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Molecular Study to Assess the Relationship or Association of Insulin Like Growth Factor 1 Gene Polymorphism with Type 1 and Type 2 Diabetes Mellitus and Diabetic Nephropathy Patients

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Abstract **Review Article**

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease worldwide. The present study was performed for the Molecular detection of insulin-like growth factor 1 single nucleotide polymorphisms (rs10860860) of patients with type 1 and type 2 diabetes mellitus and diabetic nephropathy in Al-Qadisiyah Province. To achieve this aim, 100 samples were collected, divided into 2 groups: those who are 40 Type 1 diabetes patients (20 T1DM with nephropathy and 20 T1DM without nephropathy); 40 Type II diabetes patients (20 T2DM with nephropathy and 20 T2DM without nephropathy); and healthy controls. The DNA was extracted from whole blood for all specimens. Genotyping was carried out by the polymerase chain reaction-sequencing technique. The digested products were analyzed by agarose gel electrophoresis. The genotypes of insulin-like growth factor 1 showed a significant association ($P < 0.05$) with type 1 and type 2 diabetes mellitus and diabetic nephropathy. Genotypes and allele frequencies related among T1DM with nephropathy and healthy controls, risk analysis revealed that homozygous TT genotype was a significant risk factor (OR=9.75), and heterozygous A/T genotype was also a risk factor significant with an odds ratio of 6.5, which means that patients with a heterozygous TT genotype are approximately ten times more likely to develop the disease than those with other genotypes. Genotype and allele frequencies between T1DM without nephropathy and healthy controls, risk analysis revealed that homozygous TT genotype was a significant risk factor (OR=6.5), and heterozygous A/T genotype was also a significant risk factor with an odds ratio of 7.8, meaning that patients with a heterozygous TT genotype are approximately seven times more likely to develop the disease than those with other genotypes. Whereas genotypes and allele frequencies related to the IGF-1 (rs10860860) SNP among T2DM with nephropathy and healthy controls, risk analysis revealed that homozygous TT genotype was a nonsignificant risk factor (OR = 3.25), heterozygous A/T genotype was also a significant A risk factor with an odds ratio of 4.76, which means that patients with a heterozygous genotype of TT are three times more likely to develop the disease than those with other genotypes. Genotypes and allele frequencies between T2DM without nephropathy and healthy controls, risk analysis revealed that homozygous TT genotype was a significant risk factor (OR=8.66), and heterozygous A/T genotype was also a significant risk factor with an odds ratio of 2.6, meaning that patients with a heterozygous TT genotype are nine times more likely to develop the disease than those with other genotypes. This implies that genetic factors, including the IGF-1 gene polymorphism, may play a role in the development of diabetic nephropathy in individuals with diabetes.

Keywords: Diabetic nephropathy (DN), Diabetes Mellitus, Insulin Like Growth Factor 1 Gene Polymorphism.

INTRODUCTION

Diabetes Mellitus (DM) is one of the most prevalent chronic diseases in the world and constitutes one of the greatest public health challenges of the 21st century (Zimmet *et al*., 2016).

Different classes of diabetes mellitus, type 1, type 2, gestational and other types are compared in terms of diagnostic criteria, etiology and genetics. The Molecular genetics of diabetes has received extensive attention in recent years. One large set of mutations and single nucleotide polymorphisms in genes that play a role in the various steps and pathways involved in glucose

metabolism and in the development, control and function of pancreatic cells have been studied (Kharroubi and Darwish, 2015).

Diabetic nephropathy is a chronic complication of both type 1 DM (beta cell destruction – absolute lack of insulin) and type 2 DM (insulin resistance and/or decreased secretion of insulin) (Vrhovac *et al*., 2008). Genetic factors in the predisposition to diabetic nephropathy have a great influence, otherwise, all patients with diabetes mellitus would develop the disease causing damage to the kidneys. Studies suggest that certain individuals have an inherited susceptibility to this complication, the pathogenesis is complex, due to various factors of metabolic disorders, they cannot confirm for sure which are the primary and secondary changes to kidney disease. The aging of the population and lifestyle habits are the main causes responsible for the increase in diabetes, consequently in nephropathy (Bello *et al*., 2014). T1DM patients are likely to develop DN 20–30% of the time, whereas T2DM patients are likely to develop DN 10–20% of the time (Dorapudi *et al*., 2019).

The IGF-1 gene (Gene ID: 3479) (Yao *et al*., 2018), is found at location 23.2 (12q23.2) on the long arm of chromosome 12 (Zidan *et al*., 2016). Each IGF1 gene in humans appears to be made up of exons and introns (Rotwein, 2017). According to ncbi.nlm.nih.gov/gene/3479, it spans 8496 bp and has seven exons and six introns.

Polymorphisms in the IGF-1 gene have recently been linked to a variety of disorders, including breast cancer, gastric cancer, and coronary artery disease (Wei *et al*., 2018). The SNP rs10860860 is found in IGF1 noncoding sequence (Rydzanicz *et al*., 2011).

Experimental

The samples were taken from 100 person, divided into 2 groups: those who are 40 Type 1 diabetes patients (20 T1DM with nephropathy and 20 T1DM without nephropathy); 40 Type II diabetes patients (20 T2DM with nephropathy and 20 T2DM without nephropathy); and healthy controls. Samples were collected from Al-Diwaniyah Teaching Hospital and Diabetes Center in Al-Qadisiyah Province from March 2022 to December 2022. Five milliliters of venous blood samples were obtained from each person under sterile conditions.

DNA Extraction

Genomic DNA from blood samples for women patients and the control group were extracted by using DNA extraction kit (Favorgen) according to the following:

General Protocol

1. 200 µl from a blood sample was transferred to a micropcentrifuge tube.
2. 20 µl Proteinase K and 200 µl FABG Buffer were added to the sample and the solution was mixed thoroughly by pulse-vortexing.
3. The mix was incubated at 60 C^{o} for 15 minutes to lyse the sample. During incubation the sample was vortexing every 3 - 5 minutes.
4. The tube was briefly spin to remove drops from the inside of the lid.
5. 200 µl ethanol (96%) was added to the sample and mixed thoroughly by pulse-vortexing for 30 seconds.
6. The tube was briefly spin to remove drops from the inside of the lid. FABG Column was placed in a collection tube. The sample mixture (including any precipitate) was transferred carefully to FABG Column. The mixture was centrifuged for 1 minute and discarded the flow-through then was placed FABG Column to a new Collection tube.
7. Immediate, FABG Column was washed with 500 µl W1 buffer by centrifuge for 1 minute, then discarded the flow-through.
8. FABG Column was washed with 750 µl Wash Buffer by centrifuge for 1 minute, then discarded the flow-through.
9. Centrifuge was repeated for an additional 3 minute to dry the column.
10. FABG Column was placed in elution tube.
11. 200 µl of elution buffer was added to the membrane center of FABG Column. FAGB Column was stood for 3 minute.
12. Centrifuge was making for 2 min to elute the DNA.
13. DNA fragment was stored at -20^{o}C in deep freeze.

The reaction mixture

The reaction mixture was prepared from the following materials to get the total size of 20 µl

A. PCR gradient

1) Bioneer Master mix (5 µl)
2) Forward primer (5 µl), Reverse primer (5 µl)
3) DNA template (5 µl)

B. PCR products

1) Bioneer Master mix (5 µl)
2) Forward primer (1.5 µl), Reverse primer (1.5 µl)
3) DNA template (2 µl)
4) Complements the size by nuclease-free water to reach the total volume to 20 µl.

All the components were collected in a special tube called Microcentrifuge tube PCR (20 µl).

Table 1: Master mix components

Item	Concentration
Top DNA polymerase	1 U/ µl
Each: dNTP (dATP, dCTP, dGTP, dTTP)	250 Mm
Tris-HCl (pH 9.0)	10 mM
KCl	30 mM
$MgCl_2$	1.5 mM
Stabilizer and tracking dye	

Table 2: Sequences of primers, PCR product size

Primers	Sequences	PCR Product Size
IGF1 Gene SNP rs10860860	**F: 5' AGGACCTGGCAAATGATG -3'**	**217 bp**
	R: 5'- TATGGCAATTACATATTGGAATG -3'	

Amplification by Polymerase Chain Reaction

Single plex was used in the present study but PCR optimization was done as a first step by using a gradient temperature ranging from 55 C° to 65 C° with 3 step differences in PCR wells. This is highly important to determine the optimum annealing temperature. After the determination of optimum annealing temperature (61.0 ºC) *IGF1* Gene SNP rs10860860, the PCR reaction mixture consisted of 20-50 ng template DNA, 250 mM of each dNTP, 5µl buffer, 1 U Taq DNA polymerase (Biooneer), 20 pmol of each primer and 30 mM $MgCl_2$ in 20 µl of total reaction volume. After determination of the optimum annealing temperature the following program was set in the thermo cycler to amplify the target DNA fragments: (for *IGF1* Gene SNP rs10860860). Amplified DNA fragments were electrophoresed on 2% agaros, (1x) TBE buffer (45 min at 100 V) and the bands visualized after staining with ethidium bromide under UV light. A 100 base-pair ladder were used as a size marker for estimation of fragment sizes.

Thermal reaction Cycling Conditions

Reaction conditions for two primers were listed in table (2.8) for *IGF1* Gene SNP rs10860860.

Table 3: The conditions of the reaction cycle for *IGF1* Gene SNP rs10860860 in thermo cycler

Stage	Temperature C°	Time/min.	Cycles
Initial denaturation	94	5	1
Denaturation	94	1	35
Annealing	61	40	35
Extension	72	1	35
Final extension	72	5	1
Storage	4	∞	

RESULTS AND DISCUSSION

Detection of *IGF-1 (rs10860860)* Polymorphism

The distribution of *IGF-1 (rs10860860)* Polymorphism was detected by sequencing technique. At this locus there are three genotypes; AA, AT and TT. The wild type homozygote genotype were showed only A allele amplification at 417 bp product size. The mutant type homozygote genotype were showed only T allele amplification at 417 bp product size. Whereas, the heterozygote genotype were showed A and T alleles amplification at 417 bp product size respectively, Figure (1). The genotype distribution had no deviation from Hardy-Weinberg equilibrium in all study groups.

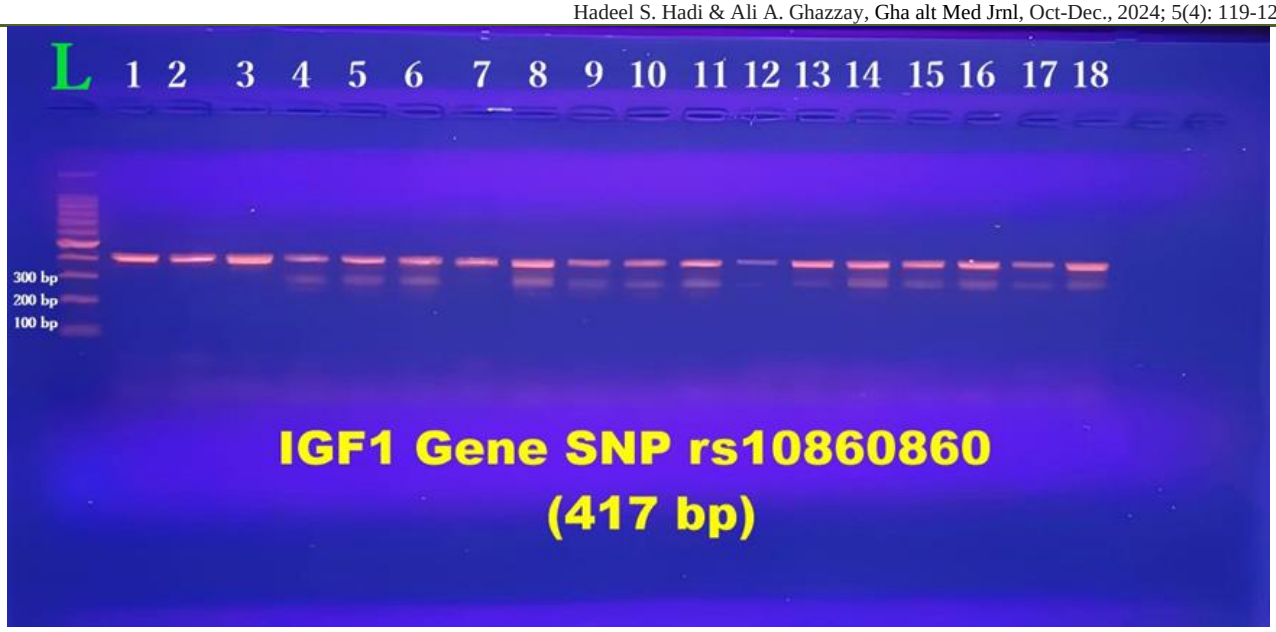

Figure 1: Gel electrophoresis of PCR products for *IGF-1*Gene rs10860860. The *IGF-1*Gene rs10860860 (417 bp) contain lane L is the 1000 bp DNA Ladder, 2% agarose gel in 1X TBE buffer containing 0.5µl ethidium bromide, lane 1,2,3 is represent T1DM with diabetic nephropathy Patients, lane 4,5,6 is represent T2DM with diabetic nephropathy Patients, lane 7,8,9,10,11 is represent T1DM Patients, lane 12,13,14,15,16 is represent T2DM Patients for *IGF-1*Gene and lane 17,18 is control.

Hardy Weinberg equation was applied to *IGF-1 (rs10860860)* genotypes, AA, AT and TT, distribution within the control group and results are shown in table 3.17. The homozygous wild genotype AA was encountered in 13 out of 20 control subjects; the heterozygous AT genotype was seen in 5 out of 20 control subjects and the homozygous mutant TT genotype was seen in 2 out of 20 control subjects, table 1. The observed distribution of control subjects according to *IGF-1 (rs10860860)* genotypes was non-significantly different from the expected one ($P = 0.448$).

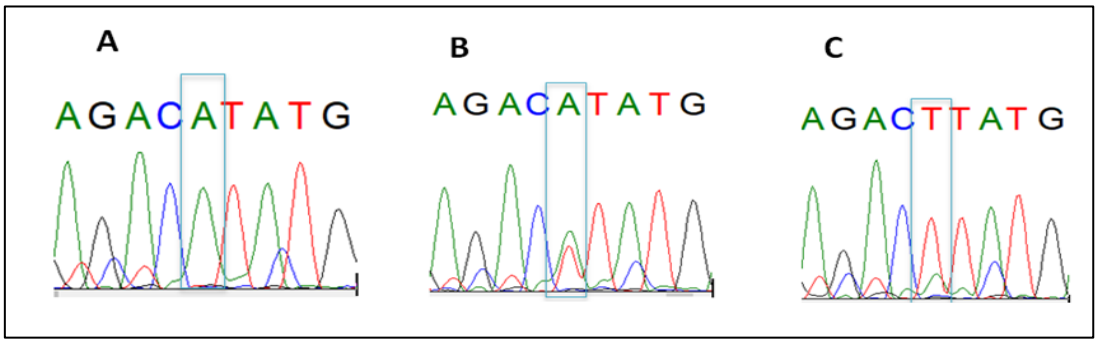

Figure 2: The DNA sequence chromatogram analysis for *rs1080860* by using molecular evolutionary genetic analysis software that showed the A: the wild type homozygote (AA) genotype, B: heterozygote (A/T) genotype and C: mutant type homozygote (TT) genotype

Table 1: Hardy Weinberg equation

Genotypes	Observed	Expected	χ^2	*P*
Homozygote reference AA	13	12.01	1.608	0.448
Heterozygote AT	5	6.97		¥
Homozygote variant TT	2	1.01		NS

¥: Chi-square test; NS: Non-significant at $P < 0.05$

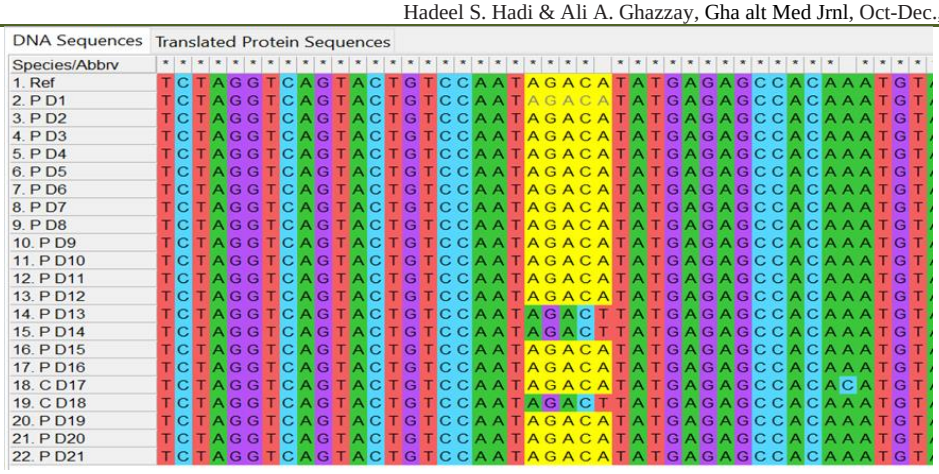

Figure 3: Multiple sequence alignment analysis of *rs1080860* gene in patient and control samples that aligned with NCBI-Genbank reference wild type sequence. The multiple alignment analysis was constructed by using molecular evolutionary genetic analysis software

Genotypic and Alleles Analysis for studied gene in T1DM with nephropathy and healthy control.

The comparison of genotypes and allele frequencies concerning *IGF-1 (rs10860860)* SNP between T1DM with nephropathy and healthy control is shown in table (2). Regarding genotypes mode, there was significant difference in the frequency distribution of genotypes between T1DM with nephropathy and healthy control (p = 0.015). Risk analysis revealed that the homozygous TT genotype was significant risk factor (OR= 9.75), and heterozygous A/T genotype was also significant risk factor with an OR of 6.5, which means that patients with heterozygous TT genotype are approximately Ten time more liable to develop disease in comparison with other genotypes.

Table 2: *IGF-1 (rs10860860)* poly genotype frequency in T1DM with nephropathy and healthy contol

IGF-1 (rs10860860)	T1DM With DN *n* = 20	Control *n* = 20	*P*	OR	95% CI
Genotype frequency					
TT	**6 (30.0%)**	**2 (10.0 %)**	**0.015 ¥ S**	**9.75**	**1.38-68.7**
A/T	**10 (50.0%)**	**5 (25.0 %)**		**6.5**	**1.37 -30.7**
AA	**4 (20.0 %)**	**13 (65.0 %)**		**Reference**	
Allele frequency					
T	**22 (55.0%)**	**9 (22.5 %)**	**0.002 ¥ S**	**4.21**	**1.59-11.1**
A	**18 (45.0 %)**	**31 (77.5 %)**		**Reference**	

Genotypic and Alleles Analysis for studied gene in T1DM without nephropathy and healthy control.

The comparison of genotypes and allele frequencies concerning *IGF-1 (rs10860860)* SNP between T1DM without nephropathy and healthy control is shown in table (3). Regarding genotypes mode, there was significant difference in the frequency distribution of genotypes between T1DM without nephropathy and healthy control (p = 0.016). Risk analysis revealed that the homozygous TT genotype was significant risk factor (OR= 6.5), and heterozygous A/T genotype was also significant risk factor with an OR of 7.8, which means that patients with heterozygous TT genotype are approximately seven time more liable to develop disease in comparison with other genotypes.

Table 3: *IGF-1 (rs10860860)* poly genotype frequency in T1DM without nephropathy and healthy control

IGF-1 (rs10860860)	T1DM Without DN *n* = 20	Control *n* = 20	*P*	OR	95% CI
Genotype frequency					
TT	**4 (20.0%)**	**2 (10.0 %)**	**0.016 ¥ S**	**6.5**	**0.85-49.8**
A/T	**12 (60.0%)**	**5 (25.0 %)**		**7.8**	**1.68 -36.06**
AA	**4 (20.0 %)**	**13 (65.0 %)**		**Reference**	
Allele frequency					
T	**20 (50.0%)**	**9 (22.5 %)**	**0.010 ¥ S**	**3.44**	**1.31-9.05**
A	**20 (50.0 %)**	**31 (77.5 %)**		**Reference**	

Genotypic and Alleles Analysis for studied gene in T2DM with nephropathy and healthy control.

The comparison of genotypes and allele frequencies concerning *IGF-1 (rs10860860)* SNP between T2DM with nephropathy and healthy control is shown in table (4). Regarding genotypes mode, there was non-significant difference in the frequency distribution of genotypes between T2DM with nephropathy and healthy control (p = 0.081). Risk analysis revealed that the homozygous TT genotype was non-significant risk factor (OR= 3.25), and heterozygous A/T genotype was also significant risk factor with an OR of 4.76, which means that patients with heterozygous TT genotype are approximately three time more liable to develop disease in comparison with other genotypes.

Table 4: *IGF-1 (rs10860860)* poly genotype frequency in T2DM with nephropathy and healthy control

IGF-1 *(rs10860860)*	T2DM With DN *n* = 20	Control *n* = 20	*P*	OR	95% CI
Genotype frequency					
TT	**3 (15.0%)**	**2 (10.0 %)**	**0.081 ¥ NS**	**3.25**	**0.42-24.6**
A/T	**11 (55.0%)**	**5 (25.0 %)**		**4.76**	**1.13 -19.9**
AA	**6 (30.0 %)**	**13 (65.0 %)**		**Reference**	
Allele frequency					
T	**19 (55.0%)**	**9 (22.5 %)**	**0.019 ¥ S**	**3.11**	**1.18-8.19**
A	**21 (45.0 %)**	**31 (77.5 %)**		**Reference**	

Genotypic and Alleles Analysis for studied gene in T2DM without nephropathy and healthy control.

The comparison of genotypes and allele frequencies concerning *IGF-1 (rs10860860)* SNP between T2DM without nephropathy and healthy control is shown in Table (5). Regarding genotypes mode, there was significant difference in the frequency distribution of genotypes between T2DM without nephropathy and healthy control (p = 0.044). Risk analysis revealed that the homozygous TT genotype was significant risk factor (OR= 8.66), and heterozygous A/T genotype was also significant risk factor with an OR of 2.6, which means that patients with heterozygous TT genotype are approximately nine time more liable to develop disease in comparison with other genotypes.

Table 5: *IGF-1 (rs10860860)* poly genotype frequency in T2DM without nephropathy and healthy control

IGF-1 (rs10860860)	T2DM Without DN *n* = 20	Control *n* = 20	*P*	OR	95% CI
Genotype frequency					
TT	**8 (40.0%)**	**2 (10.0 %)**	**0.044 ¥ S**	**8.66**	**1.39-53.8**
A/T	**6 (30.0%)**	**5 (25.0 %)**		**2.6**	**0.56 -12.02**
AA	**6(30.0 %)**	**13 (65.0 %)**		**Reference**	
Allele frequency					
T	**22 (55.0%)**	**9 (22.5 %)**	**0.002 ¥ S**	**4.21**	**1.59-11.1**
A	**18 (45.0 %)**	**31 (77.5 %)**		**Reference**	

Hegazi *et al*., found that variant genotypes AT and TT in polymorphism rs10860860 of IGF1 gene were not significantly different in control and DM groups (Hegazi *et al*., 2018). And found AT was associated with OR less than 1 in DN patients, which signifies that it could be significantly protective against DN Hegazi *et al*. also found non-significant variance regarding TT (Hegazi *et al*., 2018).

The current study is similar to other studies that have been investigated. One study conducted in Egypt found an association between genotypes of insulin-like growth factor-1 (IGF-1) and diabetic nephropathy among patients with type 2 diabetes (Hegazi *et al*., 2018). Another study investigated the involvement of IGF-1 gene polymorphism in individuals with type 2 diabetes and diabetic nephropathy. The results suggested that a specific genetic variant of the IGF-1 gene might increase the risk of both type 2 diabetes and diabetic nephropathy (AbdulAemah *et al*., 2021). A third study investigated the association of genetic polymorphisms with diabetic nephropathy and found that polymorphisms in the IGF-1 gene have been associated with the disease (Rizvi *et al*., 2014). Finally, a study conducted in China found that IGF1 gene polymorphisms were associated with diabetic retinopathy risk in the Chinese Han population (Zhang *et al*., 2017). These studies suggest that genetic factors, including IGF-1 gene polymorphisms, may play a role in the development of diabetic nephropathy and other complications in individuals with diabetes.

CONCLUSIONS

This study implies that genetic factors, including the IGF-1 gene polymorphism, may play a role

in the development of diabetic nephropathy in individuals with diabetes.

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