Angiotensinogen M235T and angiotensin-converting enzyme I/D gene polymorphism and their association with type 2 diabetes in Egypt

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Abstract: Objectives: Type2 diabetes (T2DM) is the most common subtype of diabetes mellitus. A number of studies have examined the role of genetic polymorphisms on the risk of T2DM, and several variants have been identified as potential susceptibility genes, of those angiotensin-converting enzyme (ACE I/D) and angiotensinogen (AGT M235T). The aim of the current study was to investigate the association between genetic polymorphisms of ACE I/D and AGT M235T genes and T2DM in Egyptian population. Design and methods: A case control study was performed on 138 Egyptian subjects, 58 T2DM patients without hypertension with mean disease duration 7.8 ± 2.1 years and 80 age, and sex matching unrelated healthy controls. Genotyping of the candidate genes were performed by polymerase chain reaction technique (PCR) for ACE I/D gene and by PCR followed by restriction fragment length polymorphism (RFLEP) for AGT M235T gene and the presence or absence of the genotypes was analyzed by gel electrophoresis .We examined the association between each polymorphism and the risk of T2DM by odds ratio (OR) with 95% confidence intervals (95% CI). Results; Individuals who carried the two risk genotypes ACE (DD)/AGT (TT) had 14.5 times (95%CI 1.783-118.083, p=0.012) higher risk of developing T2DM than those who carry one risk genotype. ACE (DD) genotype OR=2.444, 95% CI 1.140-5.240, p=0.022 and AGT (TT) genotype OR=3.9, 95% CI 1.773-8.597, p<0.001. Conclusion: These data indicate an evident association between genetic polymorphisms of ACE I/D and AGT M235T genes and T2DM in Egyptian population.
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Key words: Type2 diabetes, genetic polymorphisms, angiotensin-converting enzyme and angiotensinogen (AGT M235T).

1. Introduction

Type 2 diabetes mellitus (T2DM) is a prevalent, chronic condition associated with extensive morbidity, decreased quality of life, and increased utilization of health services. T2DM is a worldwide epidemic with a prevalence that is expected to double by the year 2025, affecting over 5% of the adult population [1]. The polygenic nature of T2DM has been a major challenge to identifying genes involved in the pathogenesis of this disease, knowledge that could give rise to new treatments modalities [2,3]. Screening of candidate genes for nucleotide variants that are associated with T2DM is a core component of much diabetes genetics research. Several studies have been analyzed to select the candidate genes because of known or presumed biological or physiological functions. The choice of candidates is inevitably limited by incomplete understanding of the regulation of the processes and the pathophysiology of T2DM [4].

The angiotensinogen (AGT) gene encodes the precursor of the vasoactive hormone angiotensin II, which is the effector peptide of the renin-angiotensin system. The protein encoded by this gene, pre-angiotensinogen or angiotensinogen precursor, is expressed in the liver and is cleaved by the enzyme renin in response to lowered blood pressure. The resulting product, angiotensin I, is then cleaved by angiotensin converting enzyme (ACE) primarily within the kidney to generate the physiologically active enzyme angiotensin II. ACE found in other tissues of the body has no physiological role (ACE has a high density in the lung, but activation here promotes no vasoconstriction, angiotensin II is below physiological levels of action). Angiotensin II acts as an endocrine, autocrine/paracrine, and intracrine hormone.

A polymorphism in exon 2 of the AGT gene a threonine to methionine substitution at position 235 (M235T), has been associated with essential hypertension [5], diabetic nephropathy [6] and coronary heart disease [7]. The ACE polymorphism identified in 1990 by Rigat and co-workers [8] is one of the best researched polymorphisms. This polymorphism of the ACE gene is based on the presence (insertion) or absence (deletion) of a 287-bp Alu1 element inside intron 16 of the ACE gene on chromosome 17 producing three genotypes (DD, II homotypes and ID heterotype) [9].

The highest serum ACE activity was seen in the DD genotype while and the lowest was seen in the II genotype. Since then it has been speculated that these differences in plasma ACE activity associated with the ACE genotype might affect the therapeutic response of ACE inhibitors explaining interindividual variability in cardiovascular or renal response to equivalent doses of ACE inhibitors. Several studies have investigated extent of this effect modification on response to ACE inhibitors for different indications such as hypertension [10], diabetic nephropathy [11] and coronary artery disease [12].

The aim of this study was to investigate the association between genetic polymorphisms of ACE I/D and AGT M235T genes and T2DM in Egyptian population.

2. Subjects and Methods:

Subjects

A total of 58 adult Egyptian T2DM patients (mean age 46.53±6.53 years) were recruited from the outpatient diabetes clinic of Ain Shams University Hospital in addition to 80 age, sex and BMI matched healthy unrelated Egyptian adult subjects (mean age 44.76 ± 7.21 years). Informed consent was obtained from all subjects with approval granted by Ain shams Research and Ethics Committee "Ain-shams Faculty of Medicine federal number IRB00006444, prior to sample collection. Inclusion criteria: fasting plasma glucose≥ 126 mg/dl, Haemoglobin A1c ≥6.5% and/or treatment for diabetes included diet and/or oral antidiabetic drugs to achieve glycemic control. Duration of the disease on diabetic patients was 7.8 ±2.1 years. Recruitment of patients was restricted by the following criteria: the presence of hypertension/or taking antihypertensive drugs, diabetic nephropathy defined by persistent microalbuminuria (Albumin: Creatinine Ratio in spot urine sample: 2.5–25 mg/mmol in males, 3.5–35 mg/mmol in females) checked at least on two consecutive occasions over the previous six months. Renal or liver failure, retinopathy diagnosed by funduscopy examination, cardiovascular disease and intake of hormonal replacement therapy. Taking hypoglycemic drugs and lipid lowering drugs were not considered as exclusion criteria.

Detailed medical history for each group was obtained. Weight and height were measured to calculate the body mass index (BMI), systolic BP (SBP) and diastolic BP (DBP) was measured in a sitting position using mercury column sphygmomanometer after at least five minutes of rest; two readings of SBP and DBP were taken. Using NICE hypertension guideline 2011, standards for hypertension was systolic blood pressure (SBP) ≥140 mmHg and diastolic blood pressure (DBP) ≥90mmHg [13]. Clinical and biochemical measurements of patients with type 2 diabetes were performed after eight hours of fasting. Diagnosis of diabetes was according to the criteria of American diabetes association (Fasting plasma glucose; FPG ≥126 mg/dl. Fasting is defined as no caloric intake for at least 8 hours) [14]. Control subjects did not have any abnormalities regarding their physical examination, blood pressure, family history, urinalysis, and routine laboratory blood tests; none of them were receiving any medications at the time of participation.

Method:

Venous blood samples were collected from each subject in two separate test tubes: one was use for biochemical analysis. The other was collected on EDTA for HBA1c and DNA extraction.

2.1-Biochemical measurements: Serum urea, creatinine, total Cholesterol, high density lipoprotein, low density lipoprotein, triglyceride and fasting blood sugar were done using C111 analyser Commercial kits (Roch-Diagnostics, Swizerland).

2.2-DNA Extraction: Genomic DNA was extracted from white blood cell pellets by salting out extraction method [15] using wizard genomic DNA extraction kit from Promega. Red blood cell lysis was done by using red cell lysis buffer (20 mM tris-HCL pH 7.6) followed by centrifugation. Nuclei lysis was carried by cell lysis buffer (10 mM tris-HCL pH 8.0, 1 mM EDTA pH 8.0, 0.1% (w/v) SDS) and proteinase K (20 mg/ mL) followed by centrifugation. Protein was precipitated by protein precipitation solution (60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, 28.5 mL of water) followed by centrifugation. Finally DNA was precipitated by isopropanol and then ethanol 70% and rehydrated in Tris EDTA buffer (10mM tris, 1mM EDTA pH 8.0) and stored at -20°C. DNA purity and concentration were determined by spectrophotometer measurement of absorbance at 260 and 280 nm.

2.3-Genotyping:

2.3.1- ACE I/D polymorphism by PCR:

100 ng of DNA was amplified using Gene Amp PCR system 9700 from applied biosystem. The primers used were Sense 5'CTGGAGACCA CTCCCATCCT TTCT3' and antisense 5'GATGTGGCCATCACATT CGTCAGAT3'. PCR conditions were optimized for initial heating for 3 minutes at 94°C followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing at 58°C for 45 seconds, extension at 68 °C for 2 minutes and final extension was done at 68°C for 7 minutes. Reaction mixtures consisted of 1.25 units of thermostable Taq polymerase, 0.2 mM of each dNTP, 1.5 mM mgCl , 2 mM dimethylsulphoxide (DMSO) and 0.5 µmol of each primer, made up to a final volume of 50 µL The product was separated on 2% agarose gel and visualized by ethidium bromide staining.

Subjects were classified according to the presence or absence of a 287 base pairs insertion at intron 16 of ACE gene as II, ID, DD. Preferential amplification of the smaller 190 base pairs deletion allele (D) in ID heterozygote has led to their mistyping as DD homozygote so they were retyped using an insertion (I) specific sense primer 5'-TGGGACCACAGCGC CCGCCACTAC-3'; antisense: 5'GCCAGCCCTCC CATGC CCATAA-3' (primers were supplied by Alpha DNA, 4401 Notre-Dame St.w) and were then subjected to denaturation at 94°C for 3 minutes followed by 32 cycles of 94°C for 30 seconds, 67°C for 30 seconds and 72°C for 30 seconds, followed by 67°C for 30 seconds and 72°C for 10 minutes [16]. The products were separated on separated on 2% agarose gel and visualized by ethidium bromide staining. (Figures 1,2).

3.3.2- AGT M235T polymorphism by RT-PCR followed by RFLP:

Amplification was done using the following primer sequences: sense- 5'CCGTTTGTGCAGGGCC TGGCTCTCT3' and antisense: 5'CAGGGTGCTGT CCACACTGGACCCC3'. Reactions were carried out in 25 µL volumes under standard conditions (1.5 mmol MgCl2, 50 µmol for each dNTP, 10 mmol tris/HCl, 50 mmol KCl, 1 µmol primers, 1 U Taq DNA polymerase per sample. PCR conditions were optimized for initial denaturation at 94°C for 3 minutes, followed by 30 cycles of 1 minutes denaturation at 94°C, 1 minutes annealing at 68°C and 1 minutes extension at 72°C, then final extension at 72 for 10 minutes was done. The product of PCR was subjected to digestion by restriction enzyme Tth 111I (New England Bio Labs, Missisauga, ON, Canada) for 3 hours at 65°C. The M to T point mutation creates a detection site at position 235 [17] and the digested fragments were separated on 2% agarose gel and visualized by ethidium bromide staining (Figure3).

4-Statistical analysis:

Data was analyzed using SPSS win statistical package version 15. Numerical data were expressed as mean, standard deviation and range.

Qualitative data were expressed as frequency and percentage. Results were considered statistically significant at $p<0.05$. Continuous variables are presented as mean ± standard deviation and an ANOVA was performed. AGT M235T and ACE I/D genotype frequencies were estimated by gene counting, and the differences between the studied groups were evaluated by Pearson's $\chi 2$ test and Anova test. Odds ratios (OR) and 95% confidence interval (CI) were calculated in according to Woolf's method. The distribution of alleles and genotypes between patients and controls were determined using the (goodness-of-fit) test, and statistical significance was determined at $p<0.05$. Chi-square test was used in order to test whether the frequency distribution of genotypes in T2DM or control group was in Hardy-Weinberg equilibrium (HWE).

3. Results:

Demographic data of studied groups are shown in table (1). The frequencies of the polymorphic variants of ACE I/D and AGT M235T genes were significantly different between T2DM patients and controls ($p<0.001$). Significant differences were found in DD genotype (OR=2.444, 95% CI 1.140-5.240, $p=0.022$) and TT genotype (TT: OR=3.9, 95% CI 1.773-8.597, $p<0.001$). ACE- DD/AGT-TT combined genotype was significantly higher in T2DM patients than control with marked increase in risk to disease occurrence (OR =14.510 p=0.012). Moreover, the frequencies D allele of ACE gene was not associated with increased risk of T2DM facing a high significant association with T allel of AGT gene (D: OR=1.599, 95% CI 0.986-2.59, p =0.057 and T: OR=2.104, 95% CI 1.291-3.428, p =0.003) (Table 2). Applying HWE at the ACE I/D locus and AGT M/T locus revealed that: Regarding ACE I/D locus, no deviation from Hardy-Weinberg equilibrium (HWE) was detected in both T2DM ($p^2 = 0.158$; $2pq = 0.397$; $q^2 = 0.360$) or control group ($p^2 = 0.260$; $2pq = 0.489$; $q^2 = 0.230$). Also, in AGT M/T locus no deviation from HWE was detected in both T2DM ($p^2 = 0.149$; $2pq = 0.471$; $q^2 = 0.384$) and control group ($p^2 = 0.313$; $2pq = 0.492$; $q^2 = 0.193$).

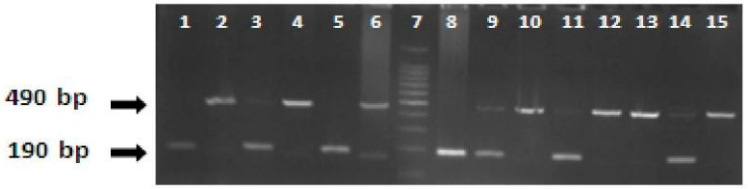

Figure 1: Gel electrophoresis of ACE I/D : lanes1,3,5,8,11 are DD homozygote with single band at 190, lanes 2,4,10,12,13 are II homozygote with one band 490, Lanes 6,9,14 are ID heterozygotes with two bands, and lane 7 is 100 bp marker.

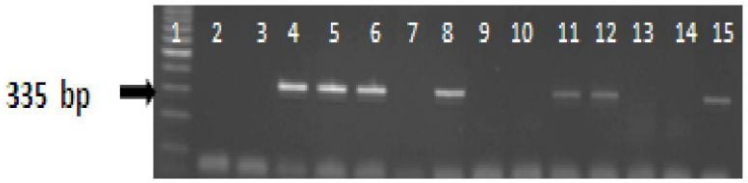

Figure 2: Gel electrophoresis using insert specific primers: lanes 4,5,6,8,11,12,15 show single bands of the insertion fragment of 335 bp so considered ID heterozygote, while lanes 2,3,7,9,10, 13, 14 no bands indicating DD homozygote, lane 1 shows 100 bp marker.

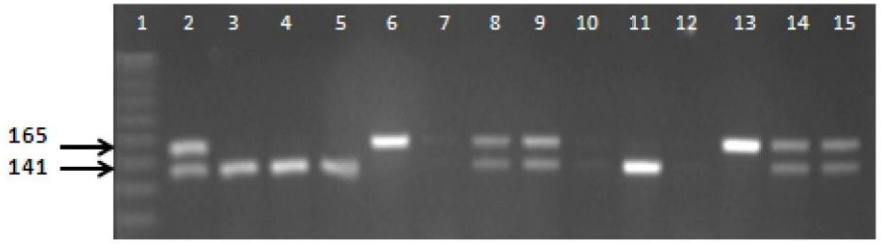

Figure 3: Gel electrophoresis of AGT T235M: samples after digestion with Tth 111 I, lanes 3,4,5,11 homozygote TT with one band 141 bp, lanes 6,13 are homozygote MM with one band 165 bp, lanes 2, 8,9,14,15 heterozygote MT with two bands at 141, and 165, lane 1 is 50 bp marker.

Table 1: demographic data of study participants—T2DM patients versus Controls

Characteristics	T2DM	Controls	p-value
Gender Male (%) Females (%)	 35 (60.34%) 23 (39.66%)	 56(70%) 24 (30%))	 0.085
Age (year)	46.53 ± 6.53	44.76 ± 7.21	0.141
Duration of diabetes	7.8 ± 2.1 years	-	-
Systolic blood pressure	136.90±18.02	132.57±13.52	0.191
Diastolic blood pressure	82.54±6.59	82.37±7.40	0.913
BMI(Kg/m^2)	28.83±3.38	27.27±3.54	0.246
Biochemical measurements:			
Fasting blood sugar (mg/dl)	163.62 ± 61.45	98.57 ± 10.19	<0.001*
HB A1C(%)	6.89±0.89	4.88±0.65	<0.001*
Cholesterol (mg/dl)	197.26 ± 41.18	187.98 ± 49.02	0.242
Triglycerides (mg/dl)	167.53 ± 91.48	128.76 ± 68.3	0.005*
HDL cholesterol (mg/dl)	38.40 ± 8.78	41.79 ± 15.06	0.299
LDL cholesterol (mg/dl)	128.85 ± 40.09	123.14 ± 43.67	0.103
Urea (mg/dl)	21.85 ± 9.08	22.22 ± 8.75	0.835
Creatinine (mg/dl)	0.91 ± 0.14	0.92 ± 0.16	0.502

***Significant p-value <0.05**

Table 2: The frequency of ACE and AGT genotypes in T2DM patients and controls.

Genotypes	T2DM No(%)	Controls No(%)	OR	95%CI	p-value
ACE I/D					
ACE -I/I	10(17.3%)	18(22.5%)	0.717	0.303-1.696	0.449
ACE -I/D	26(44.8%)	46(57.5%)	0.600	0.303-1.187	0.142
ACE -D/D	22(37.9%)	16(20%)	2.444	1.140-5.240	0.022*
D allel	0.603	0.497	1.599	0.986-2.596	0.057
AGT M235T					
AGT -M/M	11(19%)	23(28.8%)	0.580	0.257-1.311	0.191
AGT -M/T	22(37.9%)	44(55%)	0.500	0.251-0.996	0.048*
AGT -T/T	25(43.1%)	13(16.2%)	3.904	1.773-8.597	0.0007*
T allel	0.621	0.437	2.104	1.291-3.428	0.003*
Combined genotypes					
ACE -II/ AGT -MM	0(0%)	4(5%)	0.145	0.008-2.753	0.199
ACE -II/ AGT -MT	6(3%)	10(12.5%)	0.808	0.276-2.364	0.697
ACE -II/ AGT –TT	4(6.9%)	4(5%)	1.407	0.337-5.876	0.639
ACE -ID/ AGT -MM	6(10.3%)	17(21.3%)	0.428	0.157-1.163	0.096
ACE -ID/ AGT -MT	8(13.9%)	21(26.3%)	0.449	0.183-1.103	0.081
ACE -ID/ AGT –TT	12(20.6%)	8(10%)	2.348	0.892-6.182	0.084
ACE -DD/ AGT -MM	5(8.5%)	2(2.5%)	3.679	0.689-19.673	0.128
ACE -DD/ AGT -MT	8(13.8%)	13(16.3%)	0.824	0.318-2.141	0.692
ACE -DD/ AGT -TT	9(15.5%)	1(6.3%)	14.510	1.783-118.083	0.012*

*Significant p-value <0.05

4. Discussion:

Type 2 diabetes is a complex disorder accounting for about 90–95% of all diabetes syndromes. Despite numerous reports suggesting a substantial genetic contribution to the susceptibility of type 2 diabetes, no major susceptibility genes have been identified so far [18]. The aim of the current study was to investigate the association between genetic polymorphisms of ACE I/D and AGT M235T genes and T2DM in Egyptian population. To achieve this goal, ACE I/D and AGT M235T genotyping was performed by PCR and PCR-RFLP technique respectively.

In the present study, ACE I/D genotyping in T2DM revealed that 37.9% of patients had ACE DD homotype and 44.8% had ACE ID heterotypes. ACE-II genotype was higher in controls (22.5%) than T2DM patients (17.3%) suggesting a protective role for the ACE gene with decreasing incidence for T2DM. This result goes in accordance with the result of Yan Feng [19] on Chinese population with a frequency of 39.8% in diabetic patients and 44.8% in controls.

ACE-ID heterotype and D allel were detected in (44.8% and 60.3%) of T2DM compared to (57.5% and 49.7%) of controls with no statistically significant difference between the two groups p=0.142 and 0.057 respectively in agreement with the results of Yan Feng [19] and Einas et al. [20] who reported that the effect of the ID genotype did not reach a statistically significant level suggesting a recessive effect of the D allele and considering the high frequency of D allele in diabetic group an independent risk factor. Other population studies reported association of ACE I/D polymorphism with T2DM, thereby demonstrating geographical and racial/ethnic variations of ACE I/D polymorphism with T2DM [21–26].

ACE-DD homotype was higher in T2DM patients (37.9%) compared to controls (20%) with 2.4 fold risk for T2DM (OR=2.444, 95% CI 1.140-5.240, p =0.022). Similar results was obtained in previous study on Egyptian population which revealed higher frequency of DD genotype in diabetic patients (68 %) compared to controls (33.3 %) p =0.01 [27]. The positive association of ACE-DD genotype with T2DM was demonstrated in patients with diverse ethnic backgrounds such as Malaysian [28], British Caucasian [21], Arab Tunisians [29], Taiwanese [25] and a marginally significant association in a recent meta-analysis of a total of 41

studies (4708 cases and 5368 controls) in a Chinese population [30]. In contrast, negative association was found in Turkish [22] and Thailand [26]. The DD genotype is associated with twice the normal level of serum ACE activity [29]. Renin–angiotensin system (RAS) plays a central role in the regulation of sodium metabolism, vascular tone, blood pressure, renal hemodynamics, and vascular modeling. In diabetes mellitus, activation of the RAS by hyperglycemia may be the key mechanism and effects seem to be amplified with adverse consequences such as atherosclerosis and occlusive microangiopath. Suggestive evidence for this notion is the impressive beneficial effect of pharmacological interference with the RAS in large vessel disease as well as in renal and retinal microangiopathy [31].

In the current study regarding AGT/M235T gene polymorphism, AGT/MM homotype was significantly higher in control (28.7%) than in T2DM patients (19%) raising a protective role against occurrence of diabetes.

Our results have shown an association between AGT M235T polymorphism and T2DM. Then, the risk of developing diabetes seems to be higher for TT genotype and T allele compared to MM genotype and M allele. AGT-TT homotype was significantly higher in T2DM patients (43.1%) compared to controls (16.2%) p =0.0007 with 4 folds increased risk for T2DM OR=3.9, 95% CI 1.773-8.597 confirming a previous study on a Chinese population [32]. However, some studies failed to find this association [33,34]. Moreover, Chang et al. [35] reported an association between the AGT allele and diabetic nephropathy in Taiwan. But some researchers reported no obvious association between a specific AGT genotype and diabetic nephropathy [32,36]. The AGT T allele has been functionally related to increased AGT plasma levels [37]. Plasma AGT level is rather stable in one individual, but is under the long-term control of several hormones, such as glucocorticoids, estrogens, thyroid hormones, and Angiotensin II, which are known to induce AGT expression [38]

Some DNA polymorphisms may affect gene expression at both translational and transcriptional levels. However, the effect of a single polymorphism may be masked by interaction with environmental and genetic factors. AGT and ACE two important genes of RAS belonging to the same metabolic pathway may interact with each other. AGT and ACE polymorphisms affect the amount of their final products and, because of their different frequencies in various populations, may jointly contribute to a higher risk of T2DM and other diseases that is not seen with either polymorphism alone[29]. In the present study, ACE-DD/AGT-TT combined genotype was significantly higher in T2DM patients than control with marked increase in risk to disease occurrence (OR =14.510 p =0.012). This was in accordance with the results of Mehri et al.[29] and Young et al. [32] who reported a significant interaction between the AGT and ACE genes in T2DM patients. Also, previous studies revealed significant combinational effect of ACE ID and AGT M235T polymorphisms on conferring susceptibility to diabetic nephropathy in Japanese [39] and Asian Indian populations [40].

ACE-II/AGT-MM combined genotype was not present in T2DM patients and found in 22.2% of controls with decreased risk to diabetes. Thus it may have a protective role against susceptibility to T2DM. No statistically significant difference could be encountered between the patients and the controls regarding the frequency of the combined genotypes; ACE-DD/AGT-MM, ACE-DD/AGT-MT, ACE-ID/AGT-TT, ACE-ID/AGT-MM, ACE-ID/AGT-MT, ACE-II/AGT-MT and ACE-II/AGT-TT. These data strengthen the hypothesis that genotypic combinations are more important than single gene polymorphism alone. It is likely that there is an important interaction among these two RAS gene polymorphisms and T2DM since they are both part of the same metabolic pathway. However, because of their independent segregation in different populations, their contributions to the physiological and pathological processes in diabetes are likely to vary, depending on the genotypic combinations formed.

The limitation of our study was the relatively small sample size. The present study could have yielded more consistent results if included more patients and if treatment results were followed in relation to the genotyping. In conclusion, our study provides further evidence of a role for genetic variation in the ACE I/D and AGT M253T polymorphism with risk of T2DM in Egyptian population. ACE I/D and AGT M253T polymorphic markers may raise the hope to individualize ACE inhibitor therapy in order to optimize its effectiveness and to reduce adverse effects for genetically different subgroups. Diabetes remains an important focus of investigation.

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