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Curative Effect Analysis of Zoledronic Acid in the Treatment of Postmenopausal Osteoporosis with Different Bone Turnover Rates

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Abstract

Objective: To explore the clinical efficacy of Zoledronic Acid Injection in the treatment of postmenopausal osteoporosis with different bone turnover rates. **Methods:** A total of 63 patients diagnosed with postmenopausal osteoporosis were included in this study. Each patient was administrated 5 mg/100mL Zoledronic Acid (Aclasta) intravenously once and then given a one-year prescription of 600 mg/d oral Caltrate. The bone turnover parameters (PINP, β-cross, N-MID) were measured prior to the injection of Zoledronic Acid while the bone mineral density (BMD) and the pain scores of each patient were tested before treatment and after the one-year medication. On this basis, the patients were divided into several groups according to their bone turnover rates for intergroup comparison of treatment outcomes. **Results:** BMD results and pain scores of all participants were significantly improved at different levels after treatment. However, these improvements had no significant differences between the patients with high and low bone turnover rates. **Conclusion:** Zoledronic Acid Injection can relieve bone pain, enhance the quality of life and increase the BMD in patients with postmenopausal osteoporosis, regardless of the bone turnover status.

Keywords

Osteoporosis, Postmenopausal, Bone Turnover Rate, Zoledronic Acid

1. Introduction

Osteoporosis (OP) is commonly seen in the middle-aged and elderly population in China. It is caused by a variety of factors featuring a decrease in bone mass

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and a deterioration in bone microarchitecture, which elevates the risk of fracture and fracture-prone metabolic bone diseases (MBDs). The clinical manifestations of OP mainly include pain, fracture, humpback and height loss. Considering the high mortality and disability rates, OP treatment and management has become a major issue in the public health sector [1]. Anti-osteoporosis drugs are gaining wider acceptance among clinicians treating OP. As an anti-osteoporosis drug, Zoledronic Acid (Aclasta) is Novartis' once-yearly injection that provides fracture protection in postmenopausal osteoporosis by inhibiting bone reabsorption and increasing bone mineral density (BMD) [2]. In addition, the bone turnover biomarkers released from bone remodeling, combined with the widely used BMD measurements, can improve the diagnostic accuracy of osteoporosis [3] [4]. The measurements above and bone turnover parameters are clinically applied to the analysis of the curative effect of Zoledronic Acid in the treatment of postmenopausal osteoporosis with different bone turnover rates.

2. Materials and Methods

2.1. General Information

The clinical data of 63 postmenopausal patients with osteoporosis who presented at our osteoporosis outpatient clinic from January 2016 to December 2017 were collected and analyzed in this study. All participants were postmenopausal women aged between 50 and 80 (mean age = 66.0 ± 8.0). Prior to the initial diagnosis, dual-energy X-ray absorptiometry (DXA) was used to test the bone mineral density of lumbar spine and femur, with the measured values being adopted as diagnosis and inclusion criteria. Patients were eligible for inclusion if the total lumbar spine (L1 - L4) bone mineral density value was no greater than −2.5, or T-score was below −1.0 with preexisting fragility fractures, definite systemic symptoms such as back pain or height loss, and normal 25(OH)D. Exclusion criteria: 1) Previous use (within six months before treatment) of estrogen, glucocorticoid (GC), calcitonin and other bisphosphonates (BPs) that might influence bone metabolism; 2) diabetes and endocrine disorders of thyroid, parathyroid, adrenal gland or gonad; 3) secondary osteoporosis; 4) an allergy to Zoledronic Acid.

2.2. Treatment Methods

Zoledronic Acid (5 mg in 100 mL ready-to-infuse solution) is administrated intravenously, followed by the oral medication of 600 mg/d calcium carbonate D3 (Caltrate) for one year. The patients were required to get regular sun exposure, namely basking in the sun for half an hour between 11:00 and 15:00, twice a week. The Zoledronic Acid (Aclasta) prescribed for the patients is produced by Novartis. It is a once-yearly injection that provides fracture protection in postmenopausal osteoporosis.

2.3. Outcome Measures

The following parameters were documented: pre-treatment bone turnover pa-

rameters (PINP, β-cross, N-MID), bone mineral density of lumbar spine and femur and bone pains before and after treatment. All bone turnover parameters were tested after an overnight fast by the clinical laboratory of our hospital; BMD was measured by dual-photon absorptiometry (DPA) and dual-energy X-ray absorptiometry (DXA); during treatment, the patients were recommended to pay follow-up visits to the hospital to diagnose whether they had fresh fragility fractures.

In terms of bone pains, the Visual Analogue Scale (VAS) was used for pain assessment. VAS (4-point scale): 1) No pain; 2) Mild pain (without affecting sleep); 3) Moderate pain (affecting sleep at a tolerable degree); 4) Severe pain (intolerable, unable to fall asleep).

2.4. Clinical Efficacy Assessment

1) Bone pains and fresh fractures were tested after the one-year treatment.

2) The BMD (g/cm^2) and VAS scores before and after treatment were documented and analyzed.

3) The patients were divided into different groups by each bone turnover parameter according to the corresponding normal range specified by our hospital (PINP: 0.00 - 36.4 ng/ml; β-cross: 0.30 - 2.00 ng/ml; N-MID: 14.00 - 46.00 ng/ml). Specifically, the patients were respectively divided into a normal PINP group and an abnormal group with higher PINP levels, a normal β-cross group and an abnormal one with lower β-cross levels, and a normal N-MID group and an abnormal one with lower N-MID levels to analyze the improvement in BMD and VAS score.

2.5. Statistical Methods

The statistical analysis software SPSS22.0 was used for data analysis. Measurement data were expressed in the form of "mean ± standard deviation ($x \pm s$)" and the t-test was applied to the intragroup comparison. Besides, the chi-squared test was designed for the comparison of enumeration data. $P < 0.05$ indicated a difference of statistical significance.

3. Results

After being treated for one year, no fresh fractures were detected in the 63 patients during follow-up visits. As shown in Table 1, all intergroup P values are greater than 0.05, that is, the intergroup differences show no statistical significance but comparability.

As shown in Table 2, the average post-treatment VAS score was significantly lower than the pre-treatment level, which indicates that the bone pains were alleviated in the course of treatment. Also, the BMD (g/cm^2) of all patients was significantly improved after treatment. All this has proved the clinical efficacy of Zoledronic Acid to reduce bone pains and loss of bone mineral density. But Table 3 presents a statistical analysis based on the intergroup comparison of OP

patients with different bone turnover rates, which indicates no statistically significant difference ($P > 0.05$). In other words, Zoledronic Acid is efficacious in treating postmenopausal osteoporosis regardless of the bone turnover status.

Neither fresh fragility fractures nor obvious adverse reactions were detected in the participants throughout the course of treatment.

4. Discussion

Metabolic activity brings about dynamic changes and constant remodeling of bone tissue while OP is caused by an increase in osteoclasts and loss of BMD,

Table 1. Patients' general information by group.

	Number of Cases	Age	Pre-Treatment $L_{2\text{-}4}$BMD	Pre-Treatment VAS	P1	P2	P3
PINP→	37	66.7 ± 8.3	0.827 ± 0.153	2.18 ± 0.896			
PINP↑	26	65.0 ± 7.6	0.858 ± 0.110	1.88 ± 0.833	0.401	0.376	0.18
β-CROSS↓	36	67.0 ± 8.6	0.842 ± 0.150	2.19 ± 0.92			
β-CROSS→	27	64.6 ± 7.0	0.836 ± 0.119	1.89 ± 0.801	0.248	0.868	0.173
N-MID→	32	67.5 ± 8.5	0844 ± 0.142	2.19 ± 0.931			
N-MID↑	31	64.4 ± 7.3	0.836 ± 0.134	1.94 ± 0.814	0.135	0.792	0.258

Note: ↓, →, and ↑ respectively denote subnormal, normal and above-normal levels of bone turnover parameters. P1, P2 and P3 respectively express the P-values based on the comparison of the age, pre-treatment $L_{2\text{-}4}$BMD, pre-treatment VAS between groups which divided by different levels of bone turnover parameters.

Table 2. Pre and post-treatment BMD and VAS scores.

	Number of Cases	Before Treatment	After One-Year Treatment	P-Value
BMD (g/cm^2)	63	0.840 ± 0.137	0.883 ± 0.151	0.001
VAS score	63	2.06 ± 0.878	1.7 ± 0.647	0.026

Table 3. Intergroup comparison of BMD increase , VAS (post-pre) based on different bone turnover rates.

	Number of Cases	BMD Increase (g/cm^2)	VAS (Post-Pre)	P_1-Value	P_2-Value
PINP→	37	0.047 ± 0.080	−0.243 ± 0.76		
PINP↑	26	0.045 ± 0.058	−0.192 ± 0.800	0.096	0.799
β-CROSS↓	36	0.044 ± 0.084	−0.222 ± 0.800		
β-CROSS→	27	0.050 ± 0.052	−0.222 ± 0.751	0.744	0.999
N-MID→	32	0.051 ± 0.087	−0.219 ± 0.792		
N-MID↑	31	0.036 ± 0.050	−0.226 ± 0.762	0.419	0.971

Note: P_1-value, P_2-value respectively express the P-values based on the comparison of the BMD increase, VAS (post-pre) between groups which were divided by different levels of bone turnover parameters.

which impedes bone matrix formation and calcium salts deposition and ultimately leads to increased bone resorption and imbalanced bone turnover—that is, bone resorption exceeds formation. In terms of the underlying pathogenesis, OP can be classified as primary and secondary. For the patients aged 60 and above, 56% have osteoporosis. Particularly, hip fractures contribute the greatest to morbidity and mortality among all osteoporotic fractures, with 20% of the patients died within a year after the hip fracture. With the population aging, osteoporosis becomes increasingly prevalent and imposes a national threat to public health. As such, anti-osteoporosis drugs are gaining wider clinical application. In addition, a great variety of anti-osteoporosis drugs targeting at different symptoms have been developed. For instance, Teriparatide works by stimulating new bone formation; BPs have an effect of inhibiting bone reabsorption [5].

It is well established clinically that BPs can prevent and treat OP by inhibiting osteoclast formation and bone reabsorption. The BP therapy is most often used to treat metabolic bone diseases (MBDs) featuring a significant increase in bone reabsorption, especially those with a high turnover rate [6]. Zoledronic Acid, as a new-generation bisphosphonate, spreads efficiently in bone tissue when given by injection into a vein. The high-affinity medication, first of all, is carried to the bone tissue with increased bone formation and reabsorption and selectively absorbed by active osteoclasts. It inhibits bone reabsorption by selective inhibition of osteoclast activity and induction of osteoclast apoptosis. Zoledronic Acid terminates the mevalonate pathway to block cell cycle, thereby inducing the apoptosis of osteoclasts and precursors of monocytes and inhibiting bone reabsorption. With an imidazole ring side chain with two nitrogen atoms, it shows a strong binding affinity to bone mineral and a longer action period [7] [8]. Zoledronic Acid Injection is presently known as the most effective medicine among all bone resorption inhibitors [9]. By comparing the bone reabsorption before and after treatment, Zoledronic Acid Injection is significantly effective in reducing the loss of BMD and bone pains. Further, Zoledronic Acid is administrated once a year and works for one year, which therefore notably increases patient compliance [10]. In a retrospective analysis of the efficacy of and the patient compliance with Zoledronic Acid for the treatment of osteoporosis, there were 5 men and 148 postmenopausal women with osteoporosis participating in the study and 66.5% of these participants had a history of fractures and showed poor compliance with treatment. All patients were given 5 mg/100mL Zoledronic Acid intravenously and 85% received a second dose in the following year. The BMD was increased by 11% and 20.7% respectively after one and four years of treatment. Between the second and the third year of treatment, the β-cross value dropped significantly and fresh fractures were observed in only 10.4% of the participants, a marked decline in the occurrence of fractures. The therapy was so convenient that the patients showed higher compliance, which enhanced the management and clinical efficacy of OP treatment [11].

Metabolic products, namely bone metabolic markers, are released during bone

remodeling. Bone metabolic markers are clinical indicators of bone turnover status. In clinical practices, these bone metabolic markers indicate the bone formation rate greater than the bone reabsorption rate, or vice versa. Type I procollagen-N-propeptide (PINP) is a bone formation marker reflecting the changes of newly synthesized type I collagen, namely an indicator of bone formation. The bone turnover marker N-terminal midfragment osteocalcin (N-MID OC) is a bone-specific, calcium binding protein released by bone formation and resorption. β-Cross, as a bone resorption marker, is the degradation product of type I collagen C-telopeptides. β-Cross, PINP and N-MID are three bone markers recommended by the International Osteoporosis Foundation (IOF). Zoledronic Acid is a representative bone resorption inhibitor. In a study investigating the effect of Zoledronic Acid Injection on osteoporosis, it was found that Zoledronic Acid Injection, combined with regular calcium and Vitamin D supplements, could effectively reduce the N-MID, b-CTX and PINP levels and improve the bone turnover rate; apart from this, the bone reabsorption parameter N-CTX decreased more drastically than the bone formation parameter PINP, indicating a significant improvement in quality of life [12]. For patients with different bone turnover rates, appropriate anti-osteoporosis drugs should be prescribed to inhibit bone reabsorption and accelerate bone formation [13]. Chao, M. *et al.* suggested that Zoledronic Acid could reduce bone turnover parameters in the treatment of osteoporosis, which means Zoledronic Acid is effective in inhibiting bone turnover [14].

As an increasing number of bone turnover parameters extend their clinical application, it becomes a cause for concern whether the clinical efficacy of Zoledronic Acid varies in the treatment of osteoporosis with different bone turnover rates. In this study, the patients were divided into several groups by different bone turnover parameters for intergroup comparison and the differences showed no statistical significance. The study results suggested no statistically significant differences in the curative effect of Zoledronic Acid on osteoporosis with different bone turnover rates. This indicates that bone turnover parameters are neither appropriate criteria for the clinical application of Zoledronic Acid, nor reliable indicators in the assessment of its curative effect. In terms of osteoporosis, an increase in the bone turnover rate means a high bone turnover and rapid bone loss, regardless of the relationship between bone formation and bone reabsorption. This, combined with the BMD test, serves as the basis for an initial or follow-up anti-osteoporosis therapy. If relevant bone turnover parameters rise abnormally, the patient probably has secondary osteoporosis.

5. Limitations

There were only 63 patients included in this study, and all patients were under observation for one year only. The results of the VAS are inevitably affected by the inherently subjective patients. Besides, the statistical analysis might be underrepresented because a relatively small number of patients were included in each group.

6. Conclusion

Neither severe side effects nor fresh fractures occurred during the course of treatment. It was demonstrated that Zoledronic Acid is efficacious in the treatment of postmenopausal osteoporosis with different bone turnover rates by reducing the loss of BMD and bone pains in the patients without triggering serious side effects.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Study of Angiotensin Converting Enzyme Gene Polymorphism in Egyptian Type 2 Diabetes Mellitus with Diabetic Kidney Disease

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Abstract

Objective: Diabetic kidney disease DKD (Diabetic nephropathy DN) is considered one of the chronic micro vascular complications of diabetes mellitus and considered the commonest cause leading to chronic renal failure and chronic renal dialysis. Genetic susceptibility has been implicated in DKD. The angiotensin converting enzyme (ACE) is one of the key roles in the renin angiotensin system cascade by converting angiotensin I to angiotensin II which plays a key role in regulation of blood pressure as well as electrolytes and fluid balance. This study addressed the association of (ACE) gene polymorphisms with DN in Egyptian (T2DM) patients. Methods: Our research comprised of 75 cases of T2DM with diabetic kidney disease, 100 cases of T2DM without DKD and 94 healthy volunteers. Different genotypes of ACE gene were determined by SSP-PCR analysis. Results: Gene polymorphism of ACE (DD, ID, II) in diabetic patient with DKD is 44%, 52%, 4% respectively and for T2DM individuals without DKD is 23%, 72%, 5% respectively. (DD) had significant higher frequencies in T2DM patients with DKD compared to those without DKD ($p < 0.005$) and (ID) had significant higher frequencies in T2DM without DKD ($p < 0.0001$). These results indicated that there is an association between ACE gene polymorphisms and susceptibility of diabetic patients to be affected by diabetic kidney disease. Conclusion: From our results, we can conclude that genotype of ACE in Egypt DD is the genotype of cases diabetic kidney disease. So the presence of D allele has a significant relation with diabetic kidney disease. Our data confirm the role of ACE in its relationship with diabetic kidney disease in Egyptian type 2 diabetic patients.

Keywords

ACE Gene Polymorphism, Insertion/Deletion, Type 2 Diabetes Mellitus, T2DM, Diabetic Kidney Disease, Diabetic Nephropathy, Microvascular Complications of Diabetes Mellitus

1. Introduction

Diabetic kidney disease DKD (Diabetic nephropathy DN) is a clinical syndrome characterized by persistent albuminuria (>300 mg/d or >200 μg/min) that is confirmed on at least 2 occasions 3 - 6 months apart, progressive decline in the glomerular filtration rate (GFR) and elevated arterial blood pressure [1].

Diabetic kidney disease is considered one of the most common causes for chronic renal failure and chronic hemodialysis [2]. Moreover, DKD also was considered one of the micro vascular complications in diabetic individuals and one of the leading causes of high mortality among patients with diabetes [3]. There are different etiologies that carry major and a key role that affects onset and progression of DKD, of these factors genetic predisposition and environmental circumstances. A genetic susceptibility, depends on familial clustering of DKD, has been implicated in different pathogenic background of DKD in T2DM individuals [4]. One possible genetic factor is the Angiotensin converting enzyme gene (ACE).

Gene insertion (I), deletion (D) polymorphism within the human ACE gene [5], shown to be associated with predisposition to emerging different T2DM complications, including diabetic eye disease [6] and DKD [7]. ACE possesses a crucial role in the regulation of conversion process of renin angiotensin system by controlling conversion angiotensin I to II [8].

Angiotensin II (Ang II) considered a very strong vasoconstriction factor of the systemic and the local blood pressure [9]. Ang II increases systemic and glomerular blood pressure, stimulates mesangial cell proliferation and tissue growth [10].

Several polymorphisms depend on the presence or absence of a 287 base pair sequence in intron 16, three main different genotypes homozygotes (DD, II) and heterozygote ID are found [11].

2. Methodology

2.1. Aim of the Work

The aim of our research was to check for the association of ACE gene polymorphisms with the susceptibility to Diabetic kidney disease in Egyptian individuals with T2DM.

2.2. Subjects

This research has included 175 subjects with type 2 Diabetes Mellitus. They were

recruited from the Internal Medicine Hospital (Diabetes clinic), Mansoura University, Egypt in the period between May and December 2017. The ethics committee approved the study protocol and the study was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the patients included in the study.

An inclusion criterion includes type 2 Diabetic patients fulfilling criteria of diabetic nephropathy. **An exclusion criterion includes**, Type 1 diabetes mellitus, gestational diabetes, secondary diabetes, associated autoimmune diseases, non diabetic kidney diseases, patients suffering from hematuria, acute infections particularly urinary tract infections, and pregnant females were excluded from the study.

Selected Diabetic patients divided into 2 groups according to the presence of nephropathy consists of 75 subjects affected with diabetes type 2 associated with DKD compared to 94 healthy volunteers. In the Type 2 diabetic patients affected with Diabetic kidney disease, the mean ± SD age was 58.03 ± 6.34 ranges from 45 to 74 years. They were in the form of 38 (50.7%) males and 37 (49.3%) females. The other group was affected with diabetes type 2 without DKD (n = 100), their mean ± SD age was 52.1900 ± 8.31901 range from 34 to 75 years; the gender divided between 30 (30.0%) males and 70 (70.0%) (Table 1).

3. Method

All subjects were questioned about history of diabetes mellitus, hypertension, hypercholesterolemia, history of DKD in the first degree relatives. Regarding clinical examination, blood pressure, weight, heights were measured. Laboratory investigation was done and included detection of urinary albumin with the following cutoff values (microalbuminuria, (Albumin/creatinine ration (ACR) between 30 - 300 mg/g) and macroalbuminuria, (ACR more than 300) (according to national kidney foundation), detection of Glycated hemoglobin (HbA1c), lipid profile (Table 2).

Table 1. Descriptive data of all studied cases of (T2DM with nephropathy, T2DM without nephropathy and healthy people) regarding their characteristics.

Groups		N	Mean
Age	T2DM with neuropathy	75	58.03 ± 6.34
	T2DM without neuropathy	100	52.1900 ± 8.31901
	Health control	94	51.179 ± 9.217
		Male	**Female**
Sex	T2DM with neuropathy	50.7%	49.3%
	T2DM without neuropathy	30%	70%
	Health Control	46.8%	53.2%
Body mass index (kg/rn^2)	T2DM with neuropathy	75	28.3 ± 4.1
	T2DM without neuropathy	100	28.6 ± 5.2
	Health control	94	26.7 ± 3.6

Data are means and SD.

Table 2. Descriptive data of the two groups (T2DM with nephropathy and T2DM without nephropathy) regarding clinical findings.

Parameters	Groups		Mean ± SD
duration of diabetes (Years)	T2DM with neuropathy		14.85 ± 5.032
	T2DM without neuropathy		14.74 ± 4.15
HbAlc (%)	T2DM with neuropathy		8.4 ± 1.2
	T2DM without neuropathy		8.1 ± 1.6
Retinopathy	T2DM with neuropathy	Background	52/75 (69.33%)
		Proliferative	21/75 (28%)
	T2DM without neuropathy	Background	18/100 (18%)
		Proliferative	4/100 (4%)
Creatinine (mg/dl)	T2DM with neuropathy		1.2 ± 0.9
	T2DM without neuropathy		0.8 ± 0.2
Cholesterol (mg/dl)	T2DM with neuropathy		210.22 ± 69.16
	T2DM without neuropathy		155 ± 44.32
Triglyceride (TG, mg/dl)	T2DM with neuropathy		140.97 ± 88.60
	T2DM without neuropathy		133 ± 45.23
Blood Pressure	T2DM with neuropathy	Mild hypertension	35 (45.9%)
		Moderate hypertension	28 (37.9%)
		Severe hypertension	8 (10.8%)
	T2DM without neuropathy	Normal	4 (5.4%)
		Mild hypertension	31 (31%)
		Moderate hypertension	23 (23%)
		Severe hypertension	9 (9%)
		Normal	37 (37%)
High density lipoprotein (HDL, mg/dl)	T2DM with neuropathy		39.14±12.67
	T2DM without neuropathy		42.11 ± 6.32
Low density lipoprotein (LDL, mg/dl)	T2DM with neuropathy		144.35 ± 63.21
	T2DM without neuropathy		111.24 ± 24.105
Microalbuminuria/ Macroalbuminuria	T2DM with neuropathy		20/42
	T2DM without neuropathy		-

Data are means and SD.

3.1. DNA Extraction and Purification

At first taking informed consent from all diabetic individuals included in our research and healthy volunteers, venous blood samples (3 ml) were withdrawn and added on EDTA (ethylenediamine tetra acetate) containing tubes, DNA was extracted promptly using DNA extraction and purification kit (Gentra Systems, USA) according to manufacturer's instructions and then stored at −20˚C till use.

3.2. PCR Amplification

ACE genotype analysis was performed by PCR-RFLP analysis.

Genomic DNA was isolated from peripheral blood leukocytes according to a standard salting out method [12]. Amplification was carried out in a DNA thermocycler (Eppendorf Master Cycler). First, PCR was performed using 20 pmoles of each primer (flanking primer pair): Sense oligo 5'-CTG GAG ACC ACT CCC ATC CTT TCT 3' and anti-sense oligo: 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3' in a final volume of 25 µl, containing (0.5 µ g genomic DNA, 2 mM $Mgcl_2$, 50 mM kcl, 10 mM Tris-Hcl (pH = 8.3), 0.2 mM of each dNTP, and 0.5 unit of Taq polymerase. PCR was done with an initial denaturing time at 94˚C for l min. Then the DNA was amplified for 30 cycles with denaturation at 94˚C for 30 s, annealing at 58˚C for 30 s, and extension at 72˚C for l min. This was followed by final extension at 72˚C for 8 min. PCR products were directly visualized using ethidium bromide staining after electrophoresis in a 2% agarose gel [13]. The amplification product is a 190 bp fragment in the presence of the deletion (D) allele and a 490 bp fragment in the presence of the insertion (I) allele. Therefore, there were three genotypes after electrophoresis: A 490 bp band (genotype II), 190 bp band (genotype DD), or both 490 and 190 bp band (genotype ID). Mistyping of ID heterozygote as D homozygotes may occur. Thus, each sample that had the DD genotype was applied to PCR amplification using the forward: 5-TCG GAC CAC AGC GCC CGC CAC TAC-3'; and the reverse: 5'-TCG CCA GCC CTC CCA TGC CCA TAA-3' primers with identical PCR conditions except for an annealing temperature of 67˚C. The reaction yields a 335-bp amplicon only in the presence of an I allele, and no product for homozygous DD samples.

4. Statistical Analysis

Data were prepared and undergoing analysis through Statistical Package of Social Science (SPSS, version 10.0). The frequencies of different allelic polymorphisms of all studied individuals were compared between groups by using Fisher's exact test (modified Chi square test) and Odds ratio. A value of $p < 0.05$ was considered to be significant.

5. Results

Comparing studied cases of T2DM with nephropathy to that of T2DM without nephropathy regarding the gene polymorphism of ACE and alleles (**Table 3**, **Figure 1** and **Figure 2**) showed that cases of T2DM with DKD had a statistically significant lower frequencies of the ID genotype and I allele compared to cases of T2DM without DKD (52.0% vs. 72.0%, $p < 0.0001$ and 30.0% vs. 41.0%, $p < 0.045$ respectively) while they had a significantly very high frequency of the DD genotype and D allele (44.0% vs. 23.0%, $p < 0.005$ and 70.0% vs. 59.0%, $p < 0.045$ respectively). On the other hand, II genotype had no significant frequency in cases of T2DM with nephropathy or cases of diabetes type II without nephropa-

thy.

By comparing cases of T2DM without nephropathy versus healthy people (Table 4), had a significant lower frequency of genotype DD and allele D compared to healthy people (23.0% vs. 48.9%, $p < 0.0001$ and 59.0% vs. 71.8%, $p < 0.011$ respectively), while they had a statistically significant very high frequency of ID & I allele (72.0% vs. 45.7%, $p < 0.0001$, 41.0% vs. 28.2%, $p < 0.011$, respectively). But by Comparing cases of diabetes T2DM with nephropathy and healthy people had no significant frequency (Table 5).

Comparing cases albuminuria >300 with those <300 as regards the studied ACE gene polymorphisms, it is observed that cases >300 have high frequency of DD genotype (45.2% vs. 30%, OR = 1.93, p = 0.75). Also cases >300 had low frequency of ID genotype (50% vs. 65%, OR = 0.54, p = 0.403). Regarding the allele frequencies, the D allele showed higher level among cases with macroalbuminuria (70.24% vs. 62.5%, OR = 1.42, p = 0.51), while the I allele showed lower level (29.76% vs. 37.5%, OR = 0.71, p = 0.51) (Table 6).

Comparing cases with blood pressure groups regards that the studied ACE gene polymorphisms, it is observed that in DD genotype normal have high level then sever then mild and moderate is the lower one (75%, 50%, 44.1%, 39.3, p = 0.232). In addition, in ID genotype found that moderate have higher level then mild then sever and normal is lower one (57.1%, 52.9%, 50%, 0%, p = 0.232) (Table 7).

Comparing cases with hyperlipidemia with those without hyperlipidemia as regards the studied ACE gene polymorphisms, it is observed that cases with hyperlipidemia have high frequency of DD genotype (46.2% vs. 44.7%, OR = 1.06, p = 0.903). In addition, cases with hyperlipidemia had high frequency of ID genotype (53.8% vs. 48.9%, OR = 1.22, p = 0.874). Regarding the allele frequencies, the D allele showed higher level among cases with hyperlipidemia (46.2% vs. 44.7%, OR = 1.06, p = 0.903), while the I allele showed lower level (26.9% vs. 30.9%, OR = 0.83, p = 0.76) (Table 8).

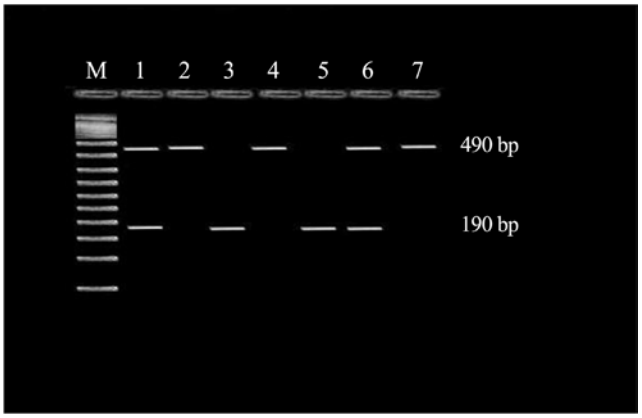

Figure 1. Detection of genetic polymorphism of ACE using PCR amplification: This figure showed 3 main different picture (DD, ID and II): DD homozygous (a single 190 base pair), ID heterozygous (190 and 490) Base pair, II homozygous (490 bp).

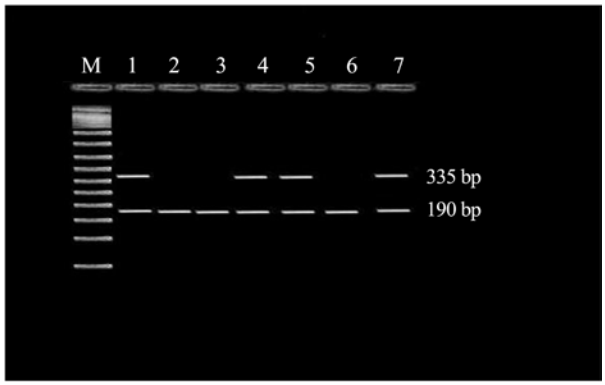

Figure 2. Second PCR amplification of ACE gene polymorphism: This figure shows, homozygous DD (a single 190 bp product) and heterozygous ID (190 and 335 bp product).

Table 3. Comparison between T2DM subjects with nephropathy and T2DM subjects without nephropathy regarding the frequency of their allele and gene polymorphism of ACE.

	Diabetic subjects with nephropathy n (%)	Diabetic subjects without nephropathy n (%)	χ^2 (P)	OR (95% CI)
	N = 75	N = 100		
DD	33 (44.0)	23 (23.0)	7.75 (0.005)*	2.6 (1.37 - 5.05)
ID	39 (52.0)	72 (72.0)	13.32 (<0.0001)**	0.27 (0.14 - 0.54)
II	3 (4.0)	5 (5.0)	0.003 (0.956)	0.792 (0.18 - 3.42)
Alleles	N = 150	N = 200		
D	105 (70)	118 (59)	4.023 (0.045)*	1.62 (1.035 - 2.54)
I	45 (30)	82 (41)	4.023 (0.045)*	0.62 (0.39 - 0.97)

Table 4. Comparison between cases T2DM without nephropathy and healthy volunteers regarding the frequency of their allele and gene polymorphism of ACE.

	Diabetic subjects without nephropathy n (%)	Healthy subjects n (%)	χ^2 (P)	OR (95% CI)
	N = 100	N = 94		
DD	23 (23.0)	46 (48.9)	13.11 (<0.0001)**	0.31 (0.17 - 0.58)
ID	72 (72.0)	43 (45.7)	12.77 (<0.0001)**	3.05 (1.7 - 5.54)
II	5 (5.0)	5 (5.3)	0.05 (0.823)	0.94 (0.26 - 3.35)
Alleles	N = 200	N = 188		
D	118 (59)	135 (71.8)	6.45 (0.011)*	0.57 (0.37 - 0.86)
I	82 (41)	53 (28.2)	6.45 (0.011)*	1.77 (1.16 - 2.71)

n = number of cases, (%) = percentage of cases, Odds ratio & 95% confidence interval = OR (95% CI). * $p < 0.05$ (significant) ** $p < 0.001$ (extremely significant): Significance using Fisher's exact test.

Table 5. Comparing between type 2 diabetes mellitus with nephropathy group and healthy control regarding their allele frequency and genotype distribution of ACE polymorphism.

	T2DM with Nephropathy n = 75 (%)	**Healthy Control n = 94 (%)**	X^2 (p)	OR 95% CI
DD	33 (44.0)	46 (48.9)	0.234 (0.629)	0.82 (0.45 - 1.51)
I D	39 (52.0)	43 (45.7)	0.43 (0.51)	1.29 (0.7 - 2.4)
I I	3 (4.0)	5 (5.3)	0.001 (0.98)	0.74 (0.17 - 3.21)
Allele	N = 150	N = 188		
D	105 (70)	135 (71.8)	0.059 (0.808)	0.92 (0.57 - 1.47)
I	45 (30)	53 (28.2)	0.059 (0.808)	1.092 (0.68 - 1.75)

Comparing cases with diabetic nephropathy with those healthy controls as regards the studied ACE gene polymorphisms, it is observed that cases with nephropathy lower frequency of DD genotype, which was not significant from that of healthy controls (44% vs. 48.9%, OR = 0.82, p = 0.629). Also cases of nephropathy had higher frequency of ID genotype (52% vs. 45.7%, OR = 1.29, p = 0.51). Regarding the allele frequencies the D allele showed lower level among cases diabetic nephropathy (70% vs. 71.8%, OR = 0.92, p = 0.808), while the I allele showed higher level (30% vs. 28.2%, OR = 1.09, p = 0.059).

Table 6. Comparison between macroalbuminuric T2DM with nephropathy group and microalbuminuric T2DM with nephropathy group regarding their allele frequency and gene polymorphism of ACE.

	Macroalbuminuria N = 42 (%)	Microalbuminuria N = 20 (%)	X^2 (p)	OR 95% CI
DD	19 (45.2)	6 (30.0)	0.75 (0.39)	1.93 (0.62 - 5.99)
I D	21 (50.0)	13 (65.0)	0.7 (0.403)	0.54 (0.18 - 1.62)
I I	2 (4.8)	1 (5.0)	0.35 (0.55)	0.95 (0.081 - 11.14)
Alleles	N = 84	N = 40		
D	59 (70.24)	25 (62.5)	0.43 (0.51)	1.42 (0.64 - 3.13)
I	25 (29.76)	15 (37.5)	0.43 (0.51)	0.71 (0.32 - 1.56)

Comparing cases albuminuria >300 with those <300 as regards the studied ACE gene polymorphisms, it is observed that cases >300 have high frequency of DD genotype (45.2% vs. 30%, OR = 1.93, p = 0.75). Also cases >300 had low frequency of ID genotype (50% vs. 65%, OR = 0.54, p = 0.403). Regarding the allele frequencies, the D allele showed higher level among cases with macroalbuminuria (70.24% vs. 62.5%, OR = 1.42, p = 0.51), while the I allele showed lower level (29.76% vs. 37.5%, OR = 0.71, p = 0.51).

Table 7. Comparing between blood pressure groups as regard ACE polymorphism in cases of diabetic nephropathy.

	Blood. p				X^2 (p)
	Mild N = 34 (%)	Moderate N = 28 (%)	Severe N = 8 (%)	Normal n = 4 (%)	
DD	15 (44.1)	11 (39.3)	4 (50.0)	3 (75.0)	
I D	18 (52.9)	16 (57.1)	4 (50.0)	0 (0)	8.090 (0.232)
I I	1 (2.9)	1 (3.6)	0 (0)	1 (25.0)	

Comparing cases with blood pressure groups regards that the studied ACE gene polymorphisms, it is observed that in DD genotype normal have high level then sever then mild and moderate is the lower one (75%, 50%, 44.1%, 39.3, p = 0.232). In addition, in ID genotype found that moderate have higher level then mild then sever and normal is lower one (57.1%, 52.9%, 50%, 0%, p = 0.232).

Table 8. Comparing between cases with hyperlipidemia and without hyperlipidemia as regard ACE polymorphism.

	With Hyperlipidemia N = 26 (%)	Without Hyperlipidemia N = 47 (%)	X^2 (p)	OR 95% CI
DD	12 (46.2)	21 (44.7)	0.015 (0.903)	1.06 (0.41 - 2.78)
I D	14 (53.8)	23 (48.9)	0.025 (0.874)	1.22 (0.47 - 3.18)
I I	0 (0)	3 (6.4)	0.22 (0.64)	0.24 (0.012 - 4.83)
Allele	N = 52	N = 94		
D	38 (73.1)	65 (69.1)	0.096 (0.76)	1.21 (0.57 - 2.57)
I	14 (26.9)	29 (30.9)	0.096 (0.76)	0.83 (0.39 - 1.75)

Comparing cases with hyperlipidemia with those without hyperlipidemia as regards the studied ACE gene polymorphisms, it is observed that cases with hyperlipidemia have high frequency of DD genotype (46.2% vs. 44.7%, OR = 1.06, p = 0.903). In addition, cases with hyperlipidemia had high frequency of ID genotype (53.8% vs. 48.9%, OR = 1.22, p = 0.874). Regarding the allele frequencies, the D allele showed higher level among cases with hyperlipidemia (46.2% vs. 44.7%, OR = 1.06, p = 0.903), while the I allele showed lower level (26.9% vs. 30.9%, OR = 0.83, p = 0.76).

6. Discussion

There are several researches indicated that development and progression of diabetic kidney disease are multifactorial including different pathophysiologic mechanisms especially environmental or genetic susceptibility. Epidemiological studies found familial clustering of diabetic kidney disease in diabetic siblings, supporting an important role of genetic defects in the pathogenesis of diabetic kidney disease [14]. ACE is an enzyme (zinc metallopeptidase enzyme) that found to be highly expressed on the epithelial and endothelial surfaces. The function of ACE is to convert angiotensin I to angiotensin II that is the highly active biochemical end product of the rennin-angiotensin system (RAS) [15] and this associated with an increased risk of vascular disease [16]. Clinical studies investigated association between diabetic kidney disease and ACE gene polymorphism showed contradictory results.

This study included (269) 75 patients with diabetic kidney disease (DKD), 100 patients T2DM patients without DKD and 94 healthy people. In selection of cases, we were keeping to have cases affected with T2DM associated with diabetic kidney diseases. Their mean age was 58.02 years, with a SD of ±6.34 years. Out of them, 94.6% having hypertension, and 35.6% with hyperlipidemia and 22.5% have consanguinity and 72% have family history to diabetes. For comparison 100 cases diabetic (T2DM) without nephropathy (mean age was 51.7 years, with a SD of ±9.4 years) (**Table 1** and **Table 2**).

This study showed that Egyptian cases of diabetic kidney disease had significantly higher frequency of genotype (DD) than cases diabetic with no DKD (44% versus 23%, p = 0.005). In addition, it's noticed that cases of diabetic nephropathy had significantly lower frequency of ID genotype than cases of diabetes without nephropathy (52% versus 72%, p < 0.0001) (**Table 3**).

Diabetic nephropathy cases showed low frequency of II genotype than diabetic without nephropathy 5.3% (4% vs. 5%). Meanwhile, total cases found to have statistically significant more frequent expression of D allele (70% vs. 59%, p = 0.045) with a significant lower level of I allele than cases of diabetic without nephropathy (30% vs. 41%, p = 0.045). Finally, in both group of cases and controls we observed that frequency D allele is higher than frequency of I allele.

In a previous study among Egyptian cases, reported that the II, ID and DD ACE genotypes was 4%, 52% and 44% in cases of diabetic nephropathy and 5%, 72% and 23% in cases without nephropathy. The ACE DD genotype shows significant association with diabetic nephropathy. We can speculate that the difference may be related to the ethnic background variations between our locations in Egypt and other countries.

Analyzing the results among Egyptian controls, this study showed that Egypt control cases (diabetic without nephropathy) had a higher frequency of ID than that of DD genotype (72% vs. 52%) with a higher frequency of II genotype (5% vs. 4%).

Also, our results showed that by comparing cases albuminuria >300 with those <300 as regards the studied ACE gene polymorphisms, it is observed that cases >300 have high frequency of DD genotype (45.2% vs. 30%, OR = 1.93, p = 0.75). Also cases >300 had low frequency of ID genotype (50% vs. 65%, OR = 0.54, p = 0.403).

Regarding the allele frequencies, the D allele showed higher level among cases with macroalbuminuria (70.24% vs. 62.5%, OR = 1.42, p = 0.51), while the I allele showed lower level (29.76% vs. 37.5%, OR = 0.71, p = 0.51) (**Table 6**). These results were in agreement with [17] who showed that females patients with abnormal excretion in urine (either micro or macro) found to have higher frequency of a genotype DD versus females with no albumin excreted in urine (DD = 27.9%, ID = 21.2% and II = 10.5%, respectively; p ≤ 0.044) in Mexico population.

These findings are supported by study carried out in India population by [18] who demonstrated that the analysis of different genotype of ACE showed the following findings: genotype DD found in (22.75%) cases with DKA, 15.42% in T2DM individuals, and 21.62% in healthy volunteers. Chi-square test between DKA group and healthy volunteers found to be non-significantly different in allele D. but, there is statistically significant difference (p < 0.05) between patients with DKD and diabetic patients.

Regarding other micro vascular complications related to diabetic kidney disease [19] demonstrated that there was highly significant correlation between diabetic retinopathy and genotype DD. Their prevalence was found to be higher in individuals affected by genotype DD (DD, ID, and II, 90.4%, 71.2%, and 70.6%; p < 0.05 respectively). individuals carry genotype DD reached the end point [s. creatinine levels more than 2.0 mg/dL (176.8 micromole/L)] more rapidly than individuals carry other different genotypes (DD, 11.38 ± 4.08 years; ID, 13.85 ±

4.04 years; II, 14.04 ± 4.06 years, respectively; $p < 0.05$) and those individuals (with DD) was progressively reach to chronic hemodialysis earlier than others (DD, 13.10 ± 4.45 years; ID, 16.21 ± 4.74 years; II, 15.13 ± 4.09 years, respectively; $p < 0.05$). Also, regarding hypertensive diabetic patients, there was a highly significant correlation between genotype DD and systolic blood pressure with progressive nature of Diabetic kidney diseases, in multiple logistic regression analysis.

Comparing cases with blood pressure groups regards that the studied ACE gene polymorphisms, it is observed that in DD genotype normal have high level then sever then mild and moderate is the lower one (75%, 50%, 44.1%, 39.3%, $p = 0.232$). In addition, in ID genotype found that moderate have higher level then mild then sever and normal is lower one (57.1%, 52.9%, 50%, 0%, $p = 0.232$) (**Table 7** and **Figure 3**).

In South Korea, subjects with genotype DD compared to others with genotype II, the OR was 3.881 (95% confidence interval, 1.564 9.628; $p = 0.003$ approximately), these results indicated that the DD genotyping of ACE gene may be considered a significant risk factor for the progressive nature of diabetic kidney disease.

Also, [20] found that diabetic individuals with a high levels of insulin resistant states, diabetic kidney disease was represented in 2/11 diabetic individuals with ACE gene (II genotype) versus 19/25 diabetic individuals represented by DD or ID genotype ($p = 0.002$). The prevalence of diabetic kidney disease was higher in the majority of individuals with both D allele plus significant insulin resistant states (19/25) compared to other patients (14. 37; OR, 5.20). These findings indicated that the ACE gene effectively can influence both onset and/or progressive nature of diabetic kidney disease in Japanese individuals with T2DM with high levels of insulin resistant state.

Also, in Tokyo, [21] concluded the following data, diabetic individuals with diabetic kidney disease have an excessive expression of genotype ID versus those individuals DKD ($p < 0.02$) and less of the genotype II versus with healthy volunteers ($p < 0.01$) and diabetic individuals without DKD ($p < 0.01$). T2DM individuals presented with genotype II have a low risk for the development of diabetic kidney disease.

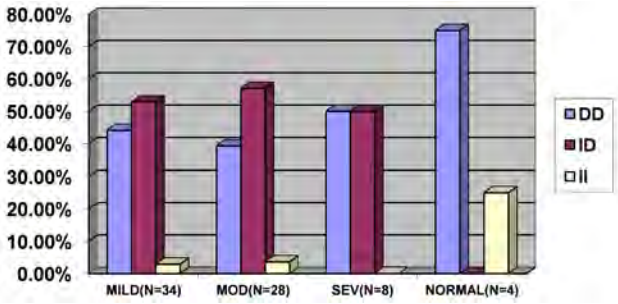

Figure 3. Comparing between blood pressure groups as regard ACE polymorphism in diabetic nephropathy cases.

Also these findings were in contrast with [22] who found that the presentation of different genotypes (II, DD and DI) did not differ significantly between T2DM individuals with or without DKD (46%; II: DD: 44%; ID: 10% vs., II: 12%, DD: 41%; ID: 47%; respectively). Also, no statistically any significantly different results between three main genotypes and allelic frequencies of the (I/D) polymorphism in all T2DM individuals versus healthy individuals with normal glycemic state (II: 11%, ID: 46%; DD: 43%; vs. II: 15%, DD: 37%; ID: 48%; respectively). So in Tunisian T2DM individuals, the (I/D) polymorphism within the ACE gene was found to be not associated with T2DM or DKD.

In addition, [23] showed that cases with DN incidence in T2DM individuals among three groups (II 45.8%, ID 52.3%, and DD 46.1%, respectively, $p > 0.05$). The increased degree of urinary excretion of albumin among the 3 groups was similar at the end-point of the study ($p > 0.05$). So, this study concluded that in china, genotype DD of ACE gene may not considered as genetic marker of clinical significance for prediction of the onset and progression of DKD in Type 2 diabetic patients in.

In addition, [24] found that there are no statistically differences in the mean eGFR according to different genotypes of ACE gene (ID: 99.5 ± 25.1 ml/min, II: 96.6 ± 19.6 ml/min, DD: 89.9 ± 28.1 ml/min). Also, they found no statistically significant changes in distribution of different genotypes diabetic individuals with different grades of albuminuria (DD:ID:II [%], normoalbuminuric patients-35:46:19, macro albuminuric patients-31:55:14, microalbuminuric patients-28:55:17). So, they concluded that, in Turkey population, ACE gene polymorphism (D/I) found to be not to be associated with eGFR in T2DM individuals.

In addition, In Poland, [25] studied (No.941) cases with renal complication of T2DM diabetic individuals. Of them 127 diabetic individuals with macroalbuminuria or end stage renal disease, 335 diabetic individuals with microalbuminuria, and a control group of 254 diabetic individuals without albuminuria with duration of diabetic state of 10 years and more. They concluded that, there were no any statistically significant differences in the different genotype D/I distribution of ACE gene or allelic frequency was found in-between different tested groups. The conclusion of this research strongly found that there was no associated link between the ACE gene D/I polymorphisms and DKD in T2DM individuals.

In addition, In Germany, [26] concluded that genetic polymorphism D/I related to ACE gene does not significantly have a major role in the onset and progression of DKD.

Comparing cases with hyperlipidemia with those without hyperlipidemia as regards the studied ACE gene polymorphisms, it is observed that cases with hyperlipidemia have high frequency of DD genotype (46.2% vs. 44.7%, OR = 1.06, $p = 0.903$). In addition, cases with hyperlipidemia had high frequency of ID genotype (53.8% vs. 48.9%, OR = 1.22, $p = 0.874$).

Regarding the allele frequencies, the D allele showed higher level among cases with hyperlipidemia (46.2% vs. 44.7%, OR= 1.06, p = 0.903), while the I allele showed lower level (26.9% vs. 30.9%, OR= 0.83, p = 0.76) (Table 8).

Possible explanation of this controversy in the results of different researches related to genetic polymorphisms may be due to multifactorial aspects, mainly the major differences in ethnic aspects of studied diabetic individuals and healthy volunteers. Other factors include the definition of nephropathy or Diabetic kidney disease and inclusion criteria of the diabetic control group without renal complication and small sample sizes in some studies.

7. Conclusion

Our findings indicated that there is a strong relation between diabetic kidney disease and genetic polymorphism of ACE gene and from our results we also found that genotype of ACE in Egypt is DD genotype of diabetic cases with diabetic kidney disease so the presence of D allele has a significant relation with diabetic kidney disease. Our data confirm the significant role of angiotensin converting enzyme gene in its relationship with diabetic kidney disease risk in Egyptian population.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

T2DM: type 2 diabetes mellitus

DN: diabetic nephropathy

ACE: Angiotensin-converting enzyme

SSP-PCR: sequence specific primer-polymerase chain reaction

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