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Molecular Study Of Angiotensin Converting Enzyme And Endothelial Nitric Oxide Gene In Patients With Type 2 Diabetes And Relationship With Nephropathy

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Abstract
This study investigates the molecular association between diabetic nephropathy (DN) and genetic variations in the angiotensin-converting enzyme (ACE) and endothelial nitric oxide synthase (eNOS) genes, both of which are critical in vascular and renal function. A total of 210 individuals were enrolled, including 120 patients with type 2 diabetes mellitus (T2DM) and 90 healthy controls. PCR and DNA sequencing were used to analyze ACE and eNOS gene polymorphisms. The ACE gene showed polymorphic bands at 175 bp and 400 bp, indicating genetic variation among diabetic and nephropathy patients. In contrast, the eNOS gene consistently appeared at 470 bp across all groups, suggesting its stable presence. DNA sequencing confirmed a 95–100% match with reference sequences from NCBI, and two novel eNOS genotypes were identified in Mosul, Iraq. These findings suggest that ACE gene polymorphisms may contribute to DN susceptibility, while eNOS gene stability may reflect its conserved role in endothelial function. The discovery of new eNOS variants highlights the potential for regional genetic diversity. Overall, this study supports the hypothesis that genetic variations in ACE and eNOS may influence DN development and progression, and further research into these markers could enhance early detection and risk assessment in diabetic populations.
Keywords: *Diabetic nephropathy, ACE gene , eNOS gene ,Type 2 diabetes mellitus (T2DM).*

INTRODUCTION

The complex chronic disease known as diabetes mellitus (DM) is typified by hyperglycemia, which may be caused by inadequate insulin secretion, action or both [1]. Evidence from the World Health Organization (WHO) suggests that type 2 diabetes mellitus (T2DM) have the highest prevalence, which represents roughly 90-95% of the cases recorded and damage, dysfunction and degeneration of many organs, blood vessels, kidneys, eyes, nerves, feet and the heart are all associated with chronic diabetes, that the main cause of renal involvement in diabetic patients is diabetic kidney disease (DKD), which is primarily diagnosed clinically [2].Diabetes is associated with long-term microvascular and macrovascular complications [3].Diabetic nephropathy (DN) appears as a severe diabetes complication that results in continuous kidney deterioration alongside organ dysfunction [4]. It is one of the frequent and harmful microvascular complications leading to CKD development because approximately one-third of type 1 diabetics and about 50% of type 2 diabetics will experience diabetic nephropathy. Mechanisms of kidney damage in diabetes can be broadly classified as metabolic, hemodynamic, inflammatory and fibrotic [5]. DN is caused by various variables, including metabolic processes, blood pressure, circulatory dynamics and genetic background [6]. DN is associated with systemic and renal inflammation involving inflammatory cells, cytokines and growth factors [7].The development process combines glomerular hypertension with advanced glycation end products, hyperfiltration and their consequences and the main contributors to diabetic nephropathy progression include poor blood sugar control, together with high blood pressure [8]. Diabetic nephropathy stands as one of the prime causes that leads people to reach end-stage renal disease while simultaneously elevating their risk of mortality [10]. The conversion of angiotensin I to vasoactive angiotensin II requires ACE as the crucial enzyme in the renin-angiotensin-aldosterone system [11]. A total of twenty-six exons, spanning 21 kb on chromosome 17q23.3 comprise the ACE gene [12]. According to recent studies, many cells, mostly those found in the epithelium of particular organs, such the lungs, can express ACE and carry out a variety of biological tasks, primarily those about homeostasis and the immune response [13].

Patients with type 2 diabetes mellitus (T2DM) may be at risk for kidney damage due to variations in the angiotensin I-converting enzyme (ACE) gene; bradykinin is rendered inactive by the vasoconstrictor

angiotensin II [14]. Bradykinin serves as a peptide substance regulating vasodilation and renal function, shows multiple roles in diabetic nephropathy through protection as well as potential harm so higher levels of plasma bradykinin result in increased glomerular size as well as expanded filtration surfaces to protect kidney structure during the early stages of diabetic nephropathy [15].The signaling molecule nitric oxide (NO) is an essential biological component that plays a significant role in various physiological processes within mammalian organisms and this molecule promotes blood vessel dilation, facilitates smooth muscle relaxation, and enables immune responses alongside neurotransmission processes, so NO synthesis occurs through the conversion of L-arginine into L-citrulline by NOS enzymes, which produce NO as a free radical compound [16]. There are three different types of NOS: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS), nNOS is mostly found in the neurological system to facilitate neuronal signaling, whereas eNOS operates in the endothelium to regulate vasodilation and blood pressure [17]. The human eNOS gene is located on chromosome 7q35-36, spanning 21 kilobases and 26 exons [18]. Dysregulation of nitric oxide systems in diabetic patients leads to damaging effects in DN development, this is due to biochemical processes reducing NO availability, leading to oxidative stress, eNOS uncoupling and eNOS activity in DN, causing hemodynamic problems and inflammatory responses [19].

Materials and methods

Study subject

Samples for this study were collected from the Al Wafaa Center for Diabetes and Endocrine Gland Disease and private laboratories in Mosul city. The study involved 210 participants, 120 patients with type 2 diabetes mellitus (64 females and 56 males). Additionally, non-diabetic participants, as a control group (53 females and 37 males). The study was conducted between October 2024 and March 2025 and the specialized physicians evaluated patients.

Collection of a blood sample

Two milliliters of blood were drawn from the venous sites of the participants for DNA extraction and placed in tubes containing EDTA anticoagulant.

DNA extraction

Genomic DNA extraction from fresh blood was done using the kit prepared by Gene Aid according to the company's instructions, by following the procedure [20]**.** Place your blood into a tube with RBC lysis buffer, leave it to settle, then spin in a centrifuge, remove and save the bottom cell pellet after discarding the top clear fluid. After the cell lysis buffer is added, vortex the tube, place it at 60ºC and move the tube in different directions every few minutes with regular (RNase A) addition, remove protein, mix with protein removal buffer, vortex, centrifuge and hold on ice if the pellet is loose. Precipitate your DNA by moving the supernatant, adding isopropanol, spinning in the centrifuge, washing twice with ethanol and drying the DNA pellet in the air. Then, rehydrate your DNA in a solution and incubate it at 60ºC to collect your eluted DNA.

DNA Concentration &Purity

Using the Nanodrop machine, researchers measured how pure and concentrated the DNA extracted from the blood. The surgery simulator uses a BioDrop-prepared 1000 spectrophotometer device**.** The device was zeroed using Tris-EDTA solution and 1 microliter of DNA was added to the device to estimate the concentration [21].

ACE gene amplification and electrophoresis

Extract DNA for polymerase chain reaction (PCR)analysis of the ACE gene using DNA primers that originated from Integrated DNA Technologies (IDT), USA as shown in Table 1. The Thermal Cycler performed three consecutive steps for each DNA sample by first denaturing them at 95 °C for 5 minutes. The amplification proceeded through thirty-five cycles following a 95 °C denaturation step lasting one minute and a 55°C annealing phase of forty-five seconds, as well as a one-minute extension at 72 °C. The experiment was continued by incubating the slides in AT solution for an extra 5 minutes at 72 °C. The generated amplicons went through gel electrophoresis. Upon completion at the reaction cycle, the samples are loaded into the agarose gel wells prepared, including red safe dye then the resulting bands are compared with the size indicator for DNA replication.

eNOS gene amplification and electrophoresis

The eNOS gene polymorphism was detected by polymerase chain reaction. The template DNA [0.5 µg per sample] was amplified using the following primers in Table 1. A total volume of 20 µL contained 0.2 µmol/L dNTPs mixed with 5 µL Cetus buffer (pH 8.3) and 5 µL DMSO alongside 0.5 units of Taq Polymerase (Cetus Perkin Elmer). The process of denaturation during the first stage took 5 minutes at 95 °C. Thirty-five cycles comprising the following steps: 95 °C denaturation for 1 minute, 25 °C annealing for 1 minute, primer extension at 72 °C for 2 minutes and finishing with a 5-minute 72 °C extension to achieve amplification. Then agarose gel analysis of PCR products is performed under ultraviolet light at room temperature.

Table 1: The primer sequences employed to amplify genes

Primer	Function	Size	Sequences 5'-3'	Reference
F	Amplification of ACE	175 bp	5'-CTGGAGACCACTCCCATCCTTTCT-3	[22]
R			5'-GATGTGGCCATCTTCGTCAGAT -3	
F	Amplification of eNOS	470 bp	5'-AGG CCC TAT GGT AGT GCC TTT-3	[23]
R			5'-TCT CTT AGT GCT GTG CTC AC-3	

RESULTS

The results of the ACE gene, as shown in Figure 1, showed that the base pairs appeared at 175bp and 400bp for the control group and one sample of a diabetic patient alone, while the gene appeared at site 175bp in the sample of a diabetic patient and patients with nephropathy without diabetes. In patients with diabetic nephropathy, one sample showed the gene at site 175bp and the other at site 400bp, which is an indication of the occurrence of genetic variation in the gene. The results in Figure 2 showed that the eNOS gene bands were observed at 470 for all samples, including control groups, diabetic patients, nephropathy patients and diabetic nephropathy patients.

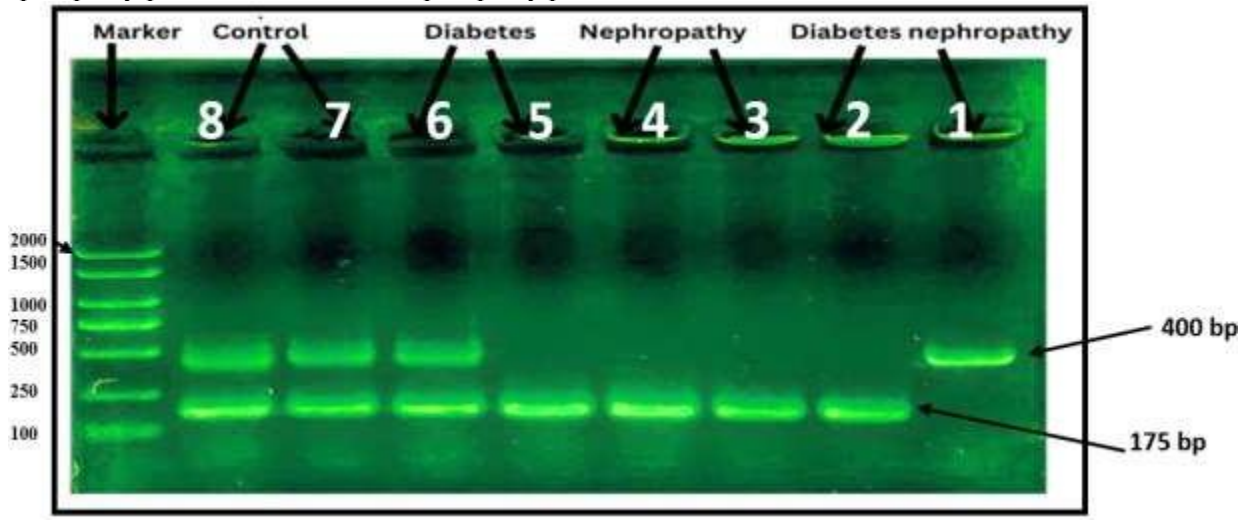

Figure 1: PCR product of DNA analyzed for the ACE gene by agarose gel electrophoresis and the size marker M with a size of 100 base pairs.

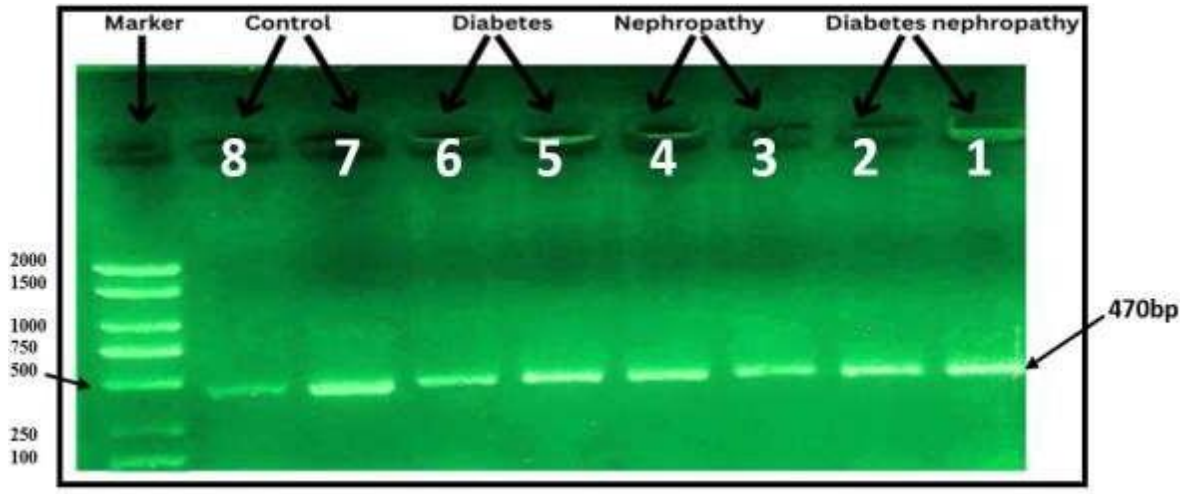

Figure 2: PCR product of DNA analyzed for eNOS gene by agarose gel electrophoresis and the size marker M with a size of 100 base pairs.

DNA Sequencing results

From the results of matching the nucleotide sequences of the ACE and eNOS genes in the samples included in our study, it was found that there was a (95%-100%) match for the ACE gene with the nucleotide sequences on the National Center for Biotechnology Information (NCBI) website, as shown in Figures 3,4 and 5. As well as a (100%) match for the eNOS gene as shown in Figures 6 and 7. The goal of conducting this test was to confirm that the primers used belong to the same genes, as well as to identify new variations for these genes that may affect their activity or contribute to the development of the disease. The study recorded two new sequences of the eNOS gene in the city of Mosul at NCBI, with the identification numbers PV102854.1 and PV102855.1.

Homo sapiens isolate ACE_Id angiotensin converting enzyme (ACE) gene, partial cds
Sequence ID: **KJ140509.1** Length: 480 Number of Matches: 1
Range 1: 394 to 480

Score	Expect	Identities	Gaps	Strand	Frame
135 bits(73)	5e-28()	83/87(95%)	3/87(3%)	Plus/Plus	

```
Query  1    GCTCCCCTTACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  394  GCTCCCCTTACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACC  453

Query  61   AGATCTGACGAA-G--ATGGCCACATC  84
            ||| ||||||||| |  ||||||||||
Sbjct  454  AGAACTGACGAATGTGATGGCCACATC  480
```

Figure [3]: Matching the nucleotides of the ACE gene to the nucleotide sequences of the NCBI site

Homo sapiens isolate ACE_Id angiotensin converting enzyme (ACE) gene, partial cds
Sequence ID: **KJ140509.1** Length: 480 Number of Matches: 1
Range 1: 343 to 480

Score	Expect	Identities	Gaps	Strand	Frame
230 bits(124)	5e-56()	134/138(97%)	3/138(2%)	Plus/Plus	

```
Query  1    GTCACTTTTATGTGGTTTCGCCAATTTTATTCCAGCTCTGAAATTCTCTGAGCTCCCCTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  343  GTCACTTTTATGTGGTTTCGCCAATTTTATTCCAGCTCTGAAATTCTCTGAGCTCCCCTT  402

Query  61   ACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCAGATCTGAC  120
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  403  ACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCAGAACTGAC  462

Query  121  GAA-G--ATGGCCACATC  135
            ||| |  ||||||||||
Sbjct  463  GAATGTGATGGCCACATC  480
```

Figure [4]: Matching the nucleotides of the ACE gene to the nucleotide sequences of the NCBI site

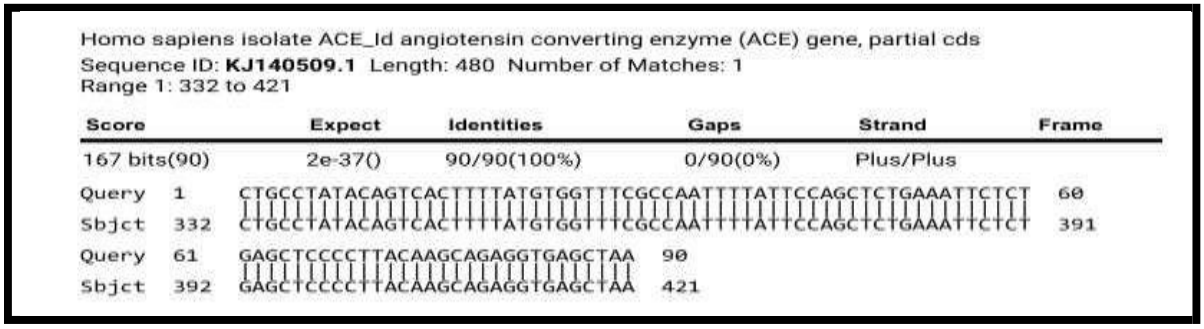

Figure [5]: Matching the nucleotides of the ACE gene to the nucleotide sequences of the NCBI site

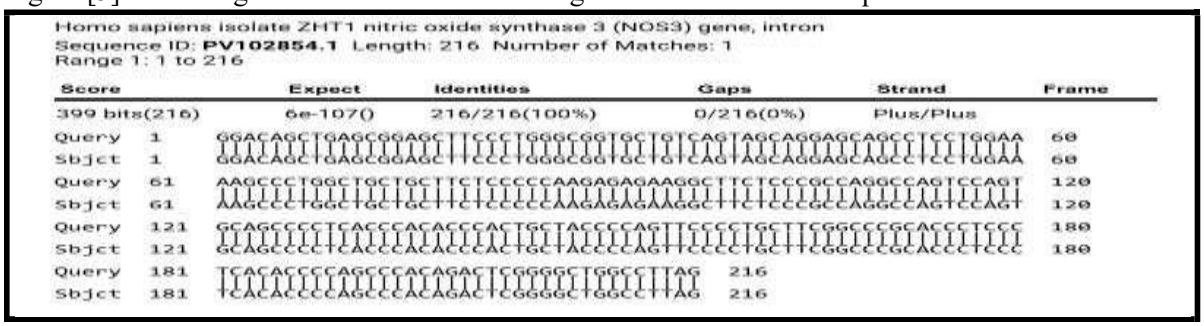

Figure [6]: Matching the nucleotides of the eNOS gene to the nucleotide sequences of the NCBI site

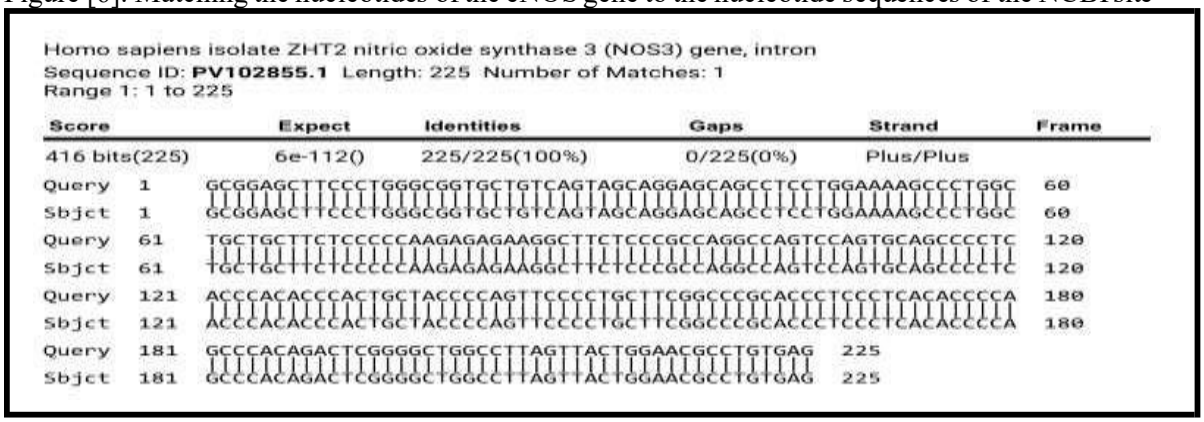

Figure [7]: Matching the nucleotides of the NOS gene to the nucleotide sequences of the NCBI site

DISCUSSION

T2D patients tend to carry the ACE gene because of various genetic and environmental influences and studies have shown that people with a variant of ACE are more likely to develop type 2 diabetes and its related problems, like hypertension and diabetic nephropathy, so the results agree with [24].

End-stage renal disease is now most often caused by diabetic nephropathy, although some diabetics develop serious kidney disease, others survive without it, despite many years with high glucose levels and since not all environmental reasons are clear, scientists have looked elsewhere for explanations and the problem can be traced to the host's genetic background [25] Gene plays a key role in how diabetic nephropathy advances number of studies suggest that genetic variation is an independent cause of diabetic nephropathy and is included in population structure analyses [26].ACE gene functions were found to play a greater role in DN, so those living with type 2 diabetes are more diverse in their cardiovascular risk profile, disease risk and chances of developing chronic kidney disease, accordingly, a relationship between ACE and urinary Albumin excretion has been found in most patients, yet not in all of them, additionally, it should be mentioned that researchers have discovered that the ACE gene plays a role in DN vulnerability type 2 diabetes is especially common among Asian people [27].ACE genes are affected by several other risks, as hyperglycemia is most often caused by alterations in genotype and one of the things

that helps complications develop as DN, so the likely effect of ACE on T2D and DN comes from its presence as a genetic variant, so research has found that the genetic variation causes a continuous high amount of ACE to be produced, so it is harmful to the kidneys in a person with high blood sugar [28].The recording of a new sequence may indicate the presence of mutations that have occurred in this gene and may thus affect its function, as the gene's performance may decrease, indicating its association with kidney disease, so researchers have looked into how the nitric oxide synthase family of genes might influence type 2 diabetes [T2DM] and a rise in eNOS gene expression is connected to diabetes [29]. NOS is important for different bodily functions and changes in its genes might affect the development of diabetes and heart diseases [30] Besides, having more than one NOS variant made people more likely to get T2DM, which is supported by [31]. Endothelial nitric oxide synthase is a critical factor that makes diabetic nephropathy worse through numerous pathways that increase inflammation as well as hypercoagulability [32]. eNOS exists primarily within vascular endothelial cells, where it synthesizes nitric oxide that strongly influences kidney development along with DN disease progression [33]. By lowering NO levels, variations of the eNOS gene are believed to make people more vulnerable to glomerular illness [34].Studies have revealed that when nitric oxide is produced in smaller amounts, it may lead to the formation of DN [35]. Nitric Oxide Synthase is also considered one of the potential candidate genes for diabetic nephropathy susceptibility. the variant of NOS was reported to increase the risk of macroalbuminuria, progression from microalbuminuria to macroalbuminuria, with declining glomerular filtration rate as serum creatinine value rises progressively, culminating in nephropathy [36].

CONCLUSION

It seems that ACE and eNOS genes may be helpful markers for predicting diabetic nephropathy; however, more studies are necessary

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