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RESEARCH ARTICLE

# Association of *eNOS* and *ACE* gene polymorphisms as a genetic risk factor in gestational diabetes in Iranian women

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## Abstract

**Background** Gestational diabetes mellitus (GDM) is the most popular metabolic disease during pregnancy. The aim of the present study was to investigate any possible association between *eNOS* Glu298Asp and *ACE* I/D gene polymorphisms and the risk of GDM in a group of Iranian pregnant women.

**Methods** In this case-control study 204 pregnant women were recruited (94 cases and 110 controls). Genomic DNA was isolated from whole blood and genotyping was performed by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR- RFLP) and only PCR for *eNOS* and *ACE* polymorphisms respectively.

**Results** Frequencies of GT and TT genotype of *eNOS* polymorphism among women with and without GDM were 67.90% vs. 74.47 and 7.41% vs. 8.51% respectively ($P = 0.4$). Corresponding figures for DD genotype of *ACE* polymorphism among GDM patients was more than that in healthy women (51.65% vs. 63.81% respectively). Conversely, ACE heterozygote genotype was more common in diabetic women (35.16% vs. 26.67% respectively). Although these differences were not statistically significant ($P = 0.2$).

**Conclusions** Our study showed that there is no association between the presence of *eNOS* and *ACE* gene polymorphisms and developing gestational diabetes mellitus among pregnant women in our population. Further longitudinal and multicenter studies should be carried out to assess the exact metabolic effects of these polymorphisms.

**Keywords** Gestational diabetes · Polymorphism · *eNOS* · Angiotensin I converting enzyme

## Introduction

Gestational Diabetes Mellitus (GDM), recognized as glucose intolerance in during pregnancy, is one of the most common medical problems pregnancy women [1, 2]. The exact mechanisms GDM still remain unknown, and there are few studies of the genetic susceptibility to GDM [3]. Genome wide association studies have shown that association of type 2 diabetes susceptibility the genes with gestational diabetes mellitus. Therefore, a possibility of the genetic structure of type 2 diabetes and gestational diabetes are, in part, similar [3, 4]. Nitric oxide derived from the endothelium plays a key role in regulating vascular tone [5]. Reduction in the secretion of basal NO may predispose people to cardiovascular disorders, Also, additional production can also damage tissue and cells [6, 7]. In the endothelium, no is synthesized mainly by endothelial nitric oxide synthase (*eNOS*) (OMIM: 607496) isoform [6]. *eNOS* gene variants that show changes in the expression and activity, Therefore, are leading to reduce or overproduction of

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NO and is associated with the development of insulin resistance and type 2 diabetes [8, 9]. Endothelial Nitric Oxide Synthase (*eNOS*) located at 7q35–36 [10]. Among the multiple polymorphisms determined in the *eNOS* gene, the 894G/T (Glu298Asp) missense substitution in exon 7 (rs1799983), researched in relation to microvascular complications of diabetes [11]. Angiotensin Converting Enzyme (*ACE*) (OMIM: 106180) can convert angiotensin I to vasoconstrictor angiotensin II. ACE serum levels influence the renin-angiotensin system [12]. The *ACE* gene is located on 17q23 which contain an Insertion/Deletion (I/D) gene polymorphism [13]. The three different *ACE* genotype DD, ID, II shows highest, intermediate and lowest serum *ACE* level respectively [14]. *ACE* I/D polymorphism is shown to be related with several complications such as coronary artery disease, hypertension, diabetic nephropathy, type 2 diabetes mellitus [15].

In this study, we characterized for the first time the relationship between the *eNOS* and *ACE* gene polymorphisms and the risk of gestational diabetes in GDM women.

## Material and methods

### Subjects and DNA extraction

In this case control study 204 pregnant women were recruited including 94 women who had developed GDM and 110 women with normal glucose levels during pregnancy between Feb 2014 and April 2015 from two prenatal clinics in Karaj, Iran. Written informed consent was obtained from all participants. The study was approved by the ethical committee of Karaj Branch, Islamic Azad University. Oral glucose tolerance test (OGTT) 75 g between 24th–28th weeks of gestation were done for all women and it is interpreted by an internist. According American Diabetes Association (ADA) guideline 2014 inclusion criteria for GDM group were nearly diagnosed GDM, based on the results of an elevated 2 h 75 g oral glucose challenge test (OGCT), without type 1 diabetes mellitus (T1DM), T2DM and for all participants no other disease such as asthma, multiple sclerosis (MS), heart problems, psychological problems and fill out consent form. Screening and management of diabetes during pregnancy were done by a qualified physician according to the ADA guidelines 2014. Pregnant women with any history of type 1 or type 2 diabetes mellitus were excluded. Demographic, clinical and other information including family history of diabetes, history of GDM, macrosomia, abortion, obesity, gestational hypertension and fetal death was obtained through a structured questionnaire. Blood samples were taken using sample tubes containing EDTA and DNA extraction was performed according to the standard protocol of salting out.

### Genotyping for eNOS Glu298Asp polymorphism

The polymorphic site of the *eNOS* gene was amplified in a total volume of 25 μl by the use of each primer: forward primer, 5'-CATGAGGCTCAGCCCCAGAAC-3′, and reverse primer, 5'-AGTCAATCCCTTTGGTGCTCAC-3′. DNA was amplified for 30 cycles, each cycle comprising denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min with a final extension time of 5 min at 72 °C. The initial denaturation stage was carried out at 95 °C for 5 min. PCR products were digested with the restriction enzyme MboI at 37 °C for 16 h. In the presence of a T at nucleotide 894, which corresponds to Asp 298, the 206 bp PCR products were cleaved into 2 fragments of 119 and 87 bp. The fragments were separated on a 2.5% agarose gel and analyzed in gel documentation system.

### Genotyping for ACE I/D polymorphism

The polymorphic site of the *ACE* gene was amplified in a total volume of 25 μl by the use of each primer: forward primer, 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3′, and reverse primer, 5′-GAT GTG GCC ATC TTC GTC AGA T-3′. DNA was amplified for 30 cycles by a predenaturation at 95 °C and denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min with a final extension time of 5 min at 72 °C. The PCR products were run on a 2% agarose gel and analyzed in gel documentation system.

The *ACE* I/D genotypes were characterized by the length of the PCR product, where a 190 bp was obtained in the homozygous cases of the deletion (D) and 490 bp in the homozygous cases for the insertion (I) and both 190 and 490 bp bands in the heterozygotes (I/D). The validity of these results was confirmed by direct sequencing of several PCR samples with each genotype which are randomly selected.

### Statistical analysis

Categorical and continuous variables were compared between patients with and without gestational diabetes mellitus using Chi-square and independent T-tests respectively. The association between the presence of polymorphism and suffering from GDM was assessed using univariate and multivariate (adjusting for age, body mass index and familial history) logistic regression models. All data analyses were conducted using the STATA V.11 software. The *P*-value of less than 0.05 was considered statistically significant.

**Data availability** In case of contact with the author, information will be provided.

## Results

Totally, 204 pregnant women were recruited in the study with the mean (SD) age 30.02 (5.16). Of them, 96(47.06%) suffered from gestational diabetes mellitus. Other characters; familial history, BMI, and FBS was statistically analyzed too (Table 1). As illustrated in Table 2, Frequencies of GT genotype of *eNOS* polymorphism among women with and without GDM were 67.90 and 74.47%, respectively. Corresponding figures for the TT genotype were 7.41 and 8.51% respectively ($P = 0.4$). The odds ratio for the association between the presence of GT genotype and GDM was 0.63 ($P = 0.2$). As well as, the frequency of the DD genotype of *ACE* polymorphism among GDM patients was more than that in healthy women (51.65% vs. 63.81% respectively). Conversely, the heterozygote genotype was more common in diabetic women (35.16% vs. 26.67% respectively). Although these differences were not statistically significant ($P = 0.2$).

Univariate analyses showed that ID genotype decreased the odds of developing GDM about 5% while DD genotype decreased the odds more than 40%, although none of these associations were significant (OR = 0.95, $P = 0.9$ & OR = 0.58, $P = 0.2$) (Table 2).

For *eNOS* polymorphism, 41.36% of GDM patients and 45.47% of healthy pregnant women were T allele carriers ($P = 0.4$). And for *ACE* polymorphism, GDM patients had a lower frequency of the D allele compared to healthy women (69.23% vs. 77.14%, respectively), although the difference was borderline significant ($P = 0.07$) (Table 3).

**Table 1** Demographic and clinical factors among pregnant women with and without GDM

| Demographic and clinical factors | Healthy women | GMD | P-value |
|---|---|---|---|
| Abortion | | | |
| No | 107(99.07) | 92(95.83) | 0.1 |
| Yes | 1(0.93) | 4(4.17) | |
| Parity | | | |
| < 3 | 86(78.72) | 64(67.90) | 0.1 |
| > =3 | 23(21.28) | 31(32.10) | |
| Familial history | | | |
| No | 77(71.30) | 47(48.96) | 0.001 |
| Yes | 31(28.70) | 49(51.04) | |
| BMI | | | |
| Mean(SD) | 31.65(27.63) | 30.58(4.26) | 0.7 |
| FBS | | | |
| Mean(SD) | 81.73(8.39) | 102.13(23.69) | <0.0001 |
| Age | | | |
| Mean(SD) | 28.90(5.29) | 31.29(4.72) | 0.0009 |

Bold values indicate that the values have not statistical significant

GMD; Gestational diabetes mellitus

## Discussions

Genetic studies on type 2 diabetes show that it is a multifactorial disease in which multiple genes interact with environmental factors. Women with a positive family history of GDM are at an increased risk of developing type 2 diabetes. It is reasonable to assume that GDM may share some risk factors, including genetic factors with type 2 diabetes.

However, the genetic basis of GDM is little known. The *eNOS* and *ACE* activity have been associated with glucose metabolism and development of insulin resistance and type 2 diabetes respectively [8, 16].

We aimed to evaluate the association of *eNOS* Glu298Asp and *ACE* I/D gene polymorphism with GDM in Iranian GDM women. Several studies have been conducted to investigate the relationship between *eNOS* gene polymorphism and multiple diseases; Ilhan N et al. did not observe any association between the Glu298Asp polymorphism in the *eNOS* gene and end-stage Renal Diseases [17]. Martinelli NC et al. showed that the -786C/4b/Asp298 *eNOS* haplotype had a significant impact on heart failure susceptibility and prognosis, particularly in African-Brazilian patients [18]. But some association studies have also been done on *eNOS* and *ACE* gene polymorphisms and the risk of type 2 diabetes mellitus. For the *eNOS* gene Thameem et al. indicated that T-786C, Glu298Asp, and 27 bp-VNTR polymorphisms are associated with T2DM and its related traits in Mexican Americans [19]. Hou H et al. showed that there is some association between *eNOS* Glu298Asp polymorphism and the risk of T2DM [20]. Until now, there is no publication for investigating the possible association between *eNOS* polymorphisms and GDM. But, one study Atay AE et al. announced that G894 T polymorphism of the *eNOS* gene influenced on NO concentration that it seems to be an independent predictors of increased urinary excretion of albumin in patients with GDM. Determining the frequency of eNOS gene G894 T polymorphism may help to identify pregnancies at increased risk of microalbuminuria [21].

Our study showed that there is no difference between pregnant women with and without gestational diabetes mellitus regarding polymorphisms and alleles of *eNOS* gene polymorphism. Although women with GDM had higher rates of the alleles of *eNOS* gene polymorphism, these differences were not statistically significant. That was the case for different genotypes of this polymorphism. Univariate and multivariate regression models showed that homozygote and heterozygote genotypes of *eNOS* gene polymorphism decreased the risk of developing GDM as of 37 and 26% respectively. Although none of these associations were statistically significant indicating that this polymorphism cannot be considered as a protective factor for gestational diabetes mellitus. Similarly, the presence of T allele of *eNOS* gene polymorphism caused a 16% reduction in the odds of experiencing GDM. However, this negative effect was not statistically significant.

**Table 2** Crude and adjusted associations between different polymorphisms and GDM

| Polymorphism | genotype | Healthy Women | GDM | *P*-value | crude odds ratio (95% CI) | adjusted odds (95% CI) |
|---|---|---|---|---|---|---|
| *eNOS* | GG | 18(17.02) | 23(24.69) | | 1 | 1 |
| | GT | 82(74.47) | 64(67.90) | 0.4 | 0.63(0.30–1.32) | 0.74(0.331.70) |
| | TT | 10(8.51) | 7(7.41) | | 0.60(0.17–2.08) | 0.63(0.162.48) |
| *ACE* | II | 10(9.52) | 12(13.19) | | 1 | 1 |
| | ID | 28(26.67) | 32(35.16) | 0.2 | 0.95(0.36–2.54) | 0.80(0.27–2.34) |
| | DD | 67(63.81) | 47(51.65) | | 0.58(0.23–1.46) | 0.50(0.19–1.36) |

*adjusted for age, body mass index and familial history of GDM

In the case of ACE I/D polymorphism and diabetes mellitus, Makoto Daimon et al. 2003 reported that the frequency of DM in DD and ID genotypes of *ACE* I/D polymorphism was significantly different from that in the II genotypes among Japanese cases [22]. Degirmenci et al. 2005 reported no significant difference in the *ACE* I/D genotype frequencies among type 2 diabetic patients and healthy individuals [23] and the higher frequency of the DD genotype among type2 diabetes was observed in a Taiwanese population [24], but in the polish individuals, *ACE* II homozygosity was a significant predictor of obesity and T2DM [25].

There are only two studies which investigate any relation between *ACE* I/D gene polymorphism and developing GDM. A case-control study by Dostalovaz et al. on 48 GDM cases 53 healthy women did not support a significant association in the genotypes distribution or allele frequency in controls and GDM women, so the *ACE* I/D polymorphism can't be considered as a genetic risk factor for GDM [26]. In the other study by Imran a Khan et al., the genotype frequency of *ACE* I/D is significantly different between GDM and Non-GDM individuals [27].

As well as, in this study ID genotype of *ACE* gene polymorphism was more common in diabetic women ($P = 0.2$) and the frequency of the DD genotype in GDM women more than that in healthy women. But, these differences were not statistically significant ($P = 0.2$). The D allele of the *ACE* gene I/D polymorphism cannot be shown to be a protective factor for developing GDM in a population-based Iranian sample. Our results of *ACE* I/D genotype distribution in GDM women are consistent with the result of Dostavola Z et al. [26]. Totally this result obtained are inconsistent that can be proved by the impact of ethnic variation in the *ACE* alleles distribution in different population and parts may be down to small sample size. In regards, GDM and T2DM have the multifunctional nature, so it is difficult to analysis GDM susceptibility factors as the environmental factor is not directly under control and can be influenced on the penetrance of many genes. As the T2DM and GDM may be sharing the same molecular etiology. Further studies on the genetic loci in several genes responsible for insulin resistance and beta cell dysfunction pathways which have been studies on T2DM will address main gaps in the GDM molecular etiology. The identification of the variations in these genes can be favored in the prevention and management of GDM and, therefore, beneficial in some way.

Understanding the genetic basis of GDM is very important because women with a positive family history of GDM are at an increased risk of developing type 2 diabetes.

## Conclusion

In conclusion, our study showed that there is no association between the presence of *eNOS* gene polymorphism and developing gestational diabetes mellitus among pregnant women.

As well as, the result of this study suggests that gestational diabetes development is not associated with the I/D polymorphism of the *ACE* gene in our population. The difference in the result of ACE polymorphisms between studies may be related to ethnicity. Further longitudinal and multicenter studies should be carried out to assess the exact metabolic effects of these polymorphisms.

**Table 3** Association between alleles of different gene polymorphisms and GDM

| | Polymorphism | Alleles | Healthy women | GDM | *P*-value | OR (95% CI) |
|---|---|---|---|---|---|---|
| *eNOS* | G | 119(54.25) | 111(58.64) | 0.4 | 1 | |
| | T | 100(45.74) | 78(41.36) | | | 0.84(0.53–1.31) |
| ACE | I | 49(22.86) | 58(30.77) | 0.07 | 1 | |
| | D | 169(77.14) | 132(69.23) | | | 0.67(0.411.07) |

Data are mean ± SD

[a]Student's *t*- test versus controls

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**Authors' contributions** MM: conceived and designed the present study and gave final approval and critically revised the manuscript; MH: designed the Genetic study; MS: technical Genetic laboratory; MA: Statistical analysis; DA: sample collection & clinical study; ZA: sample collection; ZB: sample collection; AB: sample collection.

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## Compliance with ethical standards

**Competing interests** The authors declare that there is no conflict of interest.

**Consent for publication** Oral and written consent were obtained from the patients.

**Abbreviations** T2D, Type 2 Diabetes; GDM, Gestational Diabetes Mellitus; *eNOS*, endothelial Nitric Oxide Synthase; *ACE*, Angiotensin Converting Enzyme; OGCT, Oral Glucose Challenge Test; T1DM, Type 1 Diabetes Mellitus; BMI, Body Mass Index; ADA, American Diabetes Association; I/D, Insertion/Deletion.

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