP, the resulted fragment (353bp). The resulted PCR product was incubated with CviAII restriction enzyme for one hour then the product was visualized with 2% agarose gel electrophoresis (Figure):

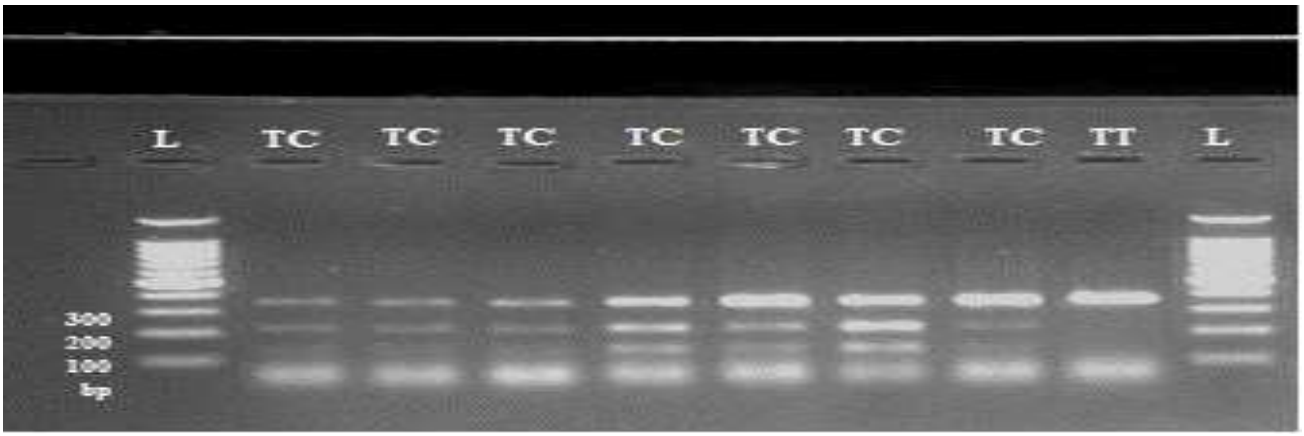

Figure 1: E-Selectin rs5368 (C468T) PCR Product after Treatment with CviAII Restriction Enzyme (One band: 353bp =TT; Two bands: 219 + 134bp = CC; Three bands: 353+ 219 + 134bp = CT), 2 % agarose gel electrophoresis for 3hrs, 300 volts

E-Selectin rs5368 (C468T) Genotype and Alleles Frequency and Distribution

Results showed that the C/C genotype was found in 35.14% of the patients compared with 30% of the control, while the C/T and CC genotypes were found in 65% and 16.22% of the patients respectively. No significant association was found between any of the E-Selectin rs5368 (C468T) genotypes and the incidence of T2DM.

Also there was no significant difference for the alleles distribution between the patients and control groups (the C allele was found 59.46% of the patients compared with 55.00% of the control and the T allele was found in 40.54% of the patients compared with 45.00%of the control), also the genotypes and allele frequencies were consistent with Hardy-Weinberg equilibrium (Tables 2 and 3).

Table 2: Distribution of E-Selectin rs5368 (C468T) Genotypes in T2DM Patients and Controls Using Fisher's Exact Test

Groups Genotype No (%)	Study groups Patients	Control	Odd Ratio	CI 95%	Fisher's exact probability *	Attributable fraction
C/C	13 (35.14%)	12 (30%)	1.26	0.44 to 3.67	0.808	20.9%
C/T	18 (65%18)	20 (50%)	0.95	0.35 to 2.54	0.999	5.3%
T/T	6 (16.22%)	8 (20%)	0.77	0.20 to 2.9	0.771	22.6%
Alleles distribution						
C n (%)	44 (59 .46%)	44(55.00%)	1.20	0.60 to 2.39	0.627	16.7%
T n (%)	30 (40.54%)	36(45.00%)	0.83	0.42 to 1.66	0.627	16.7%

$p \geq 0.05$ is not significant

Table 3: Expected Frequencies of Genotypes of the E-Selectin rs5368 (C468T) Polymorphism Using Hardy-Weinberg Equilibrium

Patients Genotypes	Observed no.	13	18	6	0.59	0.41	0.0031
	Expected no.	13.08	17.84	6.08	Not detected		
Control Genotypes	Observed no.	12	20	8	0.55	0.45	0.0041
	Expected no.	12.1	19.8	8.1	Not detected		

The expected frequencies of genotypes showed no significant differences (X^2<3.84) between observed and expected frequencies for both T2DM patients and control group.

The E-Selectin rs5368 (C468T) resulted from a missense mutation at the 9th exon of the E-Selectin gene that lead to substitution of C allele with T allele which in turn lead to change in the coding amino-acid form Histidine (H) to Tyrosine (Y).

This change has been linked to some clinical cases like, Atherosclerotic Peripheral Arterial Disease [20], Matrix metallopeptidase 9 (mmp-9) and E-selectin levels in Taiwanese individuals [21], Coal workers' pneumoconiosis (22), longitudinal blood pressure phenotypes [23] and the risk of ischemic stroke [24].

E-Selectin rs5368 (C468T) Polymorphism Sequencing Results

Ten DNA samples (6 controls and 4 patients) were sent for sequencing and the results were consistent with those obtained by the PCR-RFLP genotypes, DNA sequence was confirmed by blasting with NCBI Blast (Table 4), the score was 558 bits which indicates the great degree of similarity between both sequence, while the identities were 98% due to the high polymorphic content of the target region, also the expect was less than zero indicating a strong match between the target sequence and the reference sequence of NCBI (Figure 2, 3).

Table 4: Sequencing ID, Score, and Identities for the second targeted segment of the E-Selectin gene.

Accession	Identities	Score	Expect	Range
NG_012124.1	299/302 (98%)	558 bits(302)	3e-155	2058 to 2357

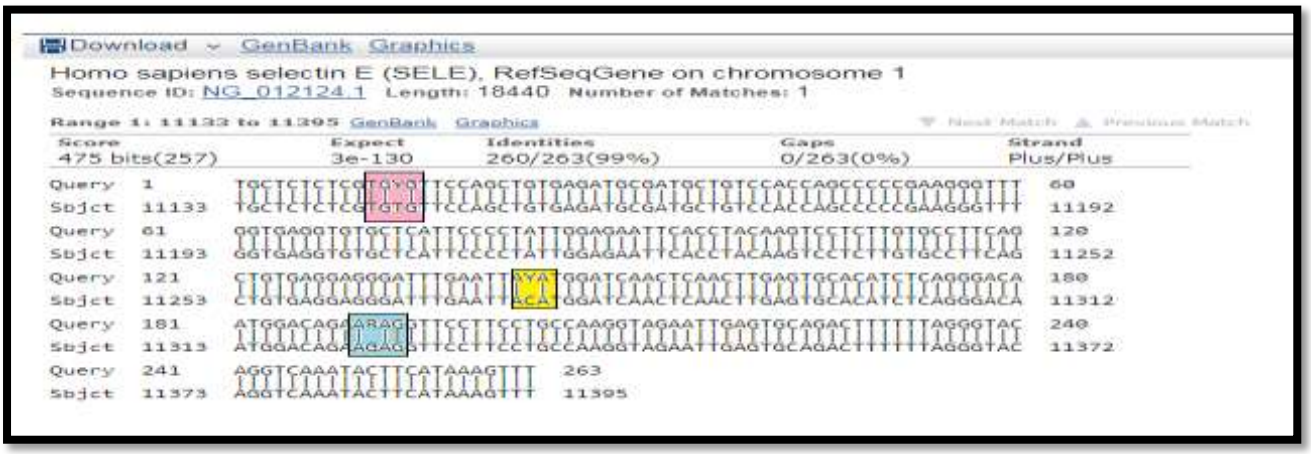

Figure 2: A representative sequence alignment of E Selectin gene amplification results with NCBI Blast. Yellow Shade is for rs5368 Y: (C/T), Blue Shade for rs768880915 R:(G /A). Pink Shade for rs5367 Y: (C/T)

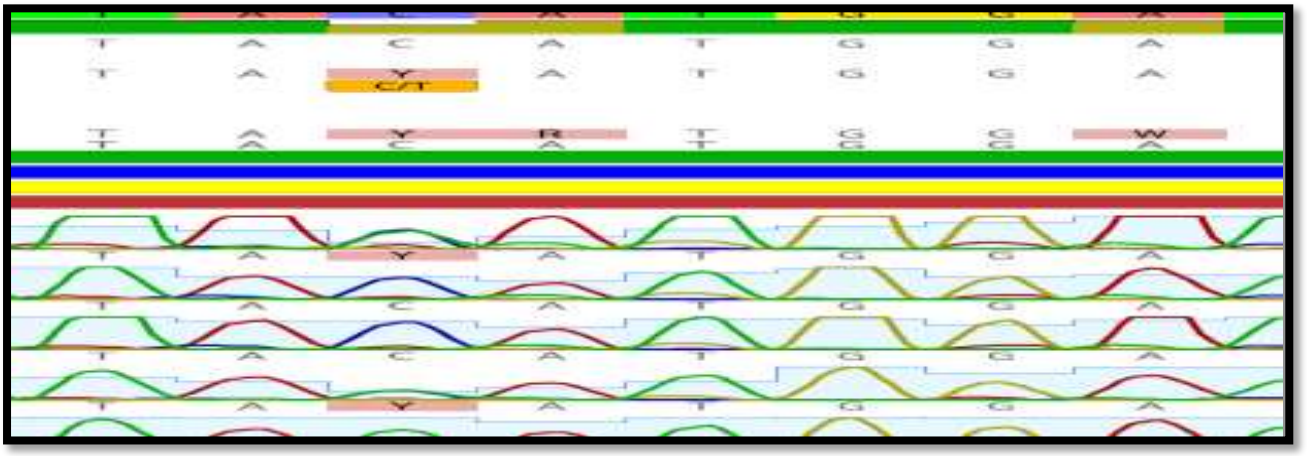

Figure 3: E-Selectin rs5368 (C468T), Y: Heterozygous C/T Locus, R: G/A by Geneious Software

Two SNPs were documented in the adjacent region of the E-Selectin rs5368 (C468T) polymorphism: rs5367 (C/T) and rs768880915 (G/A) that appeared in all the tested samples, sequence details are represented in Tables 5 and 6. Also there was a non-registered variation that appeared in all the sequenced samples that suggest the presence of new E-Selectin polymorphism in that locus, shown in Figure 4.

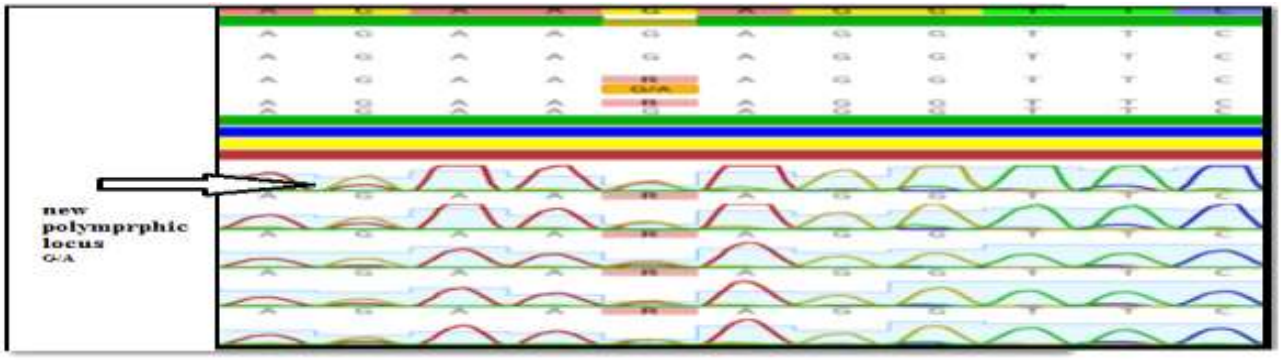

Figure 4: E-Selectin rs768880915 (G /A), R: Heterozygous locus G/A. Results Obtained by Geneious Software

Table 5: Position and Allele Information of the 3 SNPS of the E-Selectin Gene Based on NCBI Assembly Data

SNP	RefSeqGene	Gene (ID)	SNP to RefSeqGene	Position
rs5368 (C/T)	NG_012124.1	SELE (6401)	Fwd	11275
rs5367 (C/T)	NG_012124.1	SELE (6401)	Fwd	11145
rs768880915 (G /A)	NG_012124.1	SELE (6401)	rev	11323

RefSeqGene: genomic reference sequences, Gene (ID): species-specific gene identifier, Fwd: forward, rev: reverse.

Table 6: Location, Type, Effect, Frequency of the SNPs in the Targeted Region of the E-Selectin Gene

SNP	Location	Type	Effect
rs5368 (C/T)	Exon 9	Substitution	Histidine to Tyrosine
rs5367 (C/T)	splice region Exon 9	Substitution	No amino acid change
rs768880915 (G/A)	Exon 9	Substitution	Glutamic acid to Lysine

The substitution of Histidine to Tyrosine that occurs due to the E-Selectin rs5368 (C468T) polymorphism was found to be associated with some clinical disorders [20, 21, 22, 23, 24], while there was no recorded data concerning the E-Selectin rs768880915 (G/A). The substitution of an amino-acid with another (alanine with lysine), was found to have an effect on the binding affinity and ligand specificity of the E-Selectin [25], this may explain the potential consequences of these genetic variations in the E-Selectin gene.

The analysis of genotypes and alleles distribution of the E-Selectin rs5367 showed no significant difference between patients and control group. Results were consistent with Hardy-Weinberg equilibrium, which goes with the finding of the other E-Selectin SNPs. One study mentioned the E-Selectin rs5367 as non-associated genetic marker with amyotrophic lateral sclerosis [26]. The analysis of the alleles and genotype distribution of the two detected polymorphisms is illustrated in the following tables.

Table 7: Distribution of E-Selectin rs5367 Genotypes and alleles in T2DM Patients and Controls Using Fisher's Exact Test. (p>0.05 is not significant)

Groups Genotype No (%)	Study groups Patients	Control	Odd Ratio	CI 95%	Fisher's exact probability*	Attributable fraction
C/C	0	0	0	0	0	0
C/T	1 (25.00%)	2 (33.33%)	0.67	0.01 to 20.30	0.999	33.3%
T/T	3 (75.00%)	4 (66.67%)	1.50	0.05 to 117.60	0.999	33.3%
Alleles distribution						
C n (%)	1 (12.50%)	2 (16.67%)	0.71	0.01 to 16.64	0.999	28.6%
T n (%)	7 (87.50%)	10(83.33%)	1.40	0.06 to 94.23	0.999	28.6%

Table 8: Expected Frequencies of Genotypes and Alleles of the E-Selectin rs5367 Using Hardy-Weinberg Equilibrium

Groups		CC	CT	TT	C	T	X^2
Patients Genotypes	Observed no.	0	1	3	0.13	0.88	0.08
	Expected no.	0.06	0.88	3.06	Not detected		
Control Genotypes	Observed no.	0	2	6	0.13	0.88	0.16
	Expected no.	0.13	1.75	6.13	Not detected		

The expected frequencies of genotypes showed no significant differences ($X^2 < 3.84$) between observed and expected frequencies for both T2DM patients and control group. The rs768880915 genotyping analysis showed only one genotype (G/A) in all tested samples, which was not equal to those predicted by the Hardy-Weinberg Equilibrium theory (Table 9), and since the χ2 value for both patients and control is greater than 3.84 (at 0.05 significance level for 1 degree of freedom), this result suggests that the E-Selectin rs768880915 (G/A) might be under evolutionary pressure, this deviation might be due to small population sizes, assortative mating and selection that result from increased risk of fetal loss or early death. Other factors may include mutation, migration and selection [27]. These results go with the fact that Iraqi population is known to have different ethnicity groups that tend to have open marriage relations within and with other populations [28].

Table 9: Expected Frequencies of Genotypes and Allele for E-Selectin rs768880915 (G / A) using Hardy-Weinberg Equilibrium.

Patients Genotypes	Observed no.	0	4	0	0.5	0.5	4*
	Expected no.	1	2	1	Not detected		
Control Genotypes	Observed no.	0	6	0	0.5	0.5	6*
	Expected no.	1.5	3	1.5	Not detected		

* X^2>3.84 (calculated at 0.05 significance level for 1 degree of freedom)

Another E-Selectin SNP (E-Selectin rs5361 A561C) was mentioned in a local paper that considered the AC genotype as risk factor for T2DM in Iraqi population [10], while a different study found that the L-Selectin rs2229569 (pro213ser) might have a role in the development of T2DM in Iraqi Arabs patients [29].

As mentioned before, regions contain the genes of E- Selectin found to have a relationship with T2DM (16), even though the genetic polymorphism study results of the E-Selectin rs5368 (C468T)was not significantly associated with disease, many factors might influence this result; gene-environment, gene-nutrient, gene-gene interactions influence the impact of cytokine polymorphisms on the insulin sensitivity as well as serum levels of cytokines, susceptibility to disease, energy expenditure, energy derivation from dietary fats and the response to weight reduction and lipid lowering drugs [30,31].

Limitation

There are some limitations for this study (due to time limit and the precarious security situation), the study was conducted on a small size sample of population, and one polymorphism was examined. Although, larger sample size is required for more definite conclusions, this preliminary study attempted to evaluate the possible association between E-Selectin rs5368 (C468T) polymorphism and T2DM development. To the best of our knowledge, there is no published reports concerning E-Selectin rs5368 (C468T) gene polymorphism with development of T2DM locally or in surrounding countries; therefore, it was difficult to compare the results of this study to others. Also, due to the relatively small sample size, the frequencies of some homozygous variants were low or absent.

Conclusion

This study showed a success in the primers design and the PCR- RFLP protocol and techniques for the E-Selectin rs5368 (C468T) by Iraqi researcher ability, the sequence of E-Selectin rs5368 (C468T) polymorphism is found to be in concordance with the global registered NCBI sequence. While the genotyping results showed that the E-Selectin rs5368 (C468T) polymorphism is not associated with the incidence of T2DM.

Also there was no significant difference for the allele's distribution between the patients and control groups. Further studies are required with larger sample size along with E-Selectin serum level evaluation and/or Real-time PCR technique for the detection of the polymorphisms involved in this study as well as the examination of the gene expression may provide more information regarding the association with T2DM.

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