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# Angiotensin Converting Enzyme (ACE) Gene Polymorphism in Jordanian Type-1 and Type-2 Diabetic Patients

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**Abstract**

Epithelial cells of the endothelial surfaces incorporate a gene for angiotensin-converting enzyme (ACE) that encodes metallopeptidase proteins. In this work, molecular characterization of the ACE gene by investigating the presence of two specific variants of 287 bp fragment (Alu element), have been studied. Therefore, the insertion (I allele) and the deletion (D allele) of this gene fragment were controlled. A group of Jordanian patients with Type-I and Type-II Diabetes Mellitus (DM) was selected for this study. The investigated sample contains 26 subjects with Type-I DM, 45 subjects with Type-II DM in addition to a control group of 44 healthy subjects. ACE genotypes of the patients and control groups were achieved using PCR of the DNA amplification. Results reveal no significant statistical differences in ACE genotype distribution among the focus group and the control one. Therefore, there is no significant association between DM and ACE genotype distribution variants of the proposed insertion. The ACE gene polymorphism is not directly correlated to diabetes.

**Keywords:** Angiotensin; ACE gene; Diabetes mellitus, Differential expression, Alu element.

## 1. Introduction

The epithelial cells of endothelial surfaces combine genes for angiotensin-converting enzymes (ACE) that encrypt a metallopeptidase. In this research, the molecular characterization of ACE has been studied by the means of the hypothesis of presence and absence of two specific variants of 287 bp fragment based on the previous researches (Castellon and Hamdi, 2007; Hamdi and Castellon, 2003; Hamdi, *et al*., 2002). It has been based on the hypothesis of the insertion of (I allele) and the deletion of (D allele) of this gene fragment.

In order to test this hypothesis, a focus group of Jordanian patients with Type-I and Type-II Diabetes Mellitus (DM) have been used. In addition, a control group of 44 healthy subjects, and a focus group contain 26 subjects with type 1 diabetes mellitus (T1DM) and 45 with type 2 diabetes mellitus (T2DM).

Diabetes mellitus (DM) is a metabolic chronic health illness identified by a raised blood sugar levels (Zhong, *et al*., 2015). DM has been categorized into two main groups; group one is called type 1 diabetes mellitus and it has been known and denoted by (T1DM) and the second group is called type 2 diabetes mellitus and it has been known and denoted by (T2DM) (Piero, *et al*., 2015; Saucă, *et al*., 2012). Diabetes mellitus patients suffer from various health difficulties such as diabetic nephropathy, diabetic retinopathy and cardiovascular disease (CVD) (Golmohamadi, *et al*., 2006)

The angiotensin-converting enzyme (ACE) (EC 3.4.15.1) plays a vital role in inducing the conversion of angiotensin I to angiotensin II, which is the primary effector molecule of the Renin-Angiotensin-Aldosterone System (RAAS) (Golmohamadi, *et al*., 2006; Song and Lee, 2015). Angiotensin II allows for the constriction of blood vessels leading to an increment of blood pressure.

*ACE* gene polymorphism is classified based on either the presence/insertion, denoted (I), or absence/deletion, denoted (D) of 287 bp *Alu* elements at intron 16 of chromosome 17 which results in three different genotypes; DD and II homozygotes and ID heterozygote. Plasma ACE levels are 30% and 60% higher in the ID heterozygotes and DD homozygotes, respectively, when compared to the II homozygotes. Therefore, individuals who carry the DD and ID genotypes have a more active Renin-Angiotensin-Aldosterone System (RAAS) (Al-Serri, *et al*., 2015).

Various research groups have investigated the correlation of the *ACE* gene I/D polymorphism and T1DM and T2DM. However, the results are inconsistent. Some studies did not find a relative correlation between *ACE* gene polymorphism and developing DM or one or more of its complications (Jayapalan, *et al*., 2010; Kumar, *et al*., 2013; Pasha, *et al*., 2002; Schmidt, *et al*., 1995; Zhou, *et*

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*al*., 2012). On the other hand, other studies suggested that the DD genotype marks for higher risk of certain diseases like diabetic nephropathy and hypertension (Tseng, *et al*., 2011). Huang and colleagues (2001) showed that an increment in ACE gene levels is adequate to develop nephropathy in diabetic mice (Huang, *et al*., 2001). In addition, the DD genotype was abided to increase risk of severe hypoglycemia in T1D patients (Freathy, *et al*., 2007).

Various researches explored the diversity of the ACE gene haplotypes. It has been shown that the frequency distributions of the haplotypes vary among people in different countries (Farheen, *et al*., 2011). The D allele was found to be present more frequently in Africans and Caucasians, while the I allele is present more frequently among Asians (Farheen, *et al*., 2011). The frequency distributions of ACE gene haplotypes were studied in few Arab populations (Al-Hinai, *et al*., 2002; Frossard, *et al*., 1997; Motawi, *et al*., 2016) but not in Jordanians. In addition, the relation between the ACE gene polymorphism insertion/deletion and T2DM have been studied and discussed in some Arab populations but not among Jordanian DM patients (Al-Harbi, *et al*., 2012; Al-Rubeaan, *et al*., 2013; Al-Serri, *et al*., 2015; Alsafar, *et al*., 2015; Chmaisse, *et al*., 2009). Therefore, we aimed at investigating the relationship between *ACE* I/D polymorphism and T1DM and T2DM Jordanian patients. Additionally, we aimed at investigating possible roles of *ACE* expression in DM through a broad gene expression analysis of a set of available large-scale National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) datasets.

## 2. Materials and methods

### *2.1. Study subjects*

Seventy-one type 1 (n=26) and 2 diabetes mellitus (n=45) patients were recruited from three hospitals; Jordan Hospital, Dr Jameel Tutanji Hospital, and the University of Jordan Hospital, Amman, Jordan. The inclusion criteria for the current study were: T1DM and T2DM patients (who already diagnosed by consultant diabetologist), being treated for DM without any other complication(s) beside DM. All patients completed informed consent. Information regarding age, treatment and complications was collected from patients using a written questionnaire or interview. 44 subjects were recruited as Age-matched healthy control subjects (n=44). This study was approved by the Institutional Review Board (IRB), Faculty of Medicine at the University of Jordan, that conforms to the World Medical Association Declaration of Helsinki.

### *2.2. ACE genotyping*

Each participant provided a sample of 3 mL of peripheral blood which has been withdrawn on EDTA tube using a venous puncture, followed by the DNA extraction from 300 µL blood; this has been performed using a commercially kit (AccuVis Bio, UAE).

Genomic DNA from each of the control and DM patients groups analyzed by amplification of polymerase chain reaction (PCR) in 30 µL cuvette that includes various components: 1 µL of forward primers (GGACTCTGTAAGCCACTGCTGGAGACC), 1 µL of reverse primers (GGATGTGGCCATCACATTCGTCAGAT) , 8 µL of nuclease-free water, 15 µL of the master mix (New England Biolabs, USA) and finally 5 µL of DNA sample.

The PCR reaction recipe was 35 cycles of denaturation at 96 $^{o}$C for 30 seconds, followed by annealing for another 30 seconds and finally elongation at 68 $^{o}$C for 30 seconds (Hamdi, *et al*., 2002). This reaction has been conducted using a thermal cycler (MyCycler) (Bio-Rad, USA). The phase of amplification of the DNA was stopped by adding 0.25 M EDTA on ice. Electrophoresis has been performed on the amplified products on 2% (w/v) agarose gel.

### *2.3. Analysis of NCBI Gene Omnibus (GEO) datasets*

In this work, we analyzed three preprocessed and normalized DNA microarrays from the NCBI GEO project. Two T1D datasets; GSE43488 (Lietzen, *et al*., 2018) with 357 samples from 18 autoantibody-positive children (Case) and their matched controls (Control) and GSE30210 (Kallionpää, *et al*., 2014) with 247 samples from 18 prediabetes children and their matched controls. The last dataset GSE9006 (Kaizer, *et al*., 2007) is a super series with 234 samples belonging to three classes; T1DM (162 samples), T1DM (24 samples), and healthy samples (48 samples).

### *2.4. Statistical analysis*

Based on the verification of the Hardy-Weinberg equilibrium, the obtained genotypes and alleles frequencies were compared with predicted frequencies. The Statistical software, StatSoft Inc, Tulsa, OK, USA (version 7.0) has been used to perform both of Chi-square test and Fisher's exact test to obtain the polymorphism frequency. The analysis of NCBI GEO datasets was performed using the R-cran statistical environment similar to the method described in (Barghash, *et al*., 2016). Differential gene expression analysis was checked using the Kolmogorov–Smirnov (KS) test. Bear in mind, that all tests were two-sided, with the assumption of any obtained value of $p<0.05$ would be considered statistically significant.

## 3. Results

DNA was successfully extracted from 44 control subjects, 26 T1DM patients and 45 T2DM patients (Figure 1).

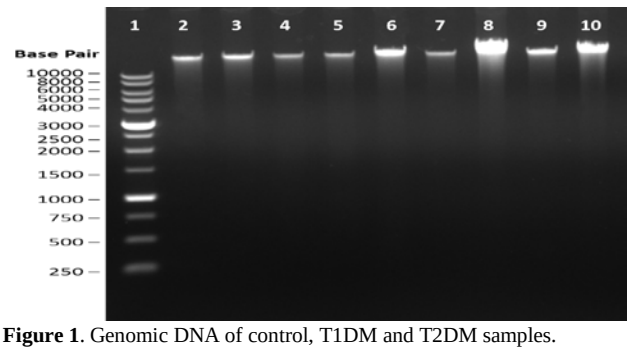


**Figure 1**. Genomic DNA of control, T1DM and T2DM samples. Lane 1: 1 kb DNA molecular weight marker; Control: Lanes 2-4; T1DM: Lanes 5-7; T2DM: Lanes 8-10.

The PCR amplification products of the 287-bp Alu region of the ACE gene are shown in Figure 2.

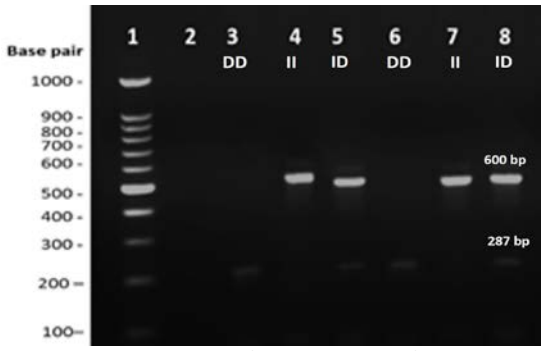


**Figure 2**. illustrates an amplified Alu segment of the ACE gene that exploits insertion (II) and/or deletion (DD) segment. Lane 1:100 bp Ladder, Lane 2: negative control, Lanes 3 and 6: Alu deletion (DD) at 287 bp, Lanes 4 and 7: Alu insertion (II) at 600 bp, Lanes 5 and 8: Alu insertion and deletion (ID) at 287 bp and 600 bp.

The PCR amplification revealed results that indicate all three genotypes, II (600bp), ID (600/287bp), DD (287bp) were observed in each of the control and DM patients. The highest genotype frequency in control and T1DM patients was the DD genotype (77.3% and 56.5%, respectively) (Table 1). The opposite way, the ID genotype was the most frequent genotype (62%) among T2DM patients (Table 1). Alternatively, the obtained results showed no significant difference in the I and D allelic frequency between the non-diabetic and diabetic groups (Table 1).

**Table1**. Genotypic and allelic distributions of the ACE gene in control and diabetic populations.

| | **Genotype** | | | **Allele** | |
|---|---|---|---|---|---|
| | **II** | **ID** | **DD** | **I** | **D** |
| **Control** | 11.4% | 11.4% | 77.2% | 0.17% | 0.83% |
| **T1DM** | 8.7% | 34.8% | 56.5% | 0.26% | 0.74% |
| **T2DM** | 2.0% | 62.0% | 36.0% | 0.20% | 0.80% |
| **P value** | 0.2501 | 0.0164 | 0.0435 | 0.3628 | 0.3628 |

Similarly, we observed that ACE did not show differential expression between the case and control samples in any of the analyzed GEO datasets where KS test consistently resulted in very high p-values. Figure 3 A and B presents a similar expression behavior in T1DM and healthy samples.

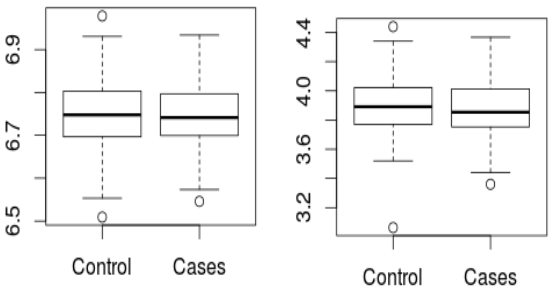


**Figure 3**. Analysis of ACE gene expression in GEO datasets GSE30210 (left) and GSE43488 (right). No differential expression is detected in ACE expression in GEO datasets GSE30210 or GSE43488.

Additionally, we analyzed GSE9006 which contains a mixture of healthy, T1DM, and T1DM samples to get an overall idea about the possible occurring change in ACE expression. However, no differential expression was detected between the analyzed sample types as presented in figure 4.

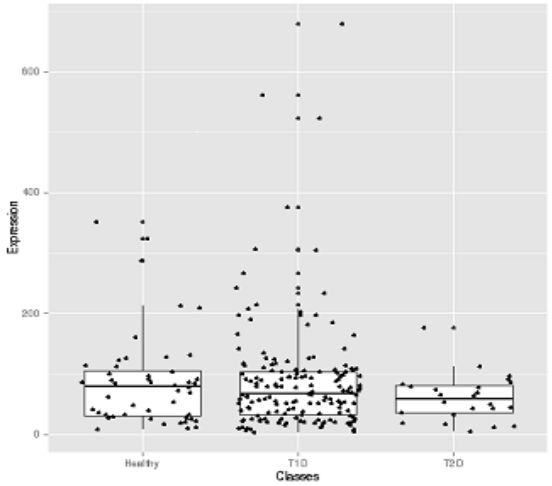


**Figure 4**. Analysis of ACE gene expression in GEO dataset GSE9006. No differential gene expression is found between the Healthy, T1D, and T2D classes

## 4. Discussion

Angiotensin-converting enzyme (ACE) cleaves angiotensin I protein converting it into the active angiotensin II hormone. Angiotensin II allows for the constriction of blood vessels; this leads an increment in blood pressure. The ACE gene is considered to be part of either the Renin-Angiotensin-Aldosterone System (RAAS) or Renin-Angiotensin System (RAS). RAAS is normally involved in regulating blood pressure and maintaining balanced body fluids and salts. As a part of RAAS, angiotensin II induces the delivery of hormone aldosterone. Aldosterone, in turn, induces the kidneys to absorb salt and water (Remuzzi, *et al*., 2005). An increment in ACE levels has been noted and correlated to induce nephropathy in diabetic mice (Huang, *et al*., 2001).

Genetic polymorphism is the presence of genetic variations that give different forms of individuals in a population of a certain species. Genetic polymorphism is one cause of diversity. In addition, polymorphism plays a leading role in diseases, such as cardiovascular and age-related diseases. The risk of developing various diseases can be increased many times by inheritance of risk alleles of the various genes at susceptibility loci (Ma, *et al*.).

An insertion/deletion polymorphism is one type of genetic polymorphism where a specific nucleotide sequence in a gene is either insertion or is deletion, i.e. presence or absence. The gene variant with the insertion of the sequence is called the insertion (I) allele, while the other variant that does not have the sequence is called the deletion (D) allele. The first report mentioning ACE I/D polymorphism has been released in 1990 by Rigat and colleagues (Rigat, *et al*., 1990). ACE I/D polymorphism involves either the insertion or deletion of 287 base pairs (*Alu* element) at intron 16 of chromosome 17. ACE I/D polymorphism is the most common type of genetic variations encountered in the RAAS system (Ma, *et al*.). Each person has two copies of the ACE gene, one from each parent. Therefore, each individual could have two D-alleles (DD), or two I-alleles (II), or one I-allele and one D-allele (ID).

Individuals that carry the DD genotype produce more ACE protein and therefore have a more active RAAS system (van Zuydam, *et al*., 2018). Many studies show that the II genotype provides a protective advantage to diabetes type 1 and 2 patients against developing nephropathy (Ha, 2014). In addition, the D allele was found to be present more frequently in Africans and Caucasians, while the I allele is present more frequently among Asians (Ma, *et al*.). It has been proposed that the DD genotype marks for higher risk of certain diseases like diabetic nephropathy and hypertension (Marre, *et al*., 1995; Tseng, *et al*., 2011), as well as increased risk of severe hypoglycemia in T1D patients (Freathy, *et al*., 2007; Pena, *et al*., 2012), but not for T2D patients (Freathy, *et al*., 2007). However, another study found that Taiwanese T2D patients who carry the DD genotype and have other risk factors like hypertension and smoking are at significate risk of peripheral arterial Disease (Tseng *et al*., 2012). The D allele of the ACE gene I/D polymorphism was found to be linked to Diabetes Type 2 in Caucasian male patients (Stephens, *et al*., 2006)

However, other researchers reported a null association between ACE I/D polymorphisms and the susceptibility of T1DM patients to nephropathy (Elhawary, *et al*., 2011; Hibberd, *et al*., 1997) or T2DM patients (Jayapalan, *et al*., 2010). It should be stated that differences in results by the different researchers regarding ACE I/D polymorphism and development of DM owe to ethnic differences, duration of sickness, and the interaction with the environment (van Zuydam, *et al*., 2018).

The results achieved in our study were found to be contradictory to most studies -up to our knowledge- that revealed an association between *ACE* DD genotype and DM (Table 1). Our result revealed a decrement in the frequency of the *ACE* DD genotype in the diabetic groups when correlated to the control group ($p<0.05$) (Table 1). This was in accordance with many studies (Doshi, *et al*., 2015; Jayapalan, *et al*., 2010; Schmidt, *et al*., 1995; Sikdar, *et al*., 2013), but against the findings of others (Baroudi, *et al*., 2009; Ergen, *et al*., 2004; Feng, *et al*., 2002) who found that *ACE* DD genotype in the T2DM is greater than the control group in Turkish, Tunisian and Chinese population, respectively. Nevertheless, the results did not provide any significant association between the I or D alleles of the *ACE* gene and DM (Table 1).

In conclusion and due to the limited number of blood samples collected, a firm understanding on the association of ACE I/D polymorphism and diabetes mellitus could not be reached. Nevertheless, there are around 450 genes associated with diabetes mellitus type 1 based on the susceptibility genes of this disease. These genes were identified using a genome-wide based association analysis, differential expression analysis, replication studies, and functional annotation clustering analysis (Qiu, *et al*., 2014). Those novel-risk genes associated with T1DM imply how important the current analysis is in either predicting or detecting disease susceptibility across the human genome.

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