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ORIGINAL ARTICLE

Angiotensin converting enzyme gene polymorphism studies: A case-control study

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Abstract Mild gestational hyperglycemia (MGH) is a very common complication of pregnancy that is characterized by intolerance to glucose. The association of angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism to MGH has been previously reported. In this study, we evaluated the association between *ACE* polymorphism and the risk of MGH in a Saudi population. We conducted a case-control study in a population of 100 MGH patients and 100 control subjects. *ACE* gene polymorphism was analyzed by the novel approach of tetraprimer amplification refractory mutation system (ARMS)-polymerase chain reaction (PCR). The frequency of *ACE* polymorphism was not associated with either alleles or genotypes in MGH patients. Glucose concentration was found to be significantly associated with the MGH group. Our study suggests that ACE genotypes were not associated with *ACE* polymorphism in a Saudi population.

1. Introduction

Mild gestational hyperglycemia (MGH) or gestational diabetes is a common complication that is characterized by glucose intolerance manifested during the initial phase of pregnancy (Gilder et al., 2014). The prevalence of MGH increases proportional to the rate of obesity and type 2 DM (T2DM), and women with MGH are at an increased risk of developing T2DM (Weinert et al., 2014). The prevalence of MGH varies in different populations or ethnic groups; in fact, the prevalence rate is 18.7% (Khan et al., 2013; Wahabi et al., 2013). The known risk factors for MGH include pre-pregnancy overweight, obesity, advanced maternal age, and a family history of T2DM or MGH (Zhang et al., 2013). The genetic background of T2DM may be applied to MGH because significant evidence demonstrates the prevalence of T2DM in women with MGH (Chon et al., 2013). MGH and T2DM are heterogeneous disorders, and the common pathophysiology and genetic variants between them can be used to determine the risk of T2DM, which may in turn be associated with the prevalence of MGH (Cho et al., 2009). Normal human pregnancy is characterized with hyperinsulinemia and a progressive decline in insulin sensitivity, which is compensated by increased insulin secretion to maintain the glucose homeostasis (Fallucca et al., 2006; Tok et al., 2006). Several studies have revealed common gene polymorphisms between MGH and T2DM (Khan et al., 2014). Recently, several parallel studies were performed to establish the relationship among functional variants

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of genome-wide association studies (GWAS), linkage scan analysis, and candidate genes in T2DM. Angiotensin-converting enzyme (*ACE*) gene is an important gene that influences the vascular structure, and abnormalities in *ACE* may result in T2DM (Al-Harbi et al., 2012), diabetic nephropathy (Rahimi, 2012), end-stage renal disease (Zhou et al., 2012), and gestational diabetes (Khan et al., 2013). *ACE* activity was strongly influenced by ACE polymorphism; specifically, I/D polymorphism of ACE accounted for 47% of the total phenotypic variance of plasma ACE. Subjects with DD (Deletion/Deletion) allele showed two-time higher ACE activity than subjects with an Insertion/Insertion (II) allele Purnamasari et al., 2012. The rennin–angiotensin system (RAS) (renin, angiotensin I, angiotensin II, and ACE) plays an important role in regulating the cardiovascular homeostasis (Xie et al., 2013). The human *ACE* is located on the chromosome 17q23 and has several variants such as rs4646994, rs8066114, and rs4461142. One of the most important ACE polymorphisms is a 287 bp Alu I/D sequence polymorphism in intron 16; it is a nonsense, repetitive DNA domain that leads to the generation of three genotypes: II, ID, and DD (Lin et al., 2014; Simsek et al., 2013). There exists no report of effect of *ACE* polymorphism on MGH risk in a Saudi population. Therefore, we aimed to investigate *ACE* polymorphism, discovered and described by Rigat et al. (1992) in pregnant women with MGH.

2. Materials and methods

2.1. Screening

A case-control study was conducted in pregnant women to estimate their blood glucose values during pregnancy to test for MGH as well as to perform insertion and polymorphism studies. The study group comprised of 100 unrelated pregnant women with MGH and 100 unrelated healthy controls selected from the Department of Gynecology and Obstetrics. All women participants in this study provided a signed written informed consent form before collection of their blood samples.

2.2. Selection

The diagnosis of MGH/GDM was based on 50 g glucose challenge test (GCT) and 100 g oral glucose tolerance test (OGTT) performed during the 24–28 weeks of pregnancy as a universal screening test. GCT was performed on all pregnant women. The plasma glucose standard of >7.8 mmol/L was considered as GCT-positive. Before OGTT, pregnant women were advised to fast overnight in addition to 3 days of an unrestricted diet. Fasting plasma samples were drawn after 1, 2, and 3 h of glucose administration to perform the 100 g OGTT test. In this study, the abnormality values established by Carpenter and Coustan criteria (Carpenter and Coustan, 1982) were followed. Pregnant women with abnormal glucose tolerance tests were included in the study. Dietary adjustments and, if necessary, insulin therapy were used to attain glycemic control in MGH women (Beneventi et al., 2014).

2.3. Blood test

A total of 6 mL peripheral blood sample was collected, of which 3 mL blood was collected in a fluoride vacutainer tube to separate the serum from the blood and 3 mL blood was collected in an ethylenediaminetetraaceticacid (EDTA) vacutainer tube for DNA separation. Serum samples were used to evaluate the fasting blood sugar (FBS), postprandial blood glucose (PPBG), GCT and OGTT values (Al-Daghri et al., 2014). The body mass index (BMI) was calculated as weight/height^2 (kg/m^2). The Bio-Rad DNA Extraction Kit was used to extract DNA from the collected peripheral blood leucocytes, and the DNA was stored at -80 °C until use.

2.4. Analysis of tetraprimer amplification refractory mutation system (ARMS)-polymerase chain reaction (PCR)

The oligonucleotide sequences for PCR primers (tetraprimers) for I/D alleles were selected from prior studies conducted in different populations (Rigat et al., 1992; Masud and Qureshi, 2011). Genotyping for *ACE* was accomplished by direct PCR, and successful amplification was confirmed by electrophoresis on 2% agarose gel. PCR reactions were performed in a thermal cycler with initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles of denaturation at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 5 min. Major allele indicates the 490 bp fragment that embraces 287 bp Alu sequence, known as an insertion, whereas the 190 bp fragment represents the minor allele with the deletion of Alu sequence. Heterozygosity specifies the combination of both major and minor fragments i.e. 490/190 bp.

2.5. Statistical analysis

Statistical package for the social sciences (SPSS) software for windows (version 21.0., Chicago, IL, USA) was used to perform the statistical analysis. Hardy–Weinberg Equilibrium (HWE) was tested for MGH and control women. The allelic frequencies were calculated based on the number of different alleles observed and the total number of alleles examined. The allele and genotype frequency differences between MGH patients and control subjects were tested by chi-square analysis. Odds ratios (ORs) and 95% confidence intervals were calculated by multiple logistic regression analysis. Results of continuous variables were expressed using a descriptive statistic [mean ± standard deviation (SD)] and analyzed by Student's *t*-test. Analysis of variance (ANOVA) was used to compare the genotype group differences for various parameters. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Women characteristics

Clinical and biochemical characteristics of MGH and controls were similar (Table 1), whereas the anthropometric measurements such as age, weight, height, and BMI remained the same ($p > 0.05$). Biochemical tests including FBS, PPBG, GCT, and

Table 1 Clinical characteristics of MGH cases and control subjects in Saudi women.

	MGH cases (n = 100)	Controls (n = 100)	p value
Age (Years)	31.9 ± 5.6	31.3 ± 6.0	0.55
Weight (kg)	79.1 ± 13.4	74.1 ± 12.1	0.12
Height (m^2)	155.1 ± 5.9	157.1 ± 5.3	0.08
BMI (kg/m^2)	34.1 ± 4.2	33.2 ± 4.2	0.16
Mean gestational age	30.3 ± 5.7	NA	NA
FBS (mmol/L)	5.0 ± 0.9	4.5 ± 0.8	< 0.001
PPBG (mmol/L)	6.5 ± 2.0	4.4 ± 1.8	0.001
GCT (mmol/L)	9.5 ± 1.8	6.3 ± 1.5	< 0.001
OGTT (Fasting hour)	5.2 ± 1.18	4.5 ± 0.87	< 0.001
OGTT (1st hour)	10.7 ± 1.8	8.0 ± 1.7	< 0.001
OGTT (2nd hour)	9.2 ± 1.8	6.7 ± 1.6	< 0.001
OGTT (3rd hour)	5.6 ± 1.7	4.5 ± 1.3	< 0.001

All continuous variables represented by mean ± standard deviation. Independent sample t-test is done comparing MGH and control subjects. Categorical variables compared using chi-square analysis. $p < 0.05$ considered significant. NA = not applicable/not analyzed.

OGTT performed for all pregnant women were found to be significant ($p < 0.05$).

3.2. Molecular analysis

The distribution of the genotype frequencies of *ACE* polymorphism in control subjects was in accordance with the HWE. The distribution of genotype and allele frequencies of I/D polymorphism in MGH and controls was determined to examine their association with the risk for MGH (Table 2). The frequencies of II, ID, and DD genotypes for MGH patients and control women were 17%, 30%, and 53% and 15%, 40%, and 45%, respectively. No significant difference was noted between the allele frequencies of MGH and controls (OR 1.14 [95%CI: 0.75–1.73]; $p = 0.40$).

3.3. Correlation of I28005D genotypes with clinical and biochemical parameters

In the present study, the I28005D polymorphism was found to be correlated with the II, ID, and DD genotypes, based on the anthropometric, clinical, and biochemical parameters. Our analysis revealed that DD genotype in combination with II and ID was significantly associated with elevated PPBG and GCT ($p < 0.05$). On the contrary, DD genotype was significantly related to a decreased 3 h OGTT level as compared to II and ID genotypes (Table 3).

4. Discussion

This is the first report employing tetraprimer ARMS-PCR for genotyping *ACE* with I and D polymorphisms in pregnant women, including 200 pregnant Saudi women (100 MGH and 100 control subjects). We investigated the relationship between *ACE* (I/D) and MGH. Until date, screening and identification of MGH have been performed by the biochemical examination of serum samples, despite the advancement in genetic screening methods. No genetic test is available to identify MGH in pregnant women (Carpenter and Coustan, 1982). To the best of our knowledge, this is the first study conducted on pregnant Saudi women. In this study, we investigated the effects of I/D of the *ACE* variants. Results of our study revealed that none of the genotypes of *ACE* were significantly associated with the risk for MGH.

The RAS is primarily involved in the regulation of blood pressure and serum electrolytes. The biological mechanism of ACE includes conversion of angiotensin I to a potent vasoconstrictor angiotensin II by a metalloenzyme that leads to inactivation of bradykinin, which is a vasodilator of the kallikrein–kinin system and has major implication in the inflammatory process including osteoarthritis (Inanir et al., 2013). Although the accurate mechanism of development of MGH/ gestational diabetes during pregnancy is unknown, it can be characterized by progressive insulin resistance resulting from a combination of increased maternal adiposity and

Table 2 Dissemination of *ACE* gene I/D polymorphism and allele frequencies between MGH patients and control subjects.

Genotypes	GDM cases (n = 100)%	Non-GDM (1 = 100)	χ^2	p value	Odds ratio (95% CI)
II	17 (17%)	15 (15%)	Reference		
ID	30 (30%)	40 (40%)	0.92	0.33	0.66 (0.28–1.55)
DD	53 (53%)	45 (45%)	0.008	0.92	1.03 (0.46–2.31)
ID + II	47 (47%)	55 (55%)	0.48	0.62	0.75 (0.34–1.67)
ID + DD	83 (83%)	85 (85%)	0.14	0.69	0.86 (0.40–1.83)
II + DD	70 (70%)	60 (60%)	0.005	0.52	1.02 (0.47–2.23)
Alleles					
I	64 (0.32)	70 (0.35)	Reference		
D	136 (0.68)	130 (0.65)	0.40	0.52	1.14 (0.75–1.73)

Table 3 Distribution of MGH characteristics according to anthropometric and metabolic parameters in ACE genotypes.

Aspects	II (n = 17)	ID (n = 30)	DD (n = 53)	χ^2	p
Age (Years)	32.55 ± 6.47	33.68 ± 5.74	31.68 ± 5.43	1.62	0.44
Age of onset	30.76 ± 5.41	29.9 ± 5.57	30.22 ± 6.17	1.25	0.53
BMI (kg/m^2)	31.25 ± 4.78	31.79 ± 4.46	29.22 ± 4.52	0.21	0.89
FBS (mmol/L)	6.37 ± 2.59	5.65 ± 2.86	6.44 ± 2.29	3.88	0.14
PPBG (mmol/L)	9.82 ± 3.79	9.84 ± 2.57	9.87 ± 3.63	9.41	0.009
GCT (mmol/L)	7.91 ± 2.52	8.15 ± 2.29	8.33 ± 3.04	6.19	0.04
OGTT (Fasting hour)	5.07 ± 1.18	5.26 ± 1.16	5.11 ± 1.20	0.08	0.95
OGTT (1st hour)	8.14 ± 5.03	7.01 ± 5.60	8.34 ± 4.60	2.99	0.22
OGTT (2nd hour)	6.76 ± 4.08	5.69 ± 4.76	7.28 ± 4.08	2.02	0.36
OGTT (3rd hour)	5.12 ± 1.98	3.17 ± 3.3	3.50 ± 2.91	9.63	0.008

insulin-desensitizing effects of placental products such as human placental lactogen, estrogen, and prolactin (Khan et al., 2014). The molecular mechanism of the association between *ACE* I/D polymorphism and the risk of MGH are relatively unclear. *ACE* has 26 exons: exons 1–12 encode for the amino domain and exons 13–26 encode for the carboxyl domain. A commonly occurring variant of *ACE* has I/D polymorphism with a 287 bp Alu repetitive sequence in intron 16 (Chmaisse et al., 2009). We examined polymorphism of *ACE*, which is an important gene in the RAS, in MGH/gestational diabetes women and healthy controls in a Saudi population. The frequency of DD genotype was not significantly different among MGH patients (p = 0.92). The frequency of D allele showed no statistical difference between MGH patients and control subjects (p = 0.53). ACE may be considered as a risk factor for the development of T2DM and its complications. Although this polymorphism occurs in the intronic region of the gene, a past study showed that DD genotype is strongly associated with increased plasma or serum ACE levels, thereby predisposing individuals to T2DM and its complications (Chmaisse et al., 2009).

The relationship between *ACE* polymorphism and gestational diabetes has previously been studied mainly in the women of Brno and Indian population (Khan et al., 2013; Dostalova et al., 2006). Reports on no association of ACE polymorphism with MGH in women (Dostalova et al., 2006) are consistent with those reporting a positive association in the Brno population (Dostalova et al., 2006). Results of the present study support data in the literature, suggesting a positive association between ACE polymorphism and MGH.

ACE variants do not show single nucleotide polymorphisms (SNP), but I/D polymorphism and the insertion allele represent repetitive sequences of Alu with the difference of 287 bp between the I/D alleles. Because of the differences between the repeat sequences of the two *ACE* alleles, a conventional PCR was used instead of the tetraprimer ARMS-PCR (Masud and Qureshi, 2011). The tetraprimer ARMS-PCR employs intelligently designed primer sets consisting of two outer non-specific primer and two inner, allele-specific primers oriented opposite to each other. In a single reaction, the outer primers amplify a large fragment of the target gene, irrespective of its genotype, which serves as an internal control, while each of the inner primer combines with the respective opposite outer primer to generate smaller allele-specific amplicons. In order to increase the allele specificity, a deliberate mismatch is incorporated into the nested primers at position −2 or −3 from the 3′ terminus (Lajin et al., 2012; Etlik et al., 2011).

However, the role of ACE in MGH women or pregnant women is unclear, with very few studies addressing the association between *ACE* polymorphism and MGH. Furthermore, there are no studies on the association between ACE serum levels and MGH in women. Therefore, the contribution of ACE activity to the development of MGH warrants further investigation.

The limitation of our present study included (i) lack of measurement of the ACE levels and (ii) use of only one snip. However, our study does not report a significant association of *ACE* I/D polymorphism in MGH women. Based on these results, we concluded that MGH was not influenced by *ACE* polymorphism in Saudi women. Further studies are required to fully understand the role and determinants of ACE activities and ACE levels in different ethnic groups.

Conflict of interest

There is no conflict of interest.

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