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Genetic Polymorphisms and Haplotype Analysis of *Endothelin*-1 Gene in Diabetes Mellitus Type II Patients

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Abstract:
Introduction: *Endothelin-1*gene is an important factor that can contribute to cardiovascular and kidney disorders. Numerous single nucleotide polymorphisms (SNPs) in the *Endothelin-1*gene was related to many diseases and may be associated with complications of diabetes mellitus type II.This study aimed to investigate the relationship between *Endothelin*-1genetic polymorphism and DM type II in the Iraqi population.
Methods: Blood samples were collected from patients with type II diabetes and control. PCR was applied to detect the *Endothelin-1*gene and conducted genetic sequences for *Endothelin-1*gene to detect SNPs (rs1800543, rs2070699, rs1476046, rs1762831452, new671, rs587777231, rs587777234). Results: SNP rs1800543 showed that the odds ratio for genotype CC (1.52) and C allele (1.12) both consider as an etiological fraction positively associated with diabetic type II. SNP rs2070699.The results showed that the odds ratio for genotype GG (1.8) and G allele (1.35) were associated with a positive correlation of diabetic type II as an etiological factor. The results of rs1476046 showed that the odds ratio for the genotype GA and A allele were 1.42 and 1.22, indicating an etiological fraction as a positive association with diabetes. According to the result linkage disequilibrium, the haplotype CCTAT, TAGGC, TATGC, and TCTAT represent a risk factor that makes an individual more susceptible to diabetes mellitus type II. In conclusion, SNPs (rs1800543, rs2070699, rs1476046, rs1762831452, and new 671) affect more susceptibility to diabetes. The linkage disequilibrium results in the current study showed a strong association between rs2070699 and rs1476046 SNPs. The haplotype TAGGC represents the widespread haplotype with risky effects, making individuals more susceptible.

Keywords: *Endothelin-1*gene, rs1800543, rs2070699, rs1476046, rs1762831452

تعدد الشكل الوراثي والنمط الفرداني لجين إلاندوثيلين في ملاضى داء السكري من النوع الثاني

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الخلاصة:

المقدمة: يعد جين *Endothelin-1* عاملًا مهمًا يُمكن أن يُساهم في أمراض القلب والأوعية الدموية والكلى. ارتبطت العديد من تعدد الشكل الوراثي للنوكليوتيدات المفردة SNP في جين *Endothelin-1* بالعديد من الأمراض وقد ترتبط بمضاعفات داء السكري من النوع الثاني. هدفت هذه الدراسة إلى دراسة العلاقة بين تعدد الأشكال الجينية للإندوثيلين ومرض السكري من النوع الثاني في السكان العراقي.

طرق العمل: تم جمع العينات من مرضى السكري من النوع الثاني والسيطرة. تم تطبيق تفاعل البلمرة المتسلسل للكشف عن مورث الإندوثيلين وأجري التسلسل الوراثي لجين الاندوثلين للكشف عن تعدد الشكل الوراثي المفرد rs1800543، rs2070699، rs1476046، rs1762831452، new671، rs 587777231، rs 587777234 . النتائج: أظهرت نتائج SNP rs1800543 أن نسبة الأرجحية للطراز الوراثي CC (1.52) والأليل C (1.12) كان له ارتباط إيجابي بمرض السكري من النوع الثاني. أظهرت نتائج rs2070699 أن نسبة الأرجحية للتمط الوراثي GG (1.8) والأليل G (1.35)كانت مرتبطة ارتباط إيجابي بمرض السكري من النوع الثاني كعامل مسبب. أظهرت نتائج rs1476046 أن نسبة الأرجحية للطراز الوراثي GA والأليل A كانت 1.42 و 1.22 مما يشير الى كونه له ارتباط إيجابي بمرض السكري. وفقًا لنتائج اختلال توازن الارتباط فإن النمط الفرداني الوراثي CCTAT و TAGGC و TATGC و TCTAT يمثل عامل خطر يجعل الفرد أكثر عرضة للإصابة بمرض السكري من النوع الثاني.الاستنتاجات تُؤثر تعدد الشكل الوراثي للنوكليوتيدات المفردة (rs1800543، rs2070699، rs1476046، rs1762831452، و new 671) بشكل أكبر على الأشخاص الذين يعانون من داء السكري. أظهرت نتائج اختلال توازن الارتباط في الدراسة الحالية ارتباطًا قويًا بين تعدد الشكل للنوكليوتيدات المفردة rs2070699 وrs1476046. يُمثل النمط الفرداني TAGGC النمط الأكثر شيوعًا ذو التأثيرات الخطرة، مما يجعل الأفراد أكثر عرضة للإصابة.

1.Introduction

Diabetes Mellitus type II represents one of the metabolic disturbances in the world. The mixing of two major factors mainly occasions the development of DM type II, included the disturbance of insulin excretion by the pancreatic beta cells and the incapability of insulin-susceptible tissues to respond to the insulin [1]. Diabetes Mellitus (DM) type II is a chronic condition that causes increased glucose levels in the body, leading to problematic glucose metabolism. The rising of glucose levels chronically triggers inflammation. Moreover, DM type II causes continuous inflammation [2]. Inflammation has a significant role in the pathophysiology of diabetes [3]. Endothelin-1(ET) protein consists of long peptides from 21 amino acids that have a role as a vasoconstrictor generated from EDN-1thelial cells, vascular smooth muscle cells, kidney medulla, and macrophages. Endothelin-1protein is recognized as an effective vasoconstrictor with proliferative, profibrotic characteristics and preservation of the tone of vascular smooth muscle cells [4]. Endothelin-1protein is recognized mainly as a potent prooxidative and proinflammatory merit, and it has a unique role relative to the pathophysiology of diabetes vascular disorders [5].

*Endothelin-1*gene consists of five exons that extend approximately 6.8 Kb from genomic DNA and encode the Endothelin-1protein [1]. SNP (rs1476046), (rs1800543), (rs2070699)are located in an intronic position of EDN-1thelin, whereas rs1762831452 is located on exon five, and rs 671 is located on exon 12 (https://www.ncbi.nlm.nih.gov/snp). *Endothelin-1*gene is an important factor that can contribute to cardiovascular and kidney disorders. Numerous single nucleotide polymorphisms (SNPs) in the *Endothelin-1*gene was related to hypertension, heart attack, ventricular hypertrophy, EDN-1thelial disorders, development of atherosclerosis, and accelerated reduction in renal function in patients with glomerulonephritis. Moreover, *Endothelin*-1genetic polymorphism was associated with complications of DM type II, like retinopathy in diabetes [6-8]. Since the genetic polymorphism of *Endothelin-1*gene has not

been studied about DM type II in the Iraqi population. Moreover, this gene has an important role in many diseases, therefore, this study aimed to investigate the relationship between the genetic polymorphism of *Endothelin-1*gene and DM type II in the Iraqi population.

2.Materials and Methods

2.1.Samples collection

Five milliliters of blood samples were collected randomly from 50 patients with DM type II and 50 healthy samples. Whole blood samples were divided into EDTA tubes (2 ml) and then stored at -20.

All samples were collected from March 2023 to August 2023 at the Specialized Center for EDN-1crine Diseases and Diabetes, Al-Rusafa, Baghdad. The patients' ages for samples were 28 to 65 years. Patients were chosen based on criteria for DM type II according to the American Diabetic Mellitus Type II Association 2016 guideline, Inclusion criteria included fast blood glucose (FBG) ≥125 mg/dL (7.0 mmol/L), HBA1c ≥ 6.5% (48 mmol/mol) [9]. Besides, patients have been identified clinically with DM type II for less than two years by counseling physicians. The control group represents non-diabetic people with <95 mg/dL FB values. Moreover, the exclusion criteria included all groups that were chosen without any other diseases, without inflammation, and positive for C-reactive protein. Anthropometric characteristics in the studied groups included weight (kg), height (meter), abdominal circumference (cm), and hip circumference. The normal range of fasting blood glucose is 75–130 mg/dI, HbA1c is 4.2–6.5 mg/dI, and Insulin is 1.1–17.0 mu/mI. The concentration measurement test of insulin resistance was counted using the HOMA-IR score. HOMAIR=(insulin fasting [mU/mL]×glucose fasting [mg/dL]/450 [1/(log Insulin) +log(Glucose)].

2.2.Ethical consent

Oral consent was obtained from patients and the control group to collect blood. Moreover, the ethics committee at the Biology Department/College of Science for Women, University of Baghdad, Iraq, approved it for conducting this study. Besides, approval for sample collection was obtained from the ethical committee in the Ministry of Health (Ref: 42369, dated 20/ 2023).

2.3.Biochemical factors measurement

The Cobas C 311 analyzer (Cobas-Roche, Germany) was loaded with special reagents and applied to measure biochemical factors**.**

2.4.Genetic analysis for SNPs

Genomic DNA was extracted from blood samples by (Geneaid kit , Taiwan) and stored at - 20°C until use. The sequence primer for *EDN-1* gene was designed by the second author (F:5`-GAAACCACTCCCAGTCCAC -3`; R: 5`- AGCAAAGGAAATCCGGGCTC-3`) with product size 649 bp. Reference sequence on chr. 6, ID sequence: J505008.1, region position (7059 - 7753 bp) included exon2 and intron2 used. This primer was applied to detect SNP (rs1800543, rs2070699, rs1476046, rs1762831452, new 671) .

Also, the primer sequences for SNPs (rs 587777231 rs 587777234) were designed by the second author (F:5`- TCAGGGCCATTGATGCACAG -3`; R: 5`-ACAGAGGACA TCGGTTTG CAT-3`) . These SNPs are located on exon3 intron3 – exon 4.

PCR analysis was performed in a mixture with a final volume 25µl, which included DNA sample(5µl),each one forward and reverse primer (1µl) for each of them, free-nuclease water (13µl), and pre-mix master mix (5µl) (Bioneer/Korea).

Electrophoresis was applied to detect PCR amplicon size for *EDN-1*gene at a voltage 70 volt/cm^2 for 1 hour in agarose (1.5%) . A safe red stain was used to visualize at UV light illumination and compared with marker ladder 100bp to determine the size gene.
NCBI/Primer designing was used. PCR conditions were initial denaturation 95C^0/5 min.; denaturation 94 C^0/30 sec. (35 cycle) ; annealing 61 C^0/30 sec. (35 cycle)extension 72 C^0/40 sec.(35 cycle) ;final extension 72 C^0/1 min. Sequencing PCR amplicons were analyzed by sending to (Macrogen corporation company /Korea) also it was sent forward and reverse primers with PCR products to comparing in sequencing gene. The sequencing was carried out by ABI3730XL to detect SNPs. Gel extraction of PCR amplicons was performed according to protocol [10]and BioEdit alignment to analysis sequencing by Blast in NCBI.

2.5.Statistical analysis

The Statistical Analysis was performed by SAS System program (2018) to detect the difference between studied groups in studied parameters. T-test was utilized to compare between patients and control means. The chi-square test was applied to significantly compare between percentages (with probability 0.05 and 0.01). It was applied to wepCal to analyze H.W.E (Hardy -Weinberg equilibrium).WINPEPI version 11.65 was used to calculate the odd ratio (OR) ,etiological, preventive factors, and P value using the Fisher test. Haplotypes analysis was carried out on the website http://analysis.bio-x.cn/.

3.Results

3.1.Anthropometric characteristics in studied groups

The outcomes showed no significant differences when comparing patients versus control for BMI (46.99 ±1.64 versus 40.01 ±1.57, respectively), waist (39.58 ±1.34 versus 40.58 ±2.05, respectively), and hip circumstances (48.81±1.91 versus 46.69 ±2.55, respectively) with p-values >0.05, Table 1.

Table 1: Comparison of anthropometric characteristic between patients and control groups

Groups	Parameters Mean ± SD		
	BMI (kg/m^2)	Waist circumstances (cm)	Hip circumstances (cm)
Patients	46.99 ±1.64	39.58 ±1.34	48.81 ±1.91
Control	40.01 ±1.57	40.58 ±2.05	46.69 ±2.55
P-value	0.0469 S	0.675NS	0.501 NS
NS: Nonsignificant p >0.05 ;S: significant p <0.05			

3.2.Laboratory profiles in studied groups

The biochemical tests in Table 2 show significant differences between patients and control according to HbA1c, FBS, insulin, and HOMA-IR with a p-value ≤0.01.

Table 2: Comparison between patients and control groups in some biochemical tests

Groups	Parameters Mean ± SD			
	HbA1c (%)	FBS (mg/dl)	Insulin(Mu/Ml)	HOMA-IR
Patients	7.02 ±0.19	137.04 ±2.97	15.35 ±1.45	5.38 ±0.61
Control	4.96 ±0.07	115.02 ±2.56	4.55 ±0.34	1.419 ±0.04
P-value	0.0001	0.0001	0.0001	0.0001
Significant P≤0.05 ; Highly significant P≤0.001				

3.3.Genotyping of *Endothelin-1*gene

The *Endothelin-1gene* was amplified using the PCR technique, and electrophoresis was used to investigate the amplicon size (649bp) (Figure 1). Table (3) shows the DNA sequence alignment for the gene in NCBI (National Center of Biotechnology Information) to determine the variations within the sequence and their location. The nucleotide sequence expected for the *EDN-1* gene was zero indicating a very significant (identical) match between the query sequence and reference sequence of NCBI (ID: NG_011817.2), for the 7059 to 7753 nucleotide sequence of the *Homo sapiens EDN-1* gene, with score 1430 bits(774) which indicates similarity between both sequences.

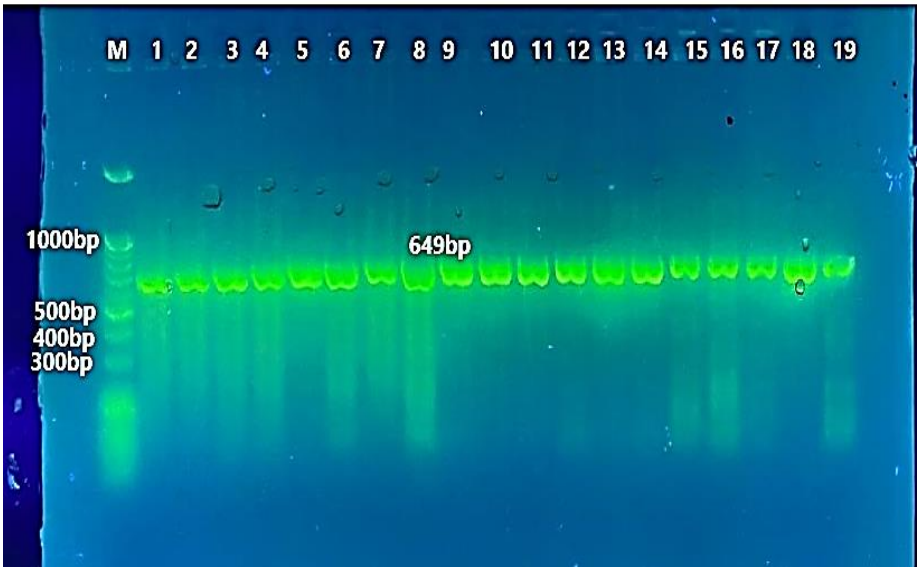

Figure 1: Electrophoresis of *Endothelin-1*gene (649bp) PCR product (lane 1-19) samples with M: ladder Marker (100bp) on agarose gel (2%) in (70 volt/cm^2, 1 hr).

Table 3: The alignment of the *Endothelin*-1gene sequence in NCBI

Sequence ID:	Identities	Gaps	Score	Expected	Range
J505008.1	790/797 (99%)	3/797 (0%)	1430 bits (774)	0.0	7059 to 7753

SNP rs1800543

The results of H.W.E for SNP rs1800543 shows higher the frequency of genotype TT (60%in patients versus 64% in control) than TC (34%in patients versus 32% in control) and CC (6% in patients versus 4% in control). The frequency of the T allele in patients and control was 0.77 and 0.8, respectively, while the C allele in patients and control was 0.23 and 0.2, respectively (Table 4). These outcome distributions were compatible with H.W.E. There are no interaction with other factors such as mutations, interbreeding, migration, gene drift or natural selection.

Table 4: Hardy Weinberg Equilibrium of SNP rs1800543

Groups rs1800543		TT	TC	CC	T	C	χ^2
Patients Genotype N=50	Observed no	30(60%)	17(34%)	3(6%)	0.77	0.23	0.08 C
	Expected no	29.6(59.2%)	17.7(35.4%)	2.6(5.2%)			
Control Genotype N=50	Observed no	31(64%)	17(32%)	2(4%)	0.8	0.2	0.03 C
	Expected no	31.2(62.4%)	16.59(33.2%)	2.21(4.42%)			
Total Observed		61	34	5			
Distribution consistent with HWE at χ^2<3.84							

Table 5 shows the results comparison genotypes and alleles between patients and control of SNP rs1800543 for genotype TT and T allele were 0.91 and 0.88, respectively, which indicates a protective fraction with a negative association for TT genotype and T allele with diabetic type II, while the odds ratio for genotype CC and C allele were 1.54 and 1.12 which indicates an etiological fraction for CC genotype and allele C with a positive association with diabetic type II. Odd ratio was close for all genotypes and alleles except heterozygote TC recorded =1.

Table 5: Comparison of genotype and alleles between patients and control for SNP rs1800543

Genotypes rs1800543 T/C	Study Group		Odds Ratio	CI 95%	Attributable Fraction(%)	Preventive Fraction(%)	P value
	Patients (50)	Control (50)					
TT	30 (60%)	31 (62%)	0.91	0.4115 to 2.0540		5	0.8
TC	17 (34%)	17 (34%)	1.00	0.4371 to 2.2876	0		1.00
CC	3 (6%)	2 (4%)	1.54	0.2448 to 9.5869		2.1	0.6
Total	50	50					
Alleles Distribution							
T	77 (77%)	79 (79%)	0.88	0.4555 to1.7386		8.7	0.7
C	23 (23%)	21 (21%)	1.12	0.5752 to 2.1953	2.5		0.7

P value <0.05 means significant differences; P value >0.05 means no significant differences; Attributable Fraction means etiological fraction when odd ration >1; Preventive fraction means protective fraction when odd ratio <1

SNP rs2070699

Table 6 shows H.W.E for SNP rs2070699(on intron 2). The genotype frequency of GT in patients (46%) and control (60%) was higher than GG (40%) in patients and 26% in control) and TT (14%) for patients and control. The frequency of the G allele in patients and control was 0.62 and 0.6, respectively. At the same time, the T allele in patients and control was 0.38 and 0.4, respectively. These outcome distributions were compatible with H.W.E.

Table 6: Hardy Weinberg Equilibrium of SNP rs2070699

Groups rs2070699		GG	GT	TT	G	T	χ^2	P-value
Patients Genotype N=50	Observed no	20(40%)	23(46%)	7(14%)	0.62	0.38	0.009 C	0.5
	Expected no	19.9(39.7%)	23.31(46.6%)	6.8(13.8%)				
Control Genotype N=50	Observed no	13(26%)	30(60%)	7(14)	0.6	0.4	2.3 C	0.2
	Expected no	15.7(13.4%)	24.64(49.3%)	9.7(19.4%)				
Total Observed		33	53	14				
Distribution consistent with HWE at χ^2<3.84 when P value >0.05 mean SNP results compatible to HWE								

Table 7 shows the results of genetic polymorphism of SNP rs2070699 for genotype GG and G allele were 1.8 and 1.35, respectively ,indicating an etiological fraction with a positive association for the GG genotype and G allele with diabetic type II. heterozygous GT recorded a negative association with diseases, so the heterozygous genotype works as a protective fraction, while the odds ratio for the T allele was 0.74, indicating a protective fraction for allele T with a negative association with DM type II. Fisher probability was close for all genotypes and alleles except homozygous TT recorded =1.

Table 7: Comparison of genotype and alleles between patients and control for SNP rs2070699

Genotypes rs 2070699	Study Group		Odds Ratio	CI 95%	Attributable Fraction(%)	Preventive Fraction(%)	P value
	Patients (50)	Control (50)					
GG	20 (40%)	13 (26%)	1.8	0.8124 to 4.4314	18.9		0.1
GT	23 (46%)	30 (60%)	0.56	0.2569 to 1.255		25.9	0.1
TT	7 (14%)	7 (14%)	1.00	0.3231 to 3.0948	0		1.0
Total	50	50					
Alleles Distribution							
G	63 (63%)	56 (56%)	1.35	0.7594 to 2.3570	15.9	15.9	0.3
T	37 (37%)	44 (44%)	0.74	0.4243 to 1.316		11.1	0.3

P value <0.05 means significant differences; P value >0.05 mean no significant differences ; Attributable Fraction means etiological fraction when odd ration >1; Preventive fraction means protective fraction when odd ratio <1

SNP rs1476046

Table 8 shows H.W.E for SNP rs1476046. The genotype frequency of GA in patients (54%) and control (46%) was higher than GG (40%) in patients and 48% in control) and AA (6%) for patients and control. The frequency of the major G allele in patients and control was 0.66 and 0.71, respectively. At the same time, G and A alleles in the patients and the control were 0.34 and 0.29, respectively. These outcomes distribution frequency were compatible with H.W.E.

Table 8: Hardy Weinberg Equilibrium of SNP rs1476046

Groups rs1476046		GG	GA	AA	G	A	χ^2
Patients Genotype N=50	Observed no	20(40%)	27(54%)	3(6%)	0.66	0.34	0.21
	Expected no	21.8(43.6%)	22.4(44.8%)	5.8(11.6%)			
Control Genotype N=50	Observed no	24(48%)	23(46%)	3(6%)	0.71	0.29	0.6
	Expected no	25.2(50.4%)	20.6(41.2%)	4.2(8.4%)			
Total Observed		44	50	6			
Distribution consistent with HWE at χ^2<3.84							

Table 9 shows the genetic polymorphism comparison of SNP rs1476046 for genotypes and alleles. The results showed that the odds ratio for genotype GA and A allele were 1.42

and 1.22, respectively, which indicates an etiological fraction with a positive association for genotype GA and G allele with diabetic type II, while the odds ratio for genotype GG and G allele were 0.72 and 0.79 which indicates negative association with disease as a protective fraction for GG genotype and allele G in diabetic type II. Odd ratio was close for all genotypes and alleles except homozygous AA recorded =1

Table 9: Comparison of genotype and alleles between patients and control for rs1476046

Genotypes rs1476046	Study Group		Odds Ratio	CI 95%	Attributable Fraction(%)	Preventive Fraction (%)	P value
	Patients (50)	Control (50)					
GG	20 (40%)	24 (48%)	0.72	0.3270 to 1.595		13.3	0.4
GA	27 (54%)	23 (46%)	1.42	0.6276 to 3.025	14.8	-	0.4
AA	3 (6%)	3 (6%)	1.00	0.1919 to 5.2102		0	1.0
Total	50	50					
Alleles Distribution							
G	67 (67.00%)	71 (71%)	0.79	0.4373 to 1.447		12.1	0.4
A	33 (33.00%)	29 (29%)	1.22	0.6908 to 2.2865	5.6	-	0.4

P value <0.05 mean significant differences; P value >0.05 mean no significant differences ; Attributable Fraction means etiological fraction when odd ration >1; Preventive fraction means protective fraction when odd ratio <1

SNP rs1762831452

Table 10 shows observed and expected numbers according to H.W.E for SNP rs1762831452. The genotype frequency of CT in patients (86%) and control (94%) was higher than CC (14%) in patients and 6% in control) and TT (0%) for patients and control. The frequency of the C allele in patients and control was 0.57 and 0.53, respectively. At the same time, the T allele in patients and control was 0.43 and 0.47, respectively. The distribution of these frequencies was not consistent with H.W.E.

Table 10: Hardy Weinberg Equilibrium of SNP rs1762831452

Groups rs176283145252		CC	CT	TT	C	T	χ^2
Patients Genotype N=50	Observed no.	7(14%)	43(86%)	0	0.57	0.43	28.4 NC
	Expected no.	16.25(32.5%)	24.51(49.02%)	9.25(18.5%)			
Control Genotype N=50	Observed no.	3(6%)	47(94%)	0	0.53	0.47	39.3 NC
	Expected no	14.05(28.1%)	24.91(49.8%)	11.05(22.1%)			
Total Observed		10	90	0			
Distribution consistent with HWE at χ^2<3.84 Distribution no consistent with HWE at χ^2>3.84 NC: Nonconsistent							

Table 11 shows the results of genotyping comparison of polymorphism for SNP rs1762831452 for genotypes and alleles. The results showed that the odds ratio for genotype CC and C allele were 2.55 and 1.17, respectively, which indicates An etiological fraction is a

positive association with DM type II for genotype CC and C allele , while the odds ratio for genotype CT and T allele were 0.39 and 0.85 which indicates a protective fraction for CT genotype and allele C with a negative association with DM type II.

Table 11: Comparison of genotype and alleles between patients and control for rs1762831452

Genotypes rs1762831452	Study Group		Odds Ratio	CI 95%	Attributable Fraction(%)	Preventive Fraction(%)	P value
	Patients (50)	Control (50)					
CC	7 (14%)	3 (6%)	2.55	0.6200 to 10.491	8.5	-	0.2
CT	43 (86%)	47 (94%)	0.39	0.0953 to 1.613		57.1	0.1
TT	**0**	**0**	**0**				
Total	**50**	**50**					
Alleles Distribution							
C	57 (57%)	53 (53%)	1.17	0.6731 to 2.05	57.1	-	0.5
T	43 (43%)	47 (47%)	0.85	0.4871 to 1.4858		7	0.5

P value <0.05 mean significant differences; P value >0.05 mean no significant differences ; Attributable Fraction means etiological fraction when odd ratio >1; Preventive fraction means protective fraction when odd ratio <1

SNP new 671

Table 12 shows the comparison between observed and expected for SNP new 671 representing a nonrecording location on the *Endothelin-1* gene in 12.293.970 nucleotides. The heterozygote genotype frequency of AC in patients of 68% and control of 86% was higher than homozygote AA (32%) and (14%) in patients and control. The current study did not record the homozygote CC for patients and control. The frequency of the A allele in patients and control was 0.66 and 0.57, respectively. At the same time, the C allele in patients and control was 0.34 and 0.43, respectively. The distribution of this frequency was not consistent with H.W.E.

Table 12: Hardy Weinberg Equilibrium of SNP new 671

Groups new671		AA	AC	CC	A	C	χ^2
Patients Genotype N=50	Observed no.	16(32%)	34(68%)	0	0.66	0.34	13.2 NC
	Expected no.	21.8(43.6%)	22.44(44.9%)	5.78(11.6%)			
Control Genotype N=50	Observed no.	7(14%)	43(86%)	0	0.57	0.43	28.4 NC
	Expected no.	16.3(32.6%)	24.51(49.02%)	9.3(18.6%)			
Total Observed		23	77	0			
Distribution consistent with HWE at χ^2<3.84 Distribution no consistent with HWE at χ^2>3.84 NC: Nonconsistent							

Table 13 shows of genotyping comparison of polymorphism for of SNP new671 for genotypes and alleles. The results showed that the odds ratio for genotype AA and A allele were 2.9 and 1.4, respectively, which indicates an etiological fraction with a positive association for genotype AA and A allele with diabetic type II, while the odds ratio for genotype AC and C allele were 0.35 and 0.36 which indicates a protective fraction for AC genotype and allele C with a negative association with diabetic type II. Fisher probability was significant differences for AA, AC genotypes except homozygous recorded =1; no one of the study individuals has CC genotype.

Table 13: Comparison of genotype and alleles between patients and control for SNP new 671

Genotypes new671	Study Group		Odds Ratio	CI 95%	Attributable Fraction(%)	Preventive Fraction (%)	P value
	Patients (50)	Control (50)					
AA	16 (32%)	7 (14%)	2.9	1.0681 to 7.8234	20.9	-	0.03
AC	34 (68%)	43 (86%)	0.35	0.1278 to 0.9362		56.2	0.03
CC	0	0	0	-	-	0	
Total	50	50					
Alleles Distribution							
A	66 (66%)	57 (57%)	1.4	0.8260 to 2.596	20.9	-	0.19
C	34 (34%)	43 (43%)	0.36	0.3852 to 1.2107	13.6	13.6	0.19

P value <0.05 mean significant differences; P value >0.05 mean no significant differences ; Attributable Fraction means etiological fraction when odd ration >1; Preventive fraction means protective fraction when odd ratio <1

SNP rs 587777231

Table 14 shows H.W.E for SNP rs587777231 (on exon 3). The only genotypes for all individuals was AA which common homozygote genotype frequency was in patients (100%) and controls (100%). The homozygote GG and heterozygote genotype AG were not recorded in the current study for patients and controls.

Table 14: Hardy Weinberg Equilibrium of SNP rs587777231

Groups rs587777231		AA	AG	GG	A	G	χ^2	P-value
Patients Genotype N=50	Observed no.	50(100%)	0	0	0.51	0.49	98 NC	<0.001
	Expected no.	25.5(51%)	49(98%)	23.5(47%)				
Control Genotype N=50	Observed no.	50(100%)	0	0	0.51	0.49	28.4 NC	<0.001
	Expected no.	25.5(51%)	49(98%)	23.5(47%)				
Total Observed		100	0	0				
Distribution consistent with HWE at χ^2<3.84 Distribution no consistent with HWE at χ^2>3.84 NC: Nonconsisstent								

Table 15: shows the results odds ratio of SNP rs58777231 for genotypes and alleles that the genotype AA is the only one recorded in all studied individuals.

Table 15: Comparison of genotype and alleles between patients and control for SNP rs 587777231

Genotypes rs587777231	Study Group		Odds Ratio	CI 95%	Attributable Fraction(%)	Preventive Fraction(%)	P value
	Patients (50)	Control (50)					
AA	50(100%)	50(100%)	-	-	-	-	-
AG	0	0	0	0	0	0	0
GG	0	0	0	0	0	0	0
Total	50	50					
Alleles Distribution							
T	100(100%)	100(100%)	-	-	-	-	-
G	0	0	0	0	0	0	0

P value <0.05 mean significant differences; P value >0.05 mean no significant differences ; Attributable Fraction means etiological fraction when odd ration >1; Preventive fraction means protective fraction when odd ratio <1

SNP rs 857777234

Table 16 shows H.W.E for SNP rs587777234 (on exon 3). The homozygote genotype frequency of TT was in patients of 100% and controls of 100%. The homozygote GG and heterozygote genotype TG were not recorded in the current study for patients and controls. These outcomes indicated the dominance of common homozygote genotypes in the Iraqi population.

Table 16: Hardy Weinberg Equilibrium of SNP rs 58777723

Groups rs587777234		TT	TG	GG	T	G	χ^2
Patients Genotype N=50	Observed no.	50(100%)	0	0	0.51	0.49	98 NC
	Expected no.	25.5(51%)	49(98%)	23.5(47%)			
Control Genotype N=50	Observed no.	50(100%)	0	0	0.51	0.49	98 NC
	Expected no.	25.5(51%)	49(98%)	23.5(47%)			
Total Observed		100	0	0			
Distribution consistent with HWE at χ^2<3.84 Distribution no consistent with HWE at χ^2>3.84 NC: Nonconsistent							

Table 17 shows of genotyping comparison of polymorphism for SNP rs587777234 for genotypes and alleles; that genotype TT was the only one recorded.

Table 17: Comparison of genotype and alleles between patients and control for SNP rs 587777234

Genotypes rs587777234	Study Group		Odds Ratio	CI 95%	Attributable Fraction(%)	Preventive Fraction(%)	P value
	Patients (50)	Control (50)					
TT	50(100%)	50(100%)	-	-	-	-	-
TG	0	0	0	0	0	0	0
GG	0	0	0	0	0	0	0
Total	50	50					
Alleles Distribution							
T	100 (100%)	100 (100%)	-	-	-	-	-
G	0	0	0	0	0	0	0

P value <0.05 mean significant differences; P value >0.05 mean no significant differences ; Attributable Fraction means etiological fraction when odd ration >1; Preventive fraction means protective fraction when odd ratio <1

Haplotype results

The linkage disequilibrium (D' value) among SNPs(rs2070699, rs1476046) was a high D' value (99%). Also, rs1762831452 and new671 recorded high D values of 99%. Moreover, SNP rs2070699 and rs1476046 were recorded at a high D value (99%).In addition, SNP new671 and rs1476046 recorded a moderate D' value of 81%. Whereas SNPs (rs1800543 and rs 671) recorded a moderate D' value (76%), another two SNPs (new671 and rs2070699) recorded low D' value of 75%, and two SNPs (rs1476046 and rs1762831452) also recorded low D' value of 74%. Also, SNPs (rs1800543 and rs1762831452);(rs2070699 and rs1762831452) also recorded low D' values (65%) and the lowest one (58%), respectively (Figure 2).

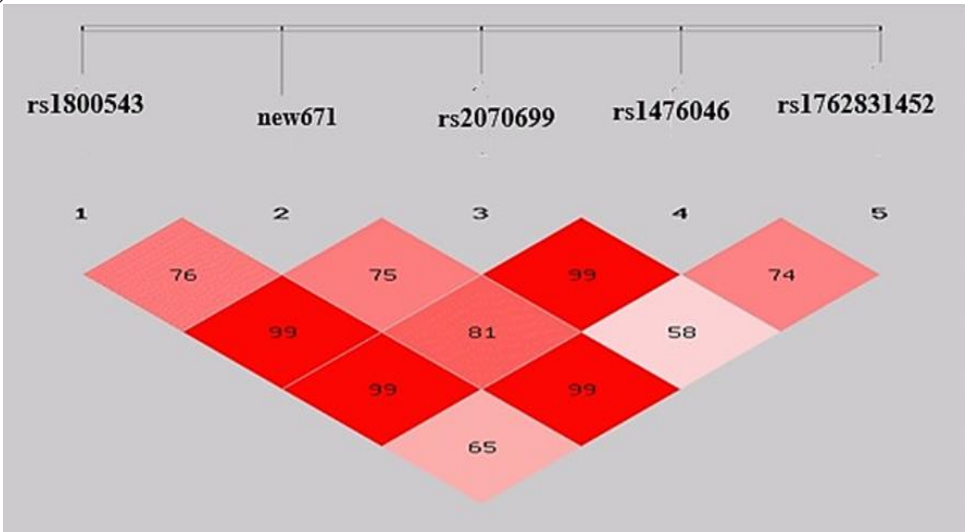

Figure 2: Analysis pairwise of Horizontal Linkage disequilibrium for SNPs (rs1800543;rs2070699; rs1476046; rs1762831452; new671) alleles. Red boxes represent pair-wise SNP correlation (D' value)

Table 18 showed haplotype results for SNPs (rs1800543; rs2070699; rs1476046; rs1762831452; new 671). The results for haplotype CCTAT, TAGGC,TAGGT, TATGC, and TCTAT showed a positive association with DM type II as a risk factor, which makes individuals more susceptible to DM type II except for other haplotypes CATAC, TCGGT that has negatively associated with disease which represent a protective factor against disease. Altogether, as results of linkage disequilibrium and haplotype represent the loci in SNPs (rs1762831452 and new671), (rs2070699 and rs1476046), and (new671 and rs1476046) affect synergistically, their coexistence causes these haplotypes to operate as a risk factor.

Table 18: Haplotype for SNPs (rs1800543;rs2070699;rs1476046;rs1762831452; new 671) alleles

Haplotypes	Frequency		χ^2	Fisher's p-value	Odds Ratio [CI 95%]
	Patients(100*)	Control(100*)			
C A T A C	4 (0.04)	7.00 (0.07)	1.790	0.1	0.35 [0.069~1.740]
C C T A T	18.0 (0.18)	11.00 (0.11)	1.715	0.1	1.86 [0.729~4.730]
T A G G C	43.00 (0.43)	44.00 (0.44)	0.383	0.5	1.23 [0.635~2.397]
T A G G T	9.00 (0.09)	7.00 (0.07)	1.069	0.3	2.09 [0.502~8.729]
T A T G C	5.0 (0.05)	1.00 (0.01)	2.309	0.1	2484.720[126.343~48865.789]
T C G G T	5.0 (0.05)	7.00 (0.07)	0.367	0.5	0.64 [0.147~2.764]
T C T A T	9.00 (0.08)	8.00 (0.08)	0.085	0.7	1.17 [0.378~3.725]

Discussion

This study showed no significant differences in anthropometric characteristics between diabetic patients and control groups except BMI in type II diabetes patients. At the same time, laboratory profile outcomes showed significant differences between patients and control according to HbA1c, FBS, Insulin, and HOMA-IR. So, this study agrees with another study that recorded increased HBA1c in type II diabetes patients [11]. Other studies showed an increased HBA1c and BMI in patients with diabetes [12-13].The current study emphasizes that all these factors are important diagnostic factors for DM2, especially since HbA1c is a critical indicator factor in controlling sugar and differs in different populations and illness statuses [14-15]. The present study agrees with other Iraqi studies, which showed an increased level of FBS and HbA1c in all diabetic patients with a significant difference, which means all of them affected with DM type II, and HBA1c is considered a good indicator to detect diabetes type II [16,17].

The *EDN-1* gene in the current study should be like other genes associated with DM2. Several genes cause DM type II in addition to various environmental agents. Previous research was conducted to determine the genetic factors related to DM type II. Recent SNP detection and protein concentration measurement will assist in understanding DM type II pathogenesis and promote diagnostics and therapy prevention [18,19].

SNP rs1800543 is compatible with HWE, which indicates this SNP is stable within a current sample of the Iraqi population through the evolution process. The homozygote genotype TT for SNP (rs1800543) was recorded a high frequency in patients and control (30+31=61). Consequently, this result makes the TT genotype common in the Iraqi population. Allele C for SNP (rs1800543) increases the risk of susceptibility for diabetics; the TC and CC genotypes may be a risk factor for diabetes. At the same time, TT makes individuals not susceptible to DM type 2.

The SNP rs 2070699 is consistent with H W E. The heterozygote genotype GT for SNP rs2070699 recorded the highest frequency in patients and control (23+30), making the GT genotype common in the Iraqi sample. The homozygote GG (20+13) and homozygote TT (7+7) represent the lowest frequency in the population study. Allele G for SNP (rs2070699) increases the risk of susceptibility for diabetics.

The heterozygote genotype GA for SNP rs1476046 was recorded at a higher frequency in both patients and control (27+23), making the GA genotype the common one in the Iraqi population. The homozygote GG (20+24) and homozygote AA (3+3) represent the lowest frequency in the population study. Allele A for SNP (rs1476046) increases the risk of susceptibility for diabetics.

One study indicated that rs1476046 for the *Endothelin-1*gene represents no risky impact on diabetes nephropathy in Caucasian patients with T2DM [7]. Another study was conducted on Jordanian people to detect the susceptibility of other SNPs, rs5370 and rs2071942, and there were no associated DM type II patients [20]. Another study demonstrated that the *Endothelin-*1gene is a potent candidate as a predisposition locus for the appearance and development of macrovascular and microvascular diabetes complications involving diabetes nephropathy [7, 20]. Therefore, current patients with risky susceptibilities may develop complications of diabetes.

Both SNPs rs1762831452 and new671 divergence from H.W.E which may be due to mutation, migration internal marriage and selection of this SNP. Other factors may involve migration, mutation, and the diverse racial groups [21]. The deviation from H.W.E. for SNP

(rs1762831452,new671) indicated that this locus may be subjected to an evolution selection in the sample studied for the Iraqi population.
Also, it may be the individual selection related to each other because the Iraqi population tends to internal marriage in close families [22].

The heterozygote genotype CT for SNP rs1762831452 recorded high frequency in patients and control (43+47), making the CT genotype common in the Iraqi population. Homozygote CC (7+3) represents the lowest frequency in the population study. At the same time, homozygote TT was not recorded in this study for patients and control. Allele C for SNP (rs1762831452) increases the risk of susceptibility for diabetics.

The heterozygote genotype AC for SNP new 671 recorded a high frequency in patients and control (34+43), may make the AC genotype the common in the Iraqi population. The homozygote AA (16+7)represents the lowest frequency in the population study. Meanwhile, the current study did not record the homozygote CC for patients and control. Allele A for SNP (new671) increases the risk of susceptibility for diabetics. The protective and risk role for the genotypes and the alleles evidenced through the odds ratio that was < 1 indicated that SNP has a protective role in response to the hazards of its incidence or exposure, while OR >1 points to the risk increase [23].
Meanwhile, the outcomes frequency for SNP rs58777731 and rs587777234, AA, and TT genotypes represent the common homozygous genotypes in the Iraqi population. The selection evolutionary process for this locus makes AA and TT genotypes dominant in this study population. Therefore, there is no association risk for diabetes.
At the same time, SNPs rs58777731 and rs 587777234 show the same genotype for all study individuals, which may be because of the natural selection in the evolution process in the Iraqi population. Also, these SNPs in NCBI recorded the dominant T allele for rs 587777234 in the world population, including African, East Asian, African American, Asian, and South Asian. At the same time, an allele for rs58777731 was recorded as dominant in the East Asian population. There are few studies about the association of these SNPs with DM2.

The small size population, mating among genotypically similar individuals, increases the homozygosity of the loci, and the mating between relatives increases the homozygosity of the whole genome [21].
Interestingly, other studies performed on the Chinese population with DM2 showed SNP rs1476046 for the *Endothelin*-1gene was related to diabetes nephropathy [24]. Besides, SNP rs1476046 is related to EDN-1thelial disorder in obese children [25]. Concerning SNP rs1762831452 results, no previous studies of this SNP with diseases exist. Therefore, the current study considers the first study on the relation of this SNP with diabetic type II and that these SNP results indicated an association with DM type II. Allele C recorded OR=1.09, meaning individuals carrying this allele will be more susceptible to diabetic type II. The heterozygote CT of SNPs rs1762831452 represents the common genotype in the current sample(control + patients)from the Iraqi population because it recorded a high frequency.
Regarding the results of SNPs rs2070699 and rs1800543, a previous study associated these SNPs with the level of nitric oxide in female patients with coronary disease [26]. Also, one Iraqi study showed no significant differences for SNP rs2070699 in infertile women [27].
Linkage disequilibrium(LD) results of this study showed strong interactions between rs2070699 and rs1476046 SNPs. Rare mutation events along an ancestral genetic background give rise to most SNP alleles, which are then passed down through the generations. SNPs often occur at tightly spaced sites on a single chromosome. Whether the particular alleles are inherited together and correlated on the population scale, this leads to allow one SNP to be

used to predict the existence of another; consequently, two loci are considered to be in LD. Linkage disequilibrium was first used to refer to shifts in genetic variation within a population. Segments of contiguous chromosomes will be broken apart by recombination events, eventually leading to the linkage equilibrium of alleles and the complete independence of each allele in a population experiencing random mating. A haplotype is the pattern of SNP blocks inherited in blocks on the same chromosome. Blocks with significant LD can differ in length among populations and the genome [28].
Whereas another study studied the association of nephrotic syndrome and (rs1476046)observed no strong association with linkage disequilibrium (56%) between (rs1476046) in children with nephrotic syndrome [29]. When linkage disequilibrium (D' value) was 99%, there were strong associations between these SNPs and diabetes. In future research, it is necessary to relate other SNPs for the *Endothelin*-1gene to diabetes. Moreover, the limitation of this study was restricted to a low sample size, so there is a need to increase the sample size in future studies, which may lead to show significant differences in SNPs studied.

The current study concluded that the SNP rs1800543 associated with DM type II as allele T was a protective factor and C allele risk factor. Moreover, allele G for rs2070699 represents a risk factor, and the T allele was a protective factor. Also, allele A for rs1476046 represents a risk factor, and the G allele was a protective factor. Moreover, allele C for rs1762831452 represents a risk factor, and the T allele is a protective factor. In addition, allele A for new671 represents a risk factor, and the C allele is a protective factor. The linkage disequilibrium emphasized a strong association among SNPs rs1762831452 and new671, recording a high D' value (99%). Moreover, SNP rs2070699 and rs1476046 were recorded with high D' values (99%) in DM type II. The results of linkage disequilibrium and haplotype represent the loci in SNPs (rs1762831452 and new671), (rs2070699 and rs1476046), and (new671 and rs1476046) work synergistically; their coexistence causes these haplotypes to operate as a risk factor. The haplotypes CCTAT, TAGGC,TAGGT, TATGC, and TCTAT represent genetic markers to the susceptibility of DM type II, associated with an increased risk (as a risk factor) in people with diabetes except CATAC, TCGGT haplotypes were associated with decreased risk (as a protective factor) in people with diabetes.

Conclusion:

The present study recorded various distributions in SNPs for the *Endothelin-1gene* in DM type II patients, which may supply data on the impact of the *Endothelin-1*gene on people with diabetes. These results of SNPs for (rs1800543, rs2070699, rs1476046, rs1762831452, and new 671) make individuals more susceptible to diabetes. The linkage disequilibrium results in the current study showed strong interactions between rs2070699 and rs1476046 SNPs. The haplotype TAGGC represents the widespread haplotype with risky effects, making individuals more susceptible. While CATAC and TCGGT represent a protective haplotype may give individuals a protect against DMT2.

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