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# Angiotensin converting enzyme insertion/deletion gene polymorphism and its relation to clinical features and prevalence of diabetic nephropathy in Egyptian population

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**Abstract:** Several reports have addressed the relation between insertion/deletion (I/D) polymorphism of angiotensin converting enzyme (ACE) gene and development of diabetic nephropathy with conflicting results. The aim of this study was to assess whether ACE gene polymorphism could impose a considerable risk for onset of nephropathy among Egyptian population with type 2 diabetes. The study included 30 normal individuals served as control and 90 age-sex matched diabetic patients, who were classified into patients without nephropathy (n=30) and others with nephropathy (n= 60), while the latter was further divided into: micro- and macro-albuminuric patients. All participants were subjected to clinical examination and routine laboratory investigations. The genotypes of ACE were determined by PCR analysis. On comparing the study subjects, frequencies of DD, ID and II genotypes showed no significant changes in all diabetic cases compared to controls (36.7 % vs. 50%, 58.9% vs. 46.7 % and 4.4% vs. 3.3% , p>0.05) respectively. Meanwhile, when all diabetic cases were compared to controls, there was no significant differences in the frequency of D and I alleles (66.1% vs. 73.3% and 33.9% vs. 26.7%) respectively. Thus, indicating no relation between ACE genetic variants and predisposition to diabetic nephropathy in the Egyptian population.
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**Key words:** Type 2 diabetes; Nephropathy; ACE gene polymorphism; Egyptian population.

## 1. Introduction

Diabetic nephropathy (DN) is a common complication among patients with established type 2 diabetes **(Sagoo and Luigi, 2020).** DN is a kidney disease characterized by persistent albuminuria and a decrease in glomerular filtration rate (GFR) with loss of renal function **(Lim ET AL., 2019)**. In late stages, kidney disease can get worse and may progress to kidney failure, also called end-stage renal disease (ESRD), which is a life threating condition **(Cheng et al., 2021)**. It is, in particular, a substantial cause of morbidity and mortality in diabetic patients (Liang et al., 2017). Metabolic disorders, hypertension, and intrarenal haemodynamic abnormalities are all involved in the pathophysiology of DN (El-Wakf et al., 2011). However, there is a strong evidence that genetic susceptibility should also be taken into account **(Fathy et al., 2019).** Despite the large number of studies looking for candidate genes, the renin-angiotensin system (RAS) genes remain the well-characterized genetic factors associated with progression of diabetic kidney disease **(MA, 2016).**

Among RAS components, angiotensin converting enzyme (ACE) is particularly important for modulating vascular tone and electrolyte balance by generating the vasoactive peptide; angiotensin II **(Sharma et al., 2019).**

ACE gene has 26 exons and 25 introns and is located on chromosome 17q23. The 16th intron of ACE gene has shown to contain insertion (I) and deletion (D) polymorphism of 287 bp fragment, resulting in three genotypes; DD, II and ID **(Habibullah et al., 2021).** Specifically, insertion/deletion(I/D) polymorphism of ACE is a genetic predictor of plasma ACE levels and consequently described to be associated with onset and severity of diabetic renal complications **( Da Silva et al., 2021).** Comparable results were found by**El-baz et al. (2018)** who indicated increased risk of DN in different population carrying the D allele (DI

and DD). The same was verified in Japanese individuals with type 2 diabetes **(Solini et al., 2002).** However, inconsistent association of ACE polymorphism with DN was shown in Indian **(Naresh et al., 2009),** Turkish **(Ergen, et al., 2004)** and American **(Zeng et al., 2021)** population, indicating variations in the distribution of ACE genotypes among different ethnic groups.

Given the above results, it was of interest to explore whether ACE gene polymorphism is a detrimental factor in onset and progression of DN among Egyptian population with type 2 diabetes.

## 2. Materials and Methods

### 2.1. Study design

Ninety- type 2 diabetic patients (mean age: 57.47 ± 0.77 year, male/female: 30/60) and 30 non-diabetic healthy volunteers served as controls (mean age: 51.17 ± 1.07 year, male/female: 8/22) were included in this study. All diabetic individuals were classified into 2 groups: individuals without nephropathy, recruited from the Internal Medicine Specialized Hospital , Mansoura University [n=30 & albumin excretion rate (AER) < 30 mg/24-hrs] and others with nephropathy, (n= 60) recruited from Urology & Nephrology Center, Mansoura University, Egypt, who were assigned into: micro-albuminuria **[**n=30 & AER= 30-300 mg/24-hrs**]** and macro-albuminuria patients with renal failure [n=30, AER> 300 mg/24-hrs & serum creatinine levels > 1.3 mg/dl]. Regarding: age (yrs), sex (M/F), weight (Kg), duration of diabetes (yrs) and clinical investigations, the study subjects were exactly matched. Individuals were considered as type 2 diabetic patients based on WHO criteria **(WHO, 2006)** and patients were diagnosed with nephropathy based on the presence of persistent albuminuria > 30 mg in at least two of three consecutive measurements in 24-hrs collected urine samples (**Rossing et al., 2005).** Measurement of blood pressure was determined using sphygmomanometer technique as recommended by **Kirkendall et al. (1980).** This study was approved by the Ethics Committee of Mansoura University and the data were collected after obtaining informed consent from all participating subjects.

### 2.2. Blood analysis

After an overnight fast, two blood samples were taken from each patient. The first (3ml) was collected in an anticoagulant tube containing EDTA solution for glycosylated haemoglobin (HbA1c) measurement, as described by **Trivelli et al. (1971)** and for DNA extraction using DNA extraction and purification kit (Gentra Systems, USA). The second (5ml ) was collected without anticoagulant for determining fasting blood sugar (FBS), total cholesterol (TC), triglycerides (TG), HDL-C and creatinine, using kits supplied by Spinreact Co. Spain. LDL-C was determined in serum, using the equation described by **Friedwald et al. (1972)**.

### 2.3. Urine analysis

Urinary albumin concentration was measured in 24-hrs collected urine samples, as described by **Orsonneau et al. (1989).**

### 2.4. Detection of angiotensin converting enzyme (ACE) genotypes

Polymerase chain reaction (PCR) was used to amplify the polymorphic region of the ACE gene. 300 ng of DNA, 200 mmol/L dNTP, and 2.5 units of Master Mix Taq DNA polymerase (Amplitaq Gold Perkin-Elmer Cetus, Norwalk, Conn), and 500 mmol/L of [5′ - CTG GAG ACC ACT CCC ATC CTT TCT -3′(forward) and 5′ - GAT GTG GCC ATC ACA TTC GTC AGA-3′(reverse)] were used in each PCR reaction . After initial DNA denaturation for 1 min at 94°C, amplification was performed in 30 cycles consisting of 30 secs at 94° C, followed by 30 secs of annealing at 58°C, and 72° C extension for 1 min, where the reaction was completed with an extension for 8 min at 72°C. On a 2% agarose gel, theamplified products were separated, stained with ethidium bromide and visualized under UV light. Because the D allele in heterozygous, sample is preferentially amplified **(Golmohamadi et al., 2006),e**ach sample had a second independent PCR amplification of the DD genotype using a primer pair [5′ - TCG GAC CAC AGC GCC CGC CAC TAC- 3′(forward) and 5′ - TCG CCA GCC CTC CCA TGC CCA TAA - 3′ (reverse)] with the exception of a 67°C annealing temperature, identifies an insertion specific sequence under identical PCR circumstances.Only in the presence of an I allele, the reaction givesa 335– bp amplicon, with no product in homozygous DD samples.

### 2.5. Statistical analysis

The SPSS statistical computer package (version 10) was used to analyze all of the data **(Snedecor and Cochran, 1980)**. The qualitative variables were reported as numbers and percentages, whereas the quantitative data were expressed as means and standard deviations (SD). Chi-square test ($\chi^2$) was used to compare qualitative data, while One Way ANOVA test was used to compare quantitative data. Odds ratio (OR) and 95% confidence interval (95% CI) were used to assess genetic risk factors by comparing the frequency of patients and controls. Statistical significance was set at $P<0.05$.

## 3. Results

### 3.1. Clinical features and biochemical characteristics of the study subjects

Clinical features and biochemical characteristics of the study subjects are presented in Table 1. The prevalence of clinical characteristics [age, gender, weight, diabetes duration and blood pressure (SBP & DBP)] did not vary significantly among diabetic patients and healthy controls according to ACE genotypes. The same was exhibited als with the measured biochemical parameters (FBS, HbA1c%, HDL-C, LDL-C, creatinine, urinary albumin).

**Table 1.** Clinical features and biochemical characteristics according to ACE genotypes in the study subjects

| Variables | DD | ID | II | P |
|---|---|---|---|---|
| Age (years) | 58.12±1.32 | 57.23±0.98 | 55.25± 4.68 | 0.71 |
| Sex (M/F) | 10/23 | 19/34 | 1/3 | 0.81 |
| Weight (kg) | 93.61±2.14 | 94.15±1.94 | 107.25± 9.22 | 0.17 |
| Duration (years) | 12.55±0.90 | 12.81±1.06 | 11.00± 3.29 | 0.87 |
| SBP (mmHg) | 149.39±5.04 | 160.38±3.63 | 147.50± 18.87 | 0.18 |
| DBP (mmHg) | 88.48±2.79 | 88.49±2.72 | 93.75± 12.48 | 0.86 |
| FBS (mg/dl) | 182.58±12.72 | 170.75±6.83 | 247.80± 53.19 | 0.06 |
| $HbA_{1c}$ % | 7.63±0.30 | 7.34±0.24 | 8.00 ± 1.03 | 0.63 |
| T-cholesterol (mg/dl) | 210.36±9.29 | 220.21±11.47 | 200.50± 16.15 | 0.76 |
| Triglyceride (mg/dl) | 162.48±16.51 | 151.08±11.24 | 141.25± 19.41 | 0.79 |
| HDL-C(mg/dl) | 39.24±3.07 | 40.13±2.29 | 33.75± 5.56 | 0.76 |
| LDL-C(mg/dl) | 138.62±7.57 | 149.86±10.85 | 138.50± 13.02 | 0.73 |
| Creatinine (mg/dl) | 1.92±0.18 | 2.11±0.33 | 2.58± 1.34 | 0.82 |
| Urinary albumin (mg/24h) | 279.91±40.18 | 254.29±40.27 | 325.00± 13.55 | 0.83 |

FBS= fasting blood sugar; HbA1c%= glycosylated hemoglobin; HDL-C= high density lipoprotein-cholesterol; LDL-C= low density lipoprotein-cholesterol; SBP= systolic blood pressure; DBP= diastolic blood pressure.

### 3.2. Distribution of ACE genotype and allele frequencies of the study subjects

Distribution of ACE genotypes and alleles among diabetic patients and healthy controls are illustrated in Tables 2 & 3. By comparing the studied subjects, frequencies of DD, ID and II genotypes showed no significant changes in all diabetic cases compared to controls (36.7 % vs. 50%, 58.9% vs. 46.7 % and 4.4% vs. 3.3%, $P>0.05$) respectively. Furthermore, there was no significant changes in the frequency of D and I alleles between diabetes patients and control subjects (66.1 % vs. 73.3 % and 33.9 % vs. 26.7%), respectively.

On comparing each of diabetic group relative to healthy control subjects, regarding their ACE gene polymorphism, no significant differences were recorded, where the frequencies of (DD, ID, II) genotypes and both (D , I alleles) were (23.3%, 73.4%, 3.3%), (60%, 40%) in diabetic patients with normo-albuminuria, and those with micro- albuminuria, (46.7%, 50% , 3.3%), (71.7%, 28.3%)
and macro-albuminuria (40%, 53.3%, 6.7%), 66.7%, 33.3%), respectively.

**Table 2.** Distribution of angiotensin converting enzyme (ACE) genotype and allele frequencies in the study subjects

| Groups | Control(n=30) | Diabetic cases (n=90) | | |
|---|---|---|---|---|
| | | DM with NOAB (n=30) | DM with MIAB (n=30) | DM with MAAB (n=30) |
| **Genotypes** | n (%) | n (%) | n (%) | n (%) |
| **DD** | 15(50) | 7(23.3) | 14(46.7) | 12(40) |
| **ID** | 14(46.7) | 22(73.4) | 15(50) | 16(53.3) |
| **II** | 1(3.3) | 1(3.3) | 1(3.3) | 2(6.7) |
| **Alleles** | n(%) | n(%) | n(%) | n(%) |
| **D** | 44(73.3) | 36(60) | 43(71.7) | 40(66.7) |
| I | 16(26.7) | 24(40) | 17(28.3) | 20 (33.3) |

n: number of cases; % = percentage of cases; I = insertion; D = deletion; DM= diabetes mellitus; NOAB= normo-albuminuria; MIAB= micro-albuminuria, MAAB= macro-albuminuria.

### 3.3. Odds ratio and 95% confidence interval in ACE polymorphism of the study subjects

From ACE gene polymorphism distribution and odds ratio, obtained results (Table 3**)** showed no significant changes, regarding (DD, ID and II) genotypes and (D and I) alleles frequencies in diabetic patients with micro- (46.7%, 50%, 3.3%, 71.7%, 28.3%) and macro-albuminuria (40%, 53.3%,6.7%, 66.7% , 33.3%) respectively, if compared to those with normo-albuminuria [23.3%, 73.4%, 3.3%, 60% and 40%]. Also, on comparing diabetic patients with macro-[40% (DD), 53.3% (ID), 6.7% (II), 66.7% (D) and 33.3% (I)] to diabetic patients with micro-albuminuria [46.7% (DD), 50% (ID), 3.3% (II) 71.7% (D) and 28.3% (I)], respectively.

### 3.4. PCR amplification for polymorphic region of ACE gene of the study subjects

On evaluating ACE gene polymorphism, DD homozygous genotype for D allele was identified by the presence of a single 190 bp product and ID heterozygous genotype was identified by the presence of both 190 bp and 490(Figure 1). The II homozygous genotype for I allele was identified by the presence of a single 490 bp product. The insertionallele can be missed in heterozygotes, thus resulting in mistyping as DD genotype. For this reason, insertion specific primers should be used to discriminate between DD and ID genotypes. The absence of 335 bp product confirms the presence of DD genotypes. Heterozygous individuals (ID genotype) were confirmed by the presence of 335 bp and 190 bp products (Figure 2).

**Table 3. Genotype and alleles distribution of ACE gene (insertion /deletion) polymorphism, Odds ratio (OR) and their 95% confidence interval (CI) in the study subjects**

| Groups | All Diabetic cases vs. CON | DM with NOAB vs CON | DM with MIAB vs. CON | DM with MAAB vs. CON | DM with MIAB vs. DM with NOAB | DM with MAAB vs. DM with NOAB | DM with MAAB vs. DM with MIAB |
|---|---|---|---|---|---|---|---|
| | OR (95%CI) | OR (95% CI) | OR (95% CI) | OR (95% CI) | OR (95% CI) | OR (95% CI) | OR (95% CI) |
| **Genotypes** | | | | | | | |
| DD | 0.58 (0.25-1.33) | 0.30 (0.10-0.92) | 0.88 (0.32-2.41) | 0.67 (0.24-1.85) | 2.88 (0.95-8.72) | 2.19 (0.72-6.70) | 0.76 (0.27-2.12) |
| ID | 1.64 (0.71-3.76) | 3.14 (1.07-9.27) | 1.14 (0.41-3.15) | 1.30 (0.47-3.60) | 0.36 (0.12-1.07) | 0.42 (0.14-1.23) | 1.14 (0.41-3.15) |
| II | 1.35 (0.14-12.57) | 1.00 (0.06-16.78) | 1.00 (0.06-16.78) | 2.07 (0.18-24.16) | 1.00 (0.06-16.78 | 2.07 (0.18-24.16) | 2.07 (0.18-24.16) |
| **Alleles** | | | | | | | |
| D | 0.71 (0.37-1.36) | 0.55 (0.25-1.18) | 0.92 (0.41-2.05) | 0.73 (0.33-1.59) | 1.67 (0.79-3.62) | 1.33 (0.63-2.80) | 0.79 (0.36-1.72) |
| I | 1.41 (0.74-2.70) | 1.83 (0.85-3.96) | 1.09 (0.49-2.42) | 1.38 (0.63-3.01) | 0.59 (0.28-1.27) | 0.75 (0.36-1.58) | 1.27 (0.58-2.75) |

OR= Odds ratio; 95% CI= 95% confidence interval; I = insertion; D = deletion; DM= diabetes mellitus; NOAB= normo-albuminuria; MIAB= micro-albuminuria, MAAB= macro-albuminuria.

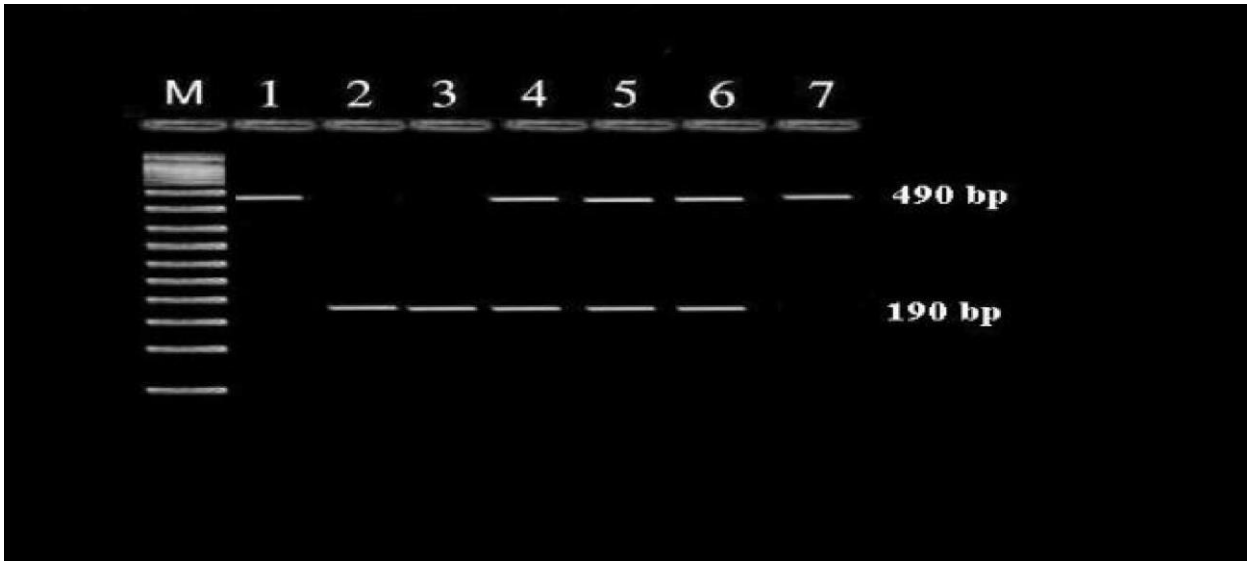


**Figure** 1. Shows initial amplification for ACE I/D polymorphism. Lane M is a marker (50 bp DNA ladder). Lanes 2 and 3 are DD homozygous genotypes for D allele, identified by the presence of a single 190 bp product. Lanes 4, 5 and 6 are ID genotypes, identified by the presence of both 190 bp and 490 bp products, and the II homozygous genotype for I allele, identified by the presence of a single 490 bp product (lanes 1 and 7). To avoid mistyping of ID heterozygotes as DD homozygotes, the DD homozygotes were validated by inserting a specific primer pair.

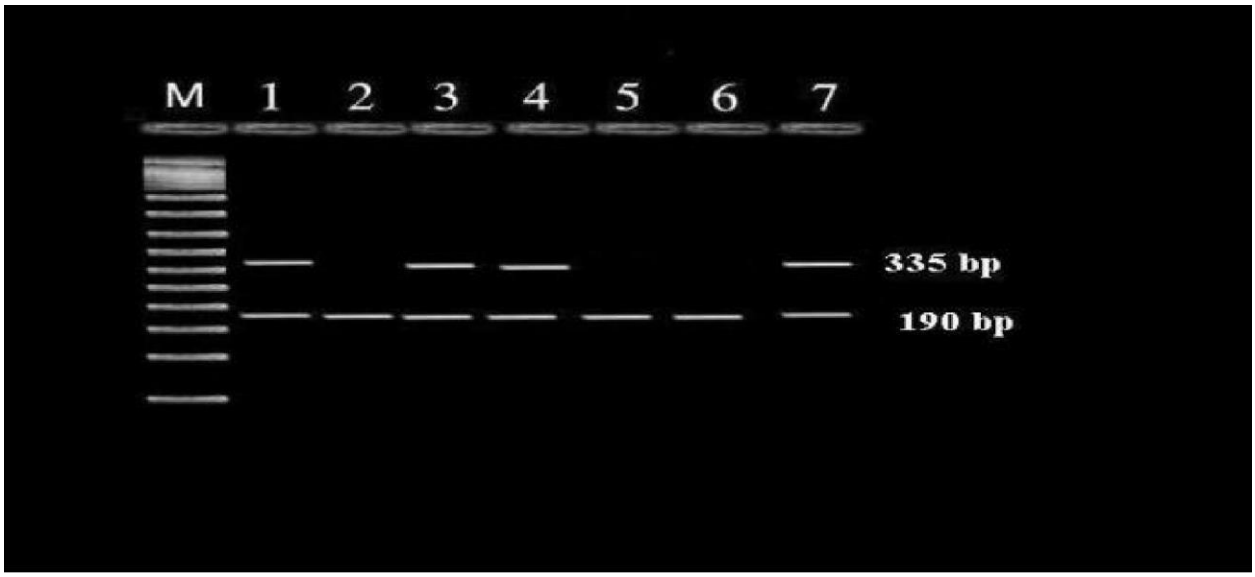


**Figure 2.** Shows PCR initial amplification for DD homozygous individuals by using insertion- specific primers. Lane M represents the 50 bp ladder. Lanes 2, 5 and 6 are DD genotypes (identified by the presence of a single 190 bp product and no presence of a single 335 bp product). Lanes 1, 3, 4 and 7 are ID genotypes (identified by the presence of both 335 bp and 190 bp products).

## 4. Discussion

Numerous reports demonstrated that genetic variations are relevant to increased susceptibility to diabetic nephropathy (DN) **(Hameed et al., 2018).**

In particular, the ACE (I/D) gene polymorphism has been linked to an increased risk for onset of DN in a variety of population **(Alharbi, 2017).** Consequent studies determined the major role of D-allele in this disease **(Maestroni and Zerbini, 2020**). The production of more angiotensin II, a vasoactive peptide, may be a proposed mechanism by which the D allele affects DN. Angiotensin II causes an increase in intraglomerular pressure and glomerular filtration rate in diabetic kidneys **(Bahreini et al., 2021).** Angiotensin II also increases the release of glomerulosclerosis-related cytokines such as, fibronectin, osteopontin, transforming growth factor and platelet-derived growth factor, all of which contribute to ESRD **(Thaha, and Widiana, 2019)**. Results from related studies indicated an association between the D- allele (ID and DD) of the ACE gene and the occurrence of DN in patients from South India, **(Wyawahare et al., 2017).** Another study demonstrated an association between the D- allele and DN in Caucasian and Asian diabetic patients **(Mahwish et al., 2020).** Two subsequent studies also showed a comparable association in the Japanese and Bangladesh diabetic subjects with nephropathy **(Ferdous et al., 2021; Shawon et al., 2021).** This is further confirmed by a study demonstrating significantly higher D-allele expression compared with the I allele among type 2 diabetic patients with nephropathy, where genotype frequencies of DD, ID, and II were 36%, 44%, and 20%, respectively **(El-baz et al., 2018),** thus indicating higher risk of DN in carriers of the D- allele than the II-genotype patients.

Although the major findings regarding the relation between ACE (DD) genotypes and DN, several studies failed to establish this relation **(Mahwish et al., 2020).** To obtain further evidence, the present study was carried out to examine the pattern of ACE genotypes distribution in Egyptian type 2 diabetic individuals with and without nephropathy. Firstly, the clinical and biochemical characteristics were determined in all the study subjects. Obtained results indicated that the clinical characters [age, gender, weight, diabetes duration, and blood pressure (SBP & DBP)] did not vary significantly in all diabetic subjects according to ACE genotypes. The same was also exhibited with respect to the measured biochemical parameters (FBS, HbA1c%, TC, TG, HDL-C, LDL-C, creatinine and urinary albumin), indicating no association between these measured variables and ACEgenetic variants in all the study subjects.

Further insights into the pathogenesis of DN may emerge from analysis of ACE gene polymorphism in the studied population, where the frequencies of DD, ID, and II genotypes showed no significant changes in all diabetic cases compared to controls (36.7 % vs.50%, 58.9 % vs. 46.7 % and 4.4% vs. 3.3%, $p > 0.05$) respectively. Furthermore, when all diabetes cases were compared to control subjects, there was no significant changes in the frequency of D- and I- alleles (66.1% vs. 73.3% and 33.9% vs. 26.7%), respectively. Thus, indicating lack of evidence for association between D-allele and development of renal complications in the studied Egyptian diabetic patients. As in agreement, **Javapalan et al. (2010)** showed no association between kidney dysfunction and polymorphism in the ACE (I/D) gene in Malaysians with type 2 diabetes. Over 1000 diabetic patients with nephropathy were studied in other research have also shown no association between nephropathy and the D allele **(Momin et al., 2019).** Additional study was carried out on diabetic patients from Iran's Kermanshah Province having a Kurdish ethnic background. In this study, the frequency of D allele showed nonsignificant increase (69.1%) in diabetic patients with nephropathy compared to patients without nephropathy (58.3%) (P=0.061). Although the presence of the DD genotype was linked to a higher risk of nephropathy, the association was not statistically significant (P=0.057). As a result, this polymorphism may not be strongly linked to the development of DN in Iranians of Kurdish ethnicity **(Lakkakula et al., 2019).** Similarly, no significant correlation between ACE gene polymorphism and development of nephropathy has been recorded between Turkish **(Kılıç et al., 2021)** and Chinese **(Miri et al., 2021)** patients with type 2 diabetes.

## 5.Conclusion

According to the above findings, it can demonstrate that ACE (I/D) polymorphism is a risk factor for diabetic nephropathy in a variety of population, but failed to exhibit a positive influence in other individuals, suggesting that ethnicity should be greatly considered. It is therefore, not surprising that in the present study the distribution of ACE genotypes or allelic frequencies did not differ significantly between controls and diabetic patients (with or without nephropathy). Also, no significant differences were displayed between diabetic patients with micro- and macro-albuminuria. So, a role for ACE gene polymorphism, as a genetic candidate in initiation and development of nephropathy cannot be ruled out in the studied Egyptian patients with type 2 diabetes.

## Conflict of interest

All authors declare no conflict ofinterest.

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