Original Research

Association of Angiotensin Converting Enzyme Gene Insertion/Deletion Polymorphism with Type 2 Diabetic Nephropathy in Gaza Strip-Palestine

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ABSTRACT

Introduction: The insertion/deletion (*I/D*) polymorphism of 287 bp *Alu* repeat sequence in intron 16 of Angiotensin Converting Enzyme (*ACE*) gene resulting in three genotypes *I/D*, *D/D* and *I/I*. *ACE* gene expression is associated with ACE levels in cells and in the plasma. It indicated that the polymorphism may modulate the expression of the *ACE* gene. The *D/D* genotype is believed to confer deleterious effect to many pathogenesis, also, it might be a cause-effect for type 2 diabetic nephropathy (T2DN). In this study, we evaluated the frequency of the different genotypes of *ACE* gene and investigated if there is an association between *ACE* gene polymorphism and T2DN by comparing the genotypes results of T2DN patients to healthy control, Type 2 diabetes mellitus (T2DM) and nephropathy patients in Gaza Strip. **Methodology**: The study included 170 subjects, consisting of 43 T2DM patients undergoing dialysis "T2DN" compared to 41 T2DM patients who were not undergoing dialysis, 43 patients undergoing dialysis without T2DM and 43 healthy individuals. Blood samples were collected in EDTA tubes for DNA extraction. Polymerase Chain Reaction (PCR) was used to detect *ACE* gene polymorphism. All subjects were asked to fill the questionnaire interview. **Results and Conclusion**: The initial results showed that there is no statistically significant association between *ACE* genotypes and T2DN, also between the T2DM and nephropathy when compared to the healthy control ($p > 0.05$). The (*D/D*) genotype was the most frequent in all study groups. Moreover, no association was observed between *ACE* genotypes and gender, diabetic nephropathy, nephropathy, diabetes, hypertension, obesity and various other diabetes complications.

Key words: Angiotensin Converting Enzyme (ACE) gene, Insertion/deletion (I/D) polymorphism, Polymerase Chain Reaction (PCR), Type 2 diabetic nephropathy (T2DN)

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is metabolic disease which can arise from different causes and characterized by insulin resistance in peripheral tissues beside to impaired secretion of insulin from pancreatic β-cells[1]. The estimated global burden of T2DM is 438 million by 2030 which was 285 million people in 2010, this indicates that an increase of 65% will happen[2]. T2DM complexity can be related to many factors which include the heterogeneity, interactions between genes and environment role[3]. T2DM, hypertension (HTN) and obesity along with dyslipidemia is termed metabolic syndrome, which is prevalent in the populations of modernized nations[4]. The genetic susceptibility of T2DM has been found to be modulated by obesity status. Insulin resistance-related genetic variants were only associated with T2DM in the obese population, while insulin secretory variants conferred a greater T2DM risk only in non-obese individuals, suggesting a potential interaction of these variants with obesity status in T2DM incidence[5].

Type 2 Diabetic Nephropathy (T2DN) is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. Microangiopathy is the earliest detectable change in the course of T2DN. The development of T2DN is characterized by glomerular hyperfiltration and an increased albumin excretion rate[6]. The amount of albumin lost in the urine has important clinical connotations, where excretion of amounts in excess of 300 mg in 24h is termed macroalbuminuria and excretion of lesser amounts of albumin, between 30 and 300 mg in 24 h, is termed microalbuminuria[7]. T2DN affects approximately one-third of diabetic patients, and it is the leading cause of end-stage renal disease (ESRD) which require dialysis or transplantation in developed countries[8], as well as in rapidly developing countries in Asia[9]. One-third of T2DM patients with excellent blood glucose control can develop to T2DN, while in most patients, even

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with anti-hypertensive therapy and suboptimal blood glucose control, T2DN is not noted. Therefore, genetic susceptibility to diabetic nephropathy has been proposed [10,11].

ACE gene is mapped on chromosome 17q23 and consists of 26 exons and 25 introns [12]. Insertion/deletion of *Alu* repeat sequence in introns 16 of the *ACE* gene leading to three genotypes "*I/I, I/D* and *D/D* genotypes" [13]. These genotypes showed different plasma expression, whereas the *D/D* genotype showed the highest plasma expression and *I/I* genotype showed the lowest plasma expression. Also, its believed that these genotypes can contribute to deleterious or protective effects. *D/D* genotype could contributes to deleterious effects on various disease pathgenetic mechanisms. On the other hand, *I/I* genotype is thought to has protective benefits [14]. Therefore, *ACEI/D* gene polymorphism has been suggested to be used to make a decision about treatment regimens in antihypertensive patients [15].

Many researchers tried to explain the association of *ACEI/D* polymorphism with T2DM risk and its related renal and cardiovascular complications in different ethnic groups, but with highly inconsistent findings. *D* allele was found to be more common in T2DM and related complications in the Tunisian [16], Indian [17] and Iranian populations [18], while others studied demonstrated no association of either allele with T2DM or related cardiovascular disease (CVD) and renal disease in Malaysia and Indonesia [19]. These differences are mostly due to multifactorial or polygenic disorders, as evidenced by the varying disease outcomes modulated by the gene-gene or gene-environment interactions [20]. Other researchers examined the response of drugs in T2DM patients and compared it through *ACE* different genotypes, *D/D* genotype patients showed less response of drugs than patients have *I/I* allele of the *ACE* gene. It seems that diabetes-associated kidney disease risk is magnified by inheriting risk alleles at several susceptibility loci [21].

METHODOLOGY

Sampling

EDTA whole blood samples were collected from 170 subjects, where 43 T2DN patients were the case group that compared to three control groups. The control groups were 43 nephropathy " undergoing dialysis" patients, 41 T2DM patients were been collected from health care centers and hospitals in the Gaza Strip and 43 healthy individuals. Self-administered questionnaires were completed by the participants. DNA extraction was performed using a Promega kit and GeneAll Kit for human DNA isolation.

PCR amplification of *ACE* gene

The sequence of the Primers for *ACE* gene used in this study was as reported [13]. PCR reaction was used to amplify the genomic DNA fragments on the intron 16 of *ACE* gene. About 150ng of extracted DNA (3 μL) was added to 7 μL master mix and 0.5 μL of each primer where all were placed in a 0.2 ml thin-walled micro-centrifuge PCR tube. The tubes were centrifuged and placed in a thermal cycler. PCR thermal cycles were performed as follow: 1): denaturation for 1 minute at 95 C°. 2): 36 cycles of melting for 15 seconds at 95 C°, Annealing for 15 seconds at 59 C°, extension for 10 seconds at 72 C°. 3): final elongation for 10 min at 72°C. PCR product was analyzed on 2% ethidium bromide-stained agarose gel electrophoresis to determine the polymorphism of the gene [22].

The amplicon generated from *ACE* gene should yield a 490 bp with *I/I* genotype and 190 bp with *D/D* genotype. Nuclease-free water (instead of the DNA template) was used as a negative control. Amplicon (PCR product) size was estimated by comparing it with DNA marker (DNA ladder 50 bp) run on the same gel.

Data Analysis

Statistical Package for Social Sciences (SPSS) program, version (20.0), was used to analyze data. Independent Samples T-test and Chi-square test were used. *P*-values < 0.05 were considered statistically significant.

RESULTS

General characteristics of study population

The study was conducted by using 170 individuals, where 43 T2DN patients (mean age 60.60±8.25) were the case group that compared to three control groups. The control groups were 43 nephropathy "undergoing dialysis" patients (mean age 58.73±11.54), 41 T2DM patients (mean age 58.73±11.54) were been collected from health care centers and hospitals in the Gaza Strip and 43 healthy individuals (mean age 49.44±7.33). In total, 80.0% of the study population were non-smokers, while 20.0% were smokers. Moreover, 48.8% of the participants were males, while about 51.2% were females. 44.1% of subjects were non-hypertensive, while 55.9% of them were hypertensive. 50.6% of the participants were non-diabetic, while 49.4% were diabetic. All of the healthy population were non-hypertensive and non-diabetic.

Table 1: Demographic characteristics of T2DN, T2DM, Nephropathy patients and healthy subjects in the studied population

	T2DN		Healthy		Nephropathy		T2DM	
	N0	%	N0	%	N0	%	N0	%
No. of participant	43	100	43	100	43	100	41	100
Male	24	55.8	22	51.2	13	30.2	24	58.5
Female	19	44.2	21	48.8	30	69.8	17	41.5
Age (mean)	60.60±8.25		49.44±7.33		58.73±11.54		58.73±11.54	

PCR Results

PCR amplification products

Once the amplicons were obtained, they were introduced to 2% agarose gel electrophoresis with ethidium bromide and the bands were visualized under UV light. With the help of a DNA ladder, D allele and *I* allele were identified at 190 and 490 bp fragments respectively.

ACE Genotypes

ACE genotypes distribution in all study population

ACE geneD/D genotype was the most frequent genotype (shown in **Figure 2**).

Distribution of ACE genotypes in study subjects

ACE genotypes distribution in all subjects was as shown in **Table 2.** No statistical significant difference was found in *ACE* genotypes among study subjects ($p > 0.05$).

ACE gene allele frequency in all study subjects

Our study showed that the *D* allele was the most frequent among the studied groups as shown in **Table 3.**

The relationship between ACE genotype and gender in all study subjects

The initial results showed that there was no statistically significant relationship between *ACE* genotypes and gender ($p > 0.05$), as shown in **Table 4.**

The relationship between ACE genotypes with diabetes duration in the diabetic population

The initial data in **Table 5** showed that there is no statistically significant association between the distribution of *ACE* genotypes and diabetes duration, where ($p > 0.05$).

The relationship between ACE genotypes with HTN and obesity state

The initial results showed that there was no statistically significant association between *ACE* genotypes with HTN and obesity state($p > 0.05$). The *D/D* genotype was the most frequent among the hypertensive and obese population (65.3% and 55.0%, respectively), as shown in **Table 6**.

The relationship between ACE genotypes with nephropathy, kidney diseases, neuropathy and retinopathy in case groups subjects

Our results found that there is no statistically significant association between *ACE* genotypes with nephropathy and kidney disease, retinopathy and neuropathy ($p > 0.05$). The *D/D* genotype was the most frequent among the nephropathy and kidney diseases, retinopathy and neuropathy patients (61.4%, 58.9%, 80.6%, respectively), as shown in **Table 7**.

The relationship between ACE genotypes with CVD and Heart attackin case group subjects

The initial results showed that there is no statistically significant association between *ACE* genotypes with CVD and heart attack ($p > 0.05$), as shown in **Table 8** .

The relationship between ACE genotypes and medical family history

The results showed that there is no statistically significant relationship between *ACE* genotypes and medical family history in all subjects ($p > 0.05$), as shown in **Table 9**.

Odds Ratio (OR) and 95% Confidence Interval (CI) of ACE genotypes between the studied groups

DISCUSSION

GenotypeFrequencies of *the ACE* gene in the Gaza Strip

ACE Genotype frequencies were 34.7% for *I/D*, 62.9% for *D/D* and 2.4% for *I/I* genotype in this study. In terms of the frequency of *I/D* polymorphism of the *ACE* gene in the current study compared to different ethnic groups in various studies, *D/D* genotype has the highest frequency in the Gaza Strip, Lebanon and Iraq, while the *I/D* genotype has the highest frequency

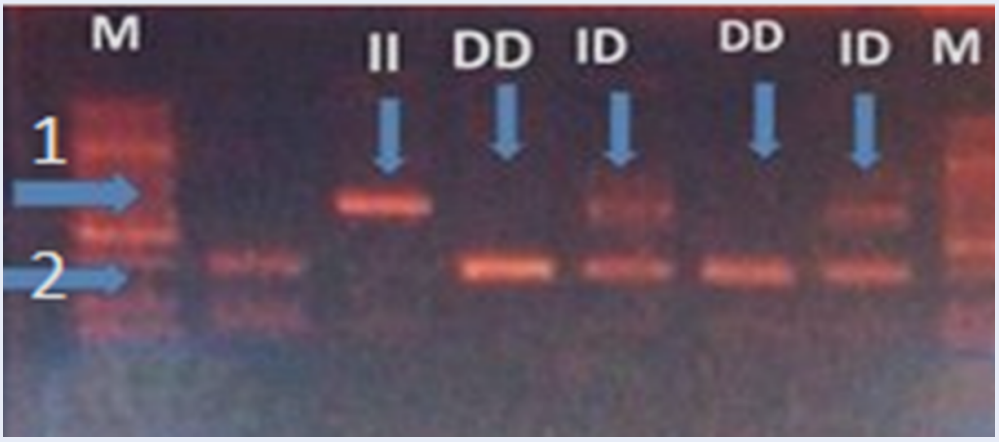

Figure 1: **PCR amplification products**; M 1 490 bp: and M 2: 190 bp; *DD* indicates to the deletion deletion polymorphism, *ID* indicates to the insertion deletion polymorphism and *II* indicates to the insertion insertion polymorphism.

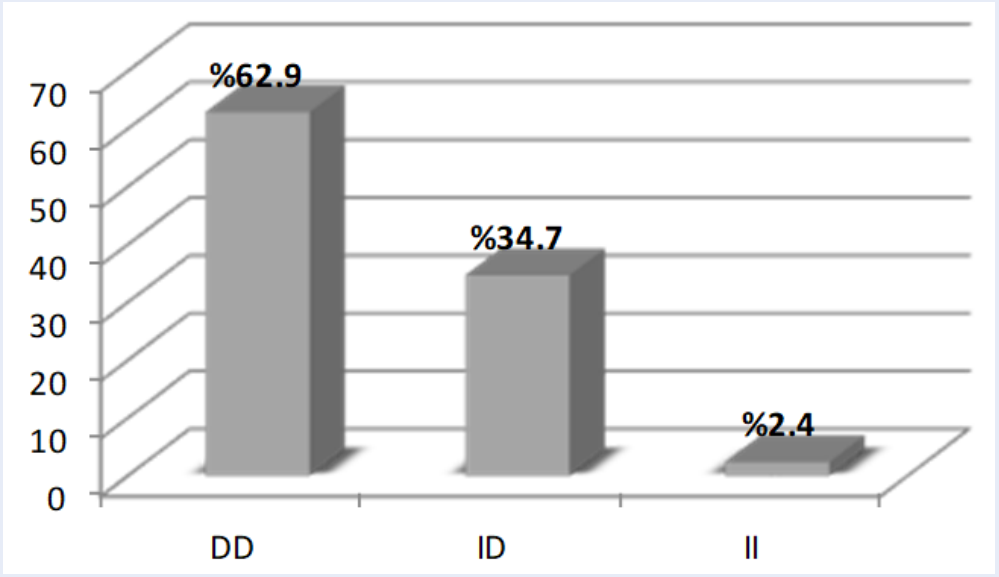

Figure 2: ***ACE* genotypes distribution in all study population.**

Table 2: Distribution of *ACE* genotype in subjects

		T2DN		Healthy		Nephropathy		T2DM		Total	
		N0	%	N0	%	N0	%	N0	%	N0	%
ACE Genotype	I/D	14	32.5	17	39.5	15	34.9	13	31.7	59	34.7
	D/D	28	65.1	26	60.5	25	58.1	28	68.3	107	62.9
	I/I	1	2.3	0	0.0	3	7.0	0	0.0	4	2.4
Total		43		43		43		41		170	
p-value						0.344					

Table 3: *ACE* gene allele frequency in all study subjects

		T2DN	Healthy	Nephropathy	T2DM	Total (%)
Allele	D	70 81.4%	69 80.2%	45 71.4%	69 84.1%	80.3
	I	16 18.6%	17 19.8%	18 28.6%	13 15.9%	19.7
Total		86	86	63	82	170

Table 4: Relationship between *ACE* genotype and gender in all study subjects

		Male No. (%)	Female No. (%)	Total	p-value
ACE Genotype	**I/D**	30 (36.1%)	29 (33.3%)	59	0.14
	D/D	53 (63.9%)	54 (62.1%)	107	
	I/I	0	4 (4.6%)	4	
Total		83	87	170	

Table 5: Relationship between *ACE* genotypes with diabetes duration in diabetic population compared to the non-diabetic population

		Non-Diabetic		Diabetic (years)					
				5-9		10-15		>15	
		n	%	n	%	n	%	n	%
ACE Genotype	I/D	15	35.7	8	19.0	6	14.3	13	31.0
	D/D	25	30.9	18	22.2	15	18.5	23	28.4
	I/I	3	75.0	0	0.0	0	0.0	1	25.0
p-value					0.635				
Allele	D	65	75.6%	44	84.6%	36	85.7%	59	79.7%
	I	21	24.4%	8	15.4%	6	14.3%	15	20.3%
Total		86		52		42		74	

* Healthy individuals were not included in the comparison

in Egypt, the UAE, China, and Pakistan, as shown in **Table 13**.

The relationship between *ACE* genotype with diabetic nephropathy

According to our initial results, there was no statistically significant association between *ACE* genotypes and T2DN, and this is similar to the data from other studies conducted in China[26], Iran[9], Tunisia[29], Turkey[30], South India[31] and others who have all failed to confirm the association of the *ACE* gene *I/D* polymorphism with diabetic nephropathy. On the other hand, a strong association between the *ACED/D* genotype and/or the *D* allele and the risk for nephropathy in T2DM was found in Egypt[23], Iraq[25], Pakistan[27], South Indian[32], Bahrain[33] and Japan[34].

The relationship between *ACE* genotype and diabetes

The current study did not find a statistically significant association between *ACE* genotypes and T2DM in the Gaza Strip, although Palestinian diabetic patients with *ACED/D* genotype have a higher prevalence of diabetes. To our knowledge, this is the first report showing the association of *ACE* gene polymorphism with diabetes and these results are in agreement

Table 6: Relationship between *ACE* genotypes with HTN and obesity state in case groups

		HTN				Obesity			
		Yes		No		Yes		No	
			%		%		%		%
ACE Genotype	I/D	29	30.5	13	40.6	15	37.5	27	31.0
	D/D	62	65.3	19	59.4	22	55.0	59	67.8
	I/I	4	4.2	0	0.0	3	7.5	1	1.1
p-value		0.330				0.102			
Allele	D	153 80.5%		51 79.7%		59 73.7%		145 83.3%	
	I	37 19.5%		13 20.3%		21 26.3%		29 16.7%	
Total		190		64		80		174	

* Healthy individuals were not included in the comparison and according to WHO the individual with BMI more than 30 was been considered as obese and those with a BMI less than 30 were grouped into non-obese populations

Table 7: Relationship between *ACE* genotypes in case group subjects with nephropathy and kidney diseases, retinopathy and neuropathy

		Nephropathy and kidney problems				Retinopathy				Neuropathy			
		Yes		No		Yes		No		Yes		No	
		N0	%	N0	%	N0	%	N0	%	N0	%	N0	%
ACE Genotype	I/D	30	34.1	12	30.8	27	37.0	15	27.8	6	19.4	36	37.5
	D/D	54	61.4	27	69.2	43	58.9	38	70.4	25	80.6	56	58.3
	I/I	4	4.5	0	0.0	3	4.1	1	1.9	0	0.0	4	4.2
p-value		0.346				0.379				0.065			
Allele	D	138 78.4%		66 84.6%		113 77.4%		91 84.3%		56 90.3%		148 77.1%	
	I	38 21.6%		1 15.4%		33 22.6%		17 15.7%		6 9.7%		44 22.9%	
Total		176		78		146		108		62		192	

* Healthy individuals were not included in the comparison

Table 8: Relationship between *ACE* genotypes with CVD and heart attack in case group subjects

		CVD				Heart attack			
		Yes		No		Yes		No	
		N0	%	N0	%	N0	%	N0	%
ACE Genotype	I/D	13	26.5	29	37.2	10	38.5	32	31.7
	D/D	35	71.4	46	59.0	16	61.5	65	64.4
	I/I	1	2.0	3	3.8	0	0.0	4	4.0
p-value		0.354				0.511			
Allele	D	83 84.7%		121 77.6%		42 80.8%		162 80.2%	
	I	15 15.3%		35 22.4%		10 19.2%		40 19.8%	
Total		98		156		52		202	

*Healthy individuals were not included in the comparison

Table 9: Relationship between *ACE* genotypes and family history to diabetes and HTN in all subjects

		Family history							
		Diabetes				HTN			
		Yes		No		Yes		No	
		N0	%	N0	%	N0	%	N0	%
ACE Genotype	I/D	28	47.5	31	52.5	23	39.0	36	61.0
	D/D	48	44.9	59	55.1	53	49.5	54	50.5
	I/I	1	25.0	3	75.0	1	25.0	3	75.0
Total		77		93		77		93	
p -value		0.676				0.303			

*Healthy individuals were not included in the comparison

with different studies. Such as a study in the UAE [19] and with a meta-analysis study in China [26]. On the other hand, the association of *D/D* genotype with increased T2DM risk was reported in Tunisia [15], Iran [17] and others.

The relationship between *ACE* genotype and Nephropathy

As we showed in our previous study [22], according to the initial results of the study no association between *ACE* genotype and nephropathy in the Gaza Strip can be approved, and this result again obtained with a different population while in a meta-analysis study by Schena *et al.* (2001), who found that the *D/D* genotype could be associated with the development of nephropathy (1.96 of risk) [35].

The relationship between *ACE* genotype and family history to diabetes and HTN

Our study showed that there is no statistically significant association between *ACE* genotypes and either Diabetes or HTN family history, but this does not conform with a study in Iraq [26] as well as a study on British Caucasians [36], which showed a significant association between genotype and a family history of diabetes ($p = 0.03$) The OR for a family history of diabetes in *D/D* compared to *I/I* subjects was 1.52 [0.89–2.60], and this disagreement may be due to the size of

Table 10: OR and 95% CI in the genotype of the ACE gene I/D polymorphism in each medical condition

Group	ACE genotype	Cases	Control group	p - value	OR	95% CI
T2DN	D/D	28	26	0.655	1.221	0.508-2.930
	I/I+I/D	15	17			
T2DM	D/D	28	26	0.454	1.408	0.574-3.457
	I/I+I/D	13	17			
Nephropathy	D/D	25	26	0.826	0.908	0.384-2.148
	I/I+I/D	18	17			
HTN	D/D	62	45	0.480	1.253	0.670-2.342
	I/I+I/D	33	30			
Obesity	D/D	28	79	0.319	0.709	0.36-01.397
	I/I+I/D	21	42			

Table 11: OR and 95% CI in the genotype of the ACE gene I/D polymorphism between T2DM and T2DN subjects

Group	ACE genotype	No.	p-value	OR	95% CI
T2DM	D/D	28	0.758	1.154	0.465-2.863
	I/I+I/D	13			
T2DN	D/D	28			
	I/I+I/D	15			

Table 12: OR and 95% CI in the genotype of the ACE gene I/D polymorphism between T2DN and nephropathy subjects

Group	ACE genotype	No.	p -value	OR	95% CI
Nephropathy	D/D	25	0.506	0.744	0.311-1.780
	I/I+I/D	18			
T2DN	D/D	28			
	I/I+I/D	15			

Table 13: OR and 95% CI in the genotype of the ACE gene I/D polymorphism between T2DN and healthy subjects

Group	ACE genotype	No.	p-value	OR	95% CI
Healthy	D/D	26	0.655	1.221	0.508-2.930
	I/I+I/D	17			
T2DN	D/D	28			
	I/I+I/D	15			

Table 14: Distribution of *ACE* gene in different human population

Country	I/D (%)	D/D (%)	I/I (%)	Reference
Egypt	39.2	31.6	29.2	[23]
Lebanon	34.8	57.5	7.7	[24]
Emiratis	52.6	41.1	6.3	[19]
Iraq	31	50	19	[25]
China	45	13.3	41.7	[26]
Pakistan	47.9	22.3	29.8	[27]
Palestine (Gaza strip)	38.2	57.5	4.7	[22]
Palestine (Gaza strip)	38.85	53.85	7.3	[28]
Palestine (Gaza strip)	34.7	62.9	2.4	The Present Study

the sample.

CONCLUSION

The present study focused on the association of *ACE* gene (*I/D*) polymorphism in the Gaza Strip and the relationship between those genotypes and T2DN in a comparison to healthy, T2DM and nephropathy subjects. *D/D* genotype was the most common in all groups. Finally, our results did not reveal any statistical association between T2DN, T2DM and nephropathy with *ACE* I/*D* polymorphism.

LIMITATIONS OF THE STUDY

The original PCR method[13] has been reported to sometimes miss extension of *I* allele, particularly in heterozygotes. Improved methods, which include a second, nested extension with allele-specific primer, have been designed for this reason. In a future study, the genotypes should be checked with such a method.

RECOMMENDATIONS

To the best of our knowledge, this is the first study to investigate the relationship between *ACEI/D* polymorphism with Diabetic Nephropathy in the Gaza Strip. Hence, additional studies considering gene-gene and gene-environment interactions should be conducted to estimate the overall risk of the *ACE* gene in the pathogenesis of diabetic nephropathy by a using larger sample size. The steady increase in the number of T2DM patients will impose a significant burden on healthcare systems, so the early identification and treatment of diabetic complications are one of the primary goals in the efforts to solve this problem. Also, we recommend that the ACE levels in plasma should be measured for all subjects and compared with the genotypes, which will help in the selection of treatment and dosage, particularly for HTN patients.

COMPETING INTERESTS

The authors declare no conflict of interest in this study.

AUTHORS' CONTRIBUTIONS

All authors participated in drafting the article and revising it critically for important intellectual content, also they all gave a final approval of the version to be submitted.

DEDICATION

The authors dedicate this work to the soul of the Palestinian Martyr " Nabil Shahwan" who kept supporting science and scientists all over his life.

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