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Interaction between angiotensin converting enzyme (ACE) insertion/deletion and aldosterone synthase (CYP11B2) -344C/T polymorphisms in relation to type 2 diabetes mellitus risk in Emiratis

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Polymorphism in Renin-Angiotensin-Aldosterone System (RAAS) genes have been studied extensively in various ethnic groups and largely with inconsistent findings on relationship with the risk of developing type 2 diabetes mellitus (T2DM). In this study, we investigated the association of Insertion/Deletion (I/D) polymorphism of angiotensin converting enzyme (ACE) and -344 C/T polymorphism of aldosterone synthase [cytochrome P450 (CYP11B2)] with T2DM in an Emirati population and interactive effects between these two gene polymorphisms on T2DM risk. A total of 243 Emirati subjects (133 healthy control and 110 T2DM patients) were selected for the study. The ACE genotypes were determined by polymerase chain reaction (PCR) followed by agarose gel electrophoresis. The CYP11B2 genotyping was performed by PCR- Restriction Fragment Length Polymorphism Analysis (RFLP). ACE genotypes were not associated with T2DM risk. The frequencies of D allele were 0.68 and 0.64 in the patients and healthy group respectively and the differences were not statistically significant. For CYP11B2 -344C/T polymorphism, CC genotype was found significantly higher in healthy subjects than in T2DM patients (22.1 Vs 9.8%, p=0.016). The subjects with CC genotype were at decreased T2DM risk in the recessive model [Odd ratio 0.38 (0.17-0.84)]. An interactive effect on T2DM risk was found between ACE-ID and CYP11B2 -CC genotypes. In the subjects with combination of ID + CC genotypes, risk of T2DM was further reduced (odd ratio 0.05 vs 1.12). The association of CC with T2DM was independent of age and gender. To date, this study is the first report on association of CYP11B2 -344C/T with T2DM and its possible interaction with ACE I/D polymorphism in an Emirati population. The results suggest that ACE I/D polymorphism does not affect T2DM risk independently however, subjects with CC genotypes either alone or in combination with ACE I/D heterozygote would be at decreased risk of developing T2DM.

Key words: Type 2 diabetes mellitus, CYP11B2, Renin-Angiotensin-Aldosterone System, angiotensin converting enzyme, genetic polymorphism.
INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a disorder characterized by hyperglycemia as a result of impaired insulin secretion and insulin resistance in peripheral tissues. The data from the IDF diabetes atlas update reported the prevalence of T2DM 18.87% in United Arab Emirates (UAE) (http://www.idf.org/diabetesatlas/5e/Update2012) which is one of the highest in the world. In addition to lifestyle and eating habits, the rising prevalence has been attributed to genetic predisposition, although most of the genes involved have not yet been determined.

Renin- Angiotensin-Aldosterone System (RAAS) is a well known circulating or hormonal system that regulates blood pressure, electrolyte and fluid homeostasis. Polymorphism in RAAS genes in relation to T2DM has been studied intensively and mostly focused on Angiotensin converting enzyme ACE along with angiotensin receptor type 1 (AT1R) and angiotensinogen (ATG) (Mehri et al., 2010; Lin et al., 2009). ACE plays an important role in the conversion of less active angiotensin I to more active angiotensin II which mediates a variety of cellular functions in different tissues. The ACE gene is mapped on chromosome 17q23 and is highly polymorphic which is characterized by the presence or absence of deletion of a 287-bp element within intron 16 leading to the D/D, I/D, and I/I genotypes (Rigat et al., 1992; Tiret et al., 1992). The frequency of D allele has been reported varying from 3% in Thai population (Nitiyanant et al., 1997; Jayapalan et al., 2008) to 75% in Arab population (Bayoumi et al., 2006; Baroudi et al., 2009). Several reports on association of ACE I/D polymorphism and T2DM with related cardiovascular and renal complication have been published but with controversial results. Whereas some have found that the D-allele is more common in T2DM and related complications (Baroudi et al., 2009; Naresh et al., 2009; Nikzamir et al., 2008), others have demonstrated no association of either allele with T2DM or related cardiovascular and renal disease (Sinorita et al., 2010; Jayapalan et al., 2010; Ramachandran et al., 2009).

Aldosterone synthase (AS) enzyme which is encoded by the cytochrome P450 11B2 (CYP11B2) gene catalyzes the final step in the synthesis of aldosterone in the zona glomerulosa of adrenal cortex, the major regulator of which angiotensin II is produced by ACE from angiotensin I. In the zona fasciculata of adrenal cortex, Cortisol is synthesized by enzyme 11-β hydroxylase which catalyzes 11- hydroxylation of 11-deoxycortisol and is highly homologous to AS and is encoded by CYP11B1 gene controlled by adrenocorticotropic hormone (corticotrophin [ACTH]). CYP11B2 and CYP11B1 are situated at chromosome 8 sharing 95% sequence similarity and are highly polymorphic. Two common variants of a single nucleotide substitution from cytosine to thymidine in the promoter region (-344 C/T) and an intron conversion in intron 2 have been reported previously in the CYP11B2 gene (White and Slutsker, 1995). Intron conversion results in two alternate forms of the gene, either the wild-type (Wt) or the conversion (Conv), which leads to replacement of part of intron 2 by the corresponding intron of the adjacent CYP11B1 gene. These variants are in tight linkage disequilibrium (LD) with each other (White and Slutsker, 1995) as well as with two CYP11B1 variants (-1889 G/T and -1859 A/G) (Barr et al., 2007). The frequencies of C and T alleles were 0.32 and 0.67, respectively in Chinese (Hai-dong et al., 2010), 0.40 and 0.60 in Tamil South Indian population (Rajan et al., 2010), 0.45 and 0.55 in Europeans and 0.44 and 0.56 in South Americans (Ganapathipillai et al., 2005). These polymorphisms in CYP11B2 have been found inconsistently associated previously with increased levels of aldosterone (Barr et al., 2007). The increased level of aldosterone has been previously demonstrated to be associated with plasma markers of insulin resistance and hyperinsulinemia independent of plasma potassium and Cortisol levels in a white hyper tension population (Colussi et al., 2007) and have been implicated in the development of cardiovascular and renal diseases in T2DM (McFarlane and Sowers, 2003). In fact recently, antihypertensive treatment with Angiotensin converting enzyme-inhibitors/angiotensin receptor blockers (ACE-i/ARB) was found protective against diabetes in a case-control study (Monami et al., 2012). The reports on association of ACE polymorphism with T2DM have been largely inconsistent and there was no report available on CYP11B2 polymorphism in T2DM. Hence, the present study was undertaken with an objective to investigate the association of ACE I/D and CYP11B2 -344 C/T polymorphisms with T2DM in Emiratis.

MATERIALS AND METHODS

Subjects

A total of 243 individuals of Arab origin were identified during their routine visit to clinics in the UAE (157 males, 86 females and 110 diabetic, 133 healthy) (Table 1). Clinical assessment and questionnaire completion were conducted at the clinic. An individual was classified as T2DM if the subject was: (1) diagnosed with T2DM by a qualified physician, (2) on a prescribed drug treatment regimen for T2DM and (3) returned biochemical test results of a fasting plasma glucose level of at least 126 mg/dl as based on the criteria laid by the World Health Organization (WHO) consultation group report (Alberti and Zimmet, 1998). Each individual provided signed, informed consent based on information provided by the ethics committee of the United Arab Emirates Ministry of Health.

DNA Extraction

After blood was drawn into EDTA tubes, genomic DNA was extracted using a Nucleic Acid Kit (Roche Applied Science, Indianapolis, IN, USA) according to the recommendations of the manufacturer. Briefly, 300 µl of whole blood from each sample was mixed with
Table 1. Demographic characteristics of Emirati samples

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy control</th>
<th>DM patient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>133</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Number of males (%)</td>
<td>99 (74.4)</td>
<td>58 (53)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Number of females (%)</td>
<td>34 (25.6)</td>
<td>52 (47)</td>
<td></td>
</tr>
<tr>
<td>&lt; 50 years of age (%)</td>
<td>114 (85.7)</td>
<td>52 (47)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;50 years of age (%)</td>
<td>19 (14.3%)</td>
<td>58 (53)</td>
<td></td>
</tr>
<tr>
<td>Mean age ± S.D</td>
<td>33±12</td>
<td>49±12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean Fasting Blood Glucose ± S.D</td>
<td>91.25±10.22</td>
<td>171.86±65</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Figure 1. PCR based diagnostic test of ACE I/D polymorphism. The PCR amplified products were run on 2.5% agarose gel at 100 V and visualized by ethidium bromide staining. Lane M represents 100 bp DNA ladder; lanes S1 and S3 demonstrating 490 bp fragments represent homozygous I/I genotype; lanes S2, S4 and S5 demonstrating 190 bp fragments represent homozygous D/D genotype.

PCR based genotyping for ACE I/D polymorphism

The ACE I/D polymorphism was determined by polymerase chain reaction (PCR) followed by agarose gel electrophoresis. PCR was carried out using the forward primer: 5'-CTGGAGACCCATCTCCATCTTTCT-3' and reverse Primer: 5'-GATGTTGGCCATCACATTGCAGAT-3' (Rigat et al., 1992) in a 50 µL reaction mixtures containing 1X PCR Taq buffer (Promega), 3 mM MgCl₂, 0.2 mM of dNTPs (Fermentas), 1 µM of each reverse and forward primer (Sigma-Aldrich), 1.5 U Taq DNA polymerase (Promega) and approximately 100 ng DNA. After initial denaturation at 95°C for 5 min, the thermostepping (Technne, U S) procedure consisted of denaturation at 95°C for 30 s, annealing at 59°C for 45 s, and extension at 72°C for 1 min, repeated for 35 cycles, and followed by a final extension at 72°C for 10 min. PCR products were separated on 2% agarose gels and visualized by Ethidium Bromide staining under UV transilluminator.

PCR-RFLP based genotyping for CYP11B2 -344C/T polymorphism

Out of the total of 243 subjects, only 215 (102 T2DM patients and 113 healthy) could be genotyped for CYP11B2 -344 C/T polymorphism. Promoter region of CYP11B2 including the -344 polymorphic site was amplified by PCR using the forward primer 5'-TGGAGGTGTTACCTGTGTCA-3' and reverse primer 5'-TCCAGGGCTCAACACACTAA-3' in a 50 µL reaction mixture using the same conditions as described above. 12 µL of PCR product was digested with 10 U of Bs uR1 (Fermentas) by incubating in water bath at 37°C overnight. The digested fragments were analyzed on 2.5% agarose gel as described earlier.

Statistical analysis

Hardy Weinberg equilibrium was tested for ACE and CYP11B2 polymorphism with chi square statistics. In case of differences of various parameters between the T2DM patients and healthy controls, two tailed student’s t test was used for checking significant differences. The differences between the T2DM patients and healthy controls with respect to genotype distributions and allele frequencies were analyzed by the Fisher's exact test. The following gene transmission models were considered: (1) recessive effect (CC Vs CT+TT), (2) a dominant effect (CC+CT Vs TT) and (3) additive effect (assigning 1, 2, 0 to CC, CT and TT respectively) of the C allele. The association was evaluated by chi square test followed by logistic regression. A p value of < 0.05 was considered for statistical significance. The interaction of ACE genotypes (II, ID, DD), age and gender with relationship of CC with T2DM was also evaluated with logistic regression using the two by four tables.

RESULTS

The demographic characteristics of participants are shown in Table 1. The mean age of subjects in the study group (n=111) was 49±12 years while that in the control group (n=133) was 33±12 years and the differences were statistically significant (p<0.0001). The male/female ratio was also significantly higher in control group.

Distribution of ACE I/D genotype within the study population

The representative gel picture for ACE gene genotyping performed by PCR and electrophoresis is shown in Figure 1. The length of amplified products in I/I and D/D genotypes were 490 and 190 bp respectively and heterozygous I/D genotypes demonstrated both 490 and 190 bp fragments.

On analyzing the ACE gene I/D polymorphism in cases and controls, I/D genotype was found most frequently present (47.1%) in all the subjects followed by DD (42.7%) and II (10.2%) with an overall frequency of 0.66 and 0.34 for D and I alleles, respectively (Table 2). Out of the 111 subjects genotyped for ACE in T2DM group, 52 (46.8%) were D/D, 48 (43.2%) were I/D and 11 (10.0%) were I/I. Thus frequencies of D and I alleles were 0.64...
Table 2. Allele and Genotype frequencies of ACE I/D polymorphism and CYP11B2 C-344T polymorphism in T2DM patients and healthy subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total number</th>
<th>T2DM patients number (%)</th>
<th>Healthy subjects number (%)</th>
<th>P value*</th>
<th>Overall frequencies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACE Genotypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>25</td>
<td>11 (10.0)</td>
<td>14 (10.5)</td>
<td>NS</td>
<td>10.2</td>
</tr>
<tr>
<td>ID</td>
<td>115</td>
<td>48 (43.2)</td>
<td>67 (50.4)</td>
<td>NS</td>
<td>47.1</td>
</tr>
<tr>
<td>DD</td>
<td>104</td>
<td>52 (46.8)</td>
<td>52 (39.1)</td>
<td>NS</td>
<td>42.7</td>
</tr>
<tr>
<td><strong>Allele frequencies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>165</td>
<td>70 (32.0)</td>
<td>95 (36.0)</td>
<td>NS</td>
<td>33.8</td>
</tr>
<tr>
<td>D</td>
<td>323</td>
<td>152 (68.0)</td>
<td>171 (64.0)</td>
<td>NS</td>
<td>66.2</td>
</tr>
<tr>
<td><strong>CYP11B2 Genotypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>35</td>
<td>10 (9.8)</td>
<td>25 (22.1)</td>
<td>0.0164</td>
<td>16.3</td>
</tr>
<tr>
<td>CT</td>
<td>101</td>
<td>52 (51.0)</td>
<td>49 (43.4)</td>
<td>Ns</td>
<td>47.0</td>
</tr>
<tr>
<td>TT</td>
<td>79</td>
<td>40 (39.2)</td>
<td>39 (34.5)</td>
<td>NS</td>
<td>36.7</td>
</tr>
<tr>
<td><strong>Allele frequencies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>171</td>
<td>72 (35.0)</td>
<td>99 (44.0)</td>
<td>0.0764</td>
<td>39.8</td>
</tr>
<tr>
<td>T</td>
<td>259</td>
<td>132 (65.0)</td>
<td>127 (56.0)</td>
<td>NS</td>
<td>60.2</td>
</tr>
</tbody>
</table>

NS-Non-significant; *Fisher Exact test.

and 0.36 respectively in this group. All the frequencies were in Hardy Weinberg equilibrium.

Distribution of CYP11B2 -344C/T genotype within the study population

A representative gel picture for CYP11B2 genotyping performed by PCR-RFLP is shown in Figure 2. PCR resulted in amplification of a 178 bp fragment which upon digestion with BsuR1 gave two fragments of 118 and 60 bp in case of CC genotype and remain undigested for TT genotypes. The heterozygotes (CT) demonstrated all three fragments. The frequencies of CC, CT and TT genotypes in T2DM patient group (n=102) were 9.8, 51.0 and 39.2%, respectively whereas in healthy control group (n=113), they were 22.1, 43.4 and 34.5%, respectively (Table 2). The frequencies of C allele were 0.35 and 0.44 and for T allele were 0.65 and 0.56 in patient and healthy group respectively (Table 2). None of the frequencies were deviated from Hardy Weinberg equilibrium.
Association between CYP11B2 -344C/T polymorphism with T2DM and its interaction with ACE I/D polymorphism

No significant differences were observed between the genotype or allele frequencies for I and D allele of ACE between T2DM patients and healthy subjects. However, CC genotype for CYP11B2 was found significantly associated with healthy subjects (p=0.0164) but the differences in C allele frequencies were not statistically significant (0.35 vs 0.44 (p=0.0764)). The results of logistic regression analysis for three gene transmission models showed that odd ratios were significant only in recessive model (Odd ratio 0.38 (0.17-0.84) p=0.017) (Table 3) demonstrating CC genotype as a protective factor for T2DM risk. Upon evaluation of interaction between CYP11B2 C-344T and ACE I/D polymorphisms in T2DM, it was found that there was significant interaction between CC with II (Odd ratio of interaction variable 20.8 (1.5-288.7), p=0.0237) and ID (odd ratio 0.05 (0.01-0.49) p=0.0103) but not with DD genotype (Table 4). The association of CC with T2DM was independent of age [Odd ratio for interaction variable 4.02 (p=0.2536)] and gender [Odd ratio for interaction variable 3.84 (p=0.1218)], however age was found independent risk factor for T2DM [Odd ratio for age variable 8.95 (4.54-17.64), p=0.0001] (Table 5).

DISCUSSION

Evidences from various clinical studies have speculated the role of RAAS in the pathogenesis and etiology of T2DM and related cardiovascular and renal diseases. The most likely mechanism by which RAAS may affects the risk of T2DM is by alteration in the level of aldosterone which has been found associated with insulin resistance and the ensuing hyperinsulinemia (Colussi et al., 2007) which are major predisposing factors to the development of T2DM. ACE produces angiotensin II the level of which depends on the availability of angiotensinogen and the potential of its action depends on the availability of the angiotensin II type 1 receptor (AT1R). Angiotensin II is the potent inducer of aldosterone synthase (CYP11B2) which catalyzes the final step in the synthesis of aldosterone. There is a lot of potential in the interaction between RAAS gene polymorphisms to affect the T2DM risk. Tietet et al. (1994) noted that risk of myocardial infarction was changed as a result of interaction between AT1R A1166C gene variation and ACE I/D polymorphism. Mehri et al. (2010) studied genotypic interaction of RAAS genes polymorphism angiotensinogen (AGT M235T), ACE I/D and AT1R (A1166C) on T2DM Tunisian patients and found that risk of T2DM was 4 and 26.2 times higher in the individuals who carried 2 and 3 risk factors, respectively than those who did not carry any risk genotypes of the RAAS genes. Most of the other studies were also carried on these 3 RAAS genes only but there was no study available on CYP11B2 either alone or along with other RAAS genes including ACE. We decided to analyze (i) the association of ACE I/D and CYP11B2 -344C/T polymorphism with T2DM individually; (ii) the effect of the association’s potential interaction with other polymorphism and finally (iii) with age and gender since the average age in controls and the ratio of male and female were unmatched with that in cases, which could have biased our results.

Individually, we did not observed any significant differences in the ACE genotype and allele frequencies between the T2DM patients and healthy subjects (Table 2). In view of the other studies available in Arabs, these were surprising results as in earlier studies DD genotype has been significantly associated with T2DM risk in Iranians (Nikazamir et al., 2008; Nakhjavani et al., 2007) and Midoun Arabs resident of Jerba Island in Tunisia (Baroudi et al., 2009). Null association has also been reported in a multiethnic Malaysian population (Jayapalan et al., 2010; Ramchandran et al., 2009) and an Indonesian population (Sinorita et al., 2010). In Chinese, I allele containing genotypes were significantly associated with metabolic syndromes (Thomas et al., 2001). The
frequency of D allele (0.64) (Table 2) in our healthy population was in concordance with that of other Arab populations such as Egyptians (0.67), Syrians (0.60) and Jordanians (0.65) (Salem and Batzer, 2009) and Emiratis (0.61) reported elsewhere (Bayouni et al., 2006).

CYP11B2 encodes a key rate limiting enzyme in the aldosterone synthesis and has been found polymorphic at -344 position with T allele associated with increased aldosterone levels and risk of essential hypertension (Barr et al., 2007). There are limited reports available on CYP11B2 -344C/T polymorphism with relation to T2DM. However, in a recent study, -344C variant was found to be significantly associated with increased fasting glucose in controls and in the subgroup containing 893 essential hypertension cases without a history of diabetes or hypoglycaemia medications. Also, the -344C variant was found to be associated with increased fasting and postprandial plasma glucose levels and decreased pancreatic β-cell function and insulin sensitivity by homeostasis model assessments (Li et al., 2011). In another study, lower levels of fasting glucose and 2 h glucose levels were observed in siblings with the T allele than their CC homozygotes for the -344 C/T polymorphism of CYP11B2 (Hsiao et al., 2012). In our study, we found CC genotype significantly higher in healthy subjects (9.8 Vs 22.1% p=0.0164) (Table 2) and associated with decrease risk of T2DM (Odd ratio 0.387 p=0.017) (Table 3) in recessive mode. In contrast, a large French cohort study found CC genotype significantly associated with high risk of T2DM in men (Bellili et al., 2010). The inconsistencies in CYP11B2
-344C/T polymorphism studies have also been observed in association with essential hypertension ((Rajan et al., 2010; Tsujita et al., 2001, Cheng and Xu, 2009) which have largely been explained based on the linkage disequilibrium of -344C/T alleles with two CYP11B1 variants -1889 G/T and -1859 A/G (Barr et al., 2007). The C allele frequency (0.44) in healthy Emirati subjects in our study was found consistent with that of Europeans (0.45) and South Americans (0.44) (Ganapathipillai et al., 2005). The combined effect of ACE I/D and CYP11B2 -344C/T polymorphism was also examined in T2DM patients. The relationship of CC genotype with T2DM was not affected by DD genotypes and the risk of T2DM was reduced further in the subjects with a combination of CC/DD genotypes (Table 4). However, surprisingly we found combination CC/II associated with increased risk of T2DM instead of expected decreased risk (Table 3). The larger odds ratios in this group might be due the smaller sample sizes in the subgroups with CC and II, because of which we were unable to evaluate this interaction with sufficient statistical power. Studies with smaller sample sizes tend to report larger odds ratios (Keavney et al., 2000). Apart from sample size, another limitation of the study is that the control and healthy groups were unmatched with respect to age and male/female ratio. Although we additionally analyzed affect of gender and age on the relation of CC genotype with T2DM and found the relationship was unaffected by age and gender (Table 5). However, we also recognized that older age is a risk factor for T2DM (Table 5). Therefore our findings should be reconfirmed and the joint effects of CC and ACE I/D genotypes should be evaluated by future population-based studies with larger sample sizes collected ideally in a prospective fashion. If we could use hyper-normal controls, such as older people known to be free of the disease of interest, it would be expected to improve the power of detecting the susceptibility variant between controls and cases.

In conclusion, our data does not support any association between ACE I/D polymorphism and occurrence of T2DM in Emirati population which may otherwise be considered associated because of high frequency of D allele and subsequently high prevalence rate of T2DM in this particular population. However, we found that CC genotypes for CYP11B2 -344C/T polymorphism alone as well as in combination with I/D are significantly associated with decreased risk of T2DM and this relationship is independent of age and gender. More investigations with large sample size may be required to extend the study report.

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