Genetic variation in ACE A2350G: association with reduction in fasting blood glucose after fluoxetine therapy in depressed patients

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Abstract
Various studies have shown that genetic factors contribute substantially to the development and progression of diabetes. Renin-angiotensin system has long been proven to have a major role in cardiovascular physiology and pathology. Its major product Angiotensin II (Ang II) with pro oxidant properties has shown to predict the future risk of diabetes. Fluoxetine, a drug of choice in management of depression, was observed to reduce fasting blood sugar (FBS). In the present study, six common polymorphisms of genes encoding for RAS components were determined in DNAs extracted from venous blood of 100 newly diagnosed depressed individuals taking 12 weeks of fluoxetine. Blood samples were collected prior and after the period of treatment in order to measure FBS. Our results indicate that carriers of GG genotype of ACE A2350G showed significantly lower FBS levels after fluoxetine treatment (P=0.043). In conclusion, this study supports the hypothesis that RAS genetic variations affect blood glucose after a course of treatment in Iranian population with depression.

Keywords: Major depressive disorder, Renin-angiotensin system, Genetic polymorphisms, Fluoxetine, Fasting blood sugar.

1. Introduction
About 19 million adults in the US and 150 million suffer from diabetes globally. Regarding a report by WHO, by the year 2025 diabetes affects around 300 million individuals worldwide (1). Hypertension and atherosclerosis are strongly affected by Insulin resistance (2). Hyperinsulinemia is observed in 50% of hypertensive individuals. On the other hand up to 75% of people with type 2 diabetes suffer from hypertension (3, 4). Abnormal glucose metabolism is seen in roughly two third of the patients diagnosed with an acute coronary syndrome (5).

The angiotensin-converting enzyme (ACE) being a key enzyme of the renin-angiotensin system (RAS) has long been known as a fundamental enzyme in the regulation of systemic blood pressure and renal electrolyte homeostasis (6, 7). Cardiovascular, renal, and adrenal function are being coordinated by this hormonal cascade. ACE catalyses the conversion of angiotensin I
Ang I) to angiotensin II (Ang II). Ang II having pro-inflammatory (8) and pro-oxidant (9) effect, results in cellular toxicity and apoptosis. Additionally, it has been suggested by prospective studies that chronic low grade systemic inflammation may predict the future risk of impaired glucose tolerance (IGT) and type 2 diabetes mellitus (T2DM) (10). Moreover, several studies imply that using ACE inhibitors and Ang II type 1 receptor blockers reduce progression from IGT to T2DM by 25-30% (11, 12) as well as beneficial effects on macrovascular and microvascular complications (13-17). Consequently, these observations imply that inflammation, mainly caused by elevated Ang-II may contribute to high levels of blood glucose and development of T2DM.

The Ang I receptor being stimulated by high insulin levels, activates RAS (18) and increases cardiac sympathetic nervous system function (19). Reports suggest that diabetic patients, specially, benefit from blockade of the RAS, with reduction of cardiovascular mortality up to 40% in a major, randomized, controlled trial (20).

Evidences indicate that the actions of Ang II is reduced by clinically active antidepressants (21). In a recent report, it was suggested that depressed patients receiving a course of treatment with fluoxetine had lower FBS levels than patients with Imipramine (22).

Response to antidepressants and progression of numerous illnesses such as diabetes mellitus and its long term macro- and micro vascular complications (23, 24), including diabetic nephropathy (25, 26) may be affected by different genetic variants of RAS.

Serum and tissue ACE levels are strongly associated with a common variant in the ACE gene, with the presence of an insertion (I) of a 287 bp fragment in intron 16 of the ACE gene being associated with lower ACE activity and the deletion (D) being associated with higher ACE activity (27, 28). Evidences suggest an association between the D allele and Type 2DM in non-Caucasian populations (29, 30). Regarding RAS polymorphisms, an association between ACE A2350G genetic variants and prevalence of diabetic retinopathy was reported in a Chinese population (31). Considering that different genetic variants of ACE gene such as ACE I/D, ACE A2350G and A-240T may affect serum ACE activity, and that fluoxetine reduces FBS levels in depressed patients, this may come into mind that aforementioned polymorphism may affect blood glucose after a period of treatment with fluoxetine in a sample of depressed patients.

2. Materials & methods

2.1 Study population

This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and Uniform Requirements for manuscripts submitted to biomedical journals. The study was approved by the local committee for ethics of medical experiments on human subjects of Shiraz University of Medical Sciences. The written consent was obtained from the participants prior to the interview.

In total, 100 newly diagnosed patients suffering from major depressive disorder (MDD) (male: 31, female: 69, mean age±SD: 33.4±11.3) were enrolled in this study. The MDD sufferers were diagnosed according to DSM-IV criteria and by an experienced psychiatrist. Psychiatric ratings by 21 item HAMD scale were used at the time of admission and after 12 weeks of treatment with 20 mg fluoxetine (FLUOXETINE-ABIDI®). Five ml of blood sample was collected prior and after the course of antidepressant treatment in order to measure changes in FBS. It is to emphasize that the enrolled patients received no oral hypoglycemic agents.

2.2 DNA extraction and genotype determination

Genomic DNAs were extracted from whole blood leukocytes using a salting out method (32). The extracted DNAs were solved in sterile distilled water and stored at 4 °C for further PCR analysis. PCR amplification/detection of ACE I/D was carried out using standard protocol (33). In order to avoid mistyping of ID as DD genotype, all DD genotypes were reconfirmed by another typing system (34). PCR amplification of A-240T and A2350G was performed using primers mentioned in Table 1 (35, 36). In each reaction, 100-200 ng of genomic DNA was amplified in 15 μl of 1× PCR master mix (67 mMTris base, pH8.8, 16.6 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.1 %
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Tween-20, 200 lM dNTPs, 5 % glycerol, 100 lM cresol red) containing 0.2-2.0 lM of each primer and 0.5 U of Taq DNA polymerase (Cinnagen Inc., Tehran, Iran). All fragments were amplified under the same procedure which is of great advantage to reduce work load (37). The program under which the amplification took place was a modified form of the previous studies. After initial denaturation at 96 ºC for 2 min, PCR was performed for 5 cycles, each one comprised of denaturation at 96 ºC for 40 s, annealing at 60 ºC for 50 s and extension at 72 ºC for 30 s followed by 25 cycles of denaturation at 96 ºC for 40 s, annealing at 55 ºC for 50 s and the extension at 72 ºC for 30 s. An Eppendorf gradient Master cycler (Hamburg, Germany) PCR machine was used as the thermal cycler. PCR products (7 µl) were digested with the specified enzymes mentioned in Table 1. Digested fragments were separated by electrophoresis on 3 % agarose (Invitrogen® UltraPure) gel after an overnight incubation and then stained with ethidium bromide and visualized in a UV transilluminator. It is to mention that all of the samples were genotyped at least twice and reconfirmed.

2.3. Statistics

Hardy-Weinberg equilibrium (HWE) for the distributions of genotypes was estimated by Arlequin 3.1 software package. A one-way analysis of variance (ANOVA procedure) was performed to detect significant differences in FBS mean scores between the genotypes after 12 weeks of treatment. Adjusted associations were investigated by logistic regression models. SPSS 15 for Windows (SPSS inc. Chicago, IL, USA) was applied for statistical analysis.

3. Results

The relationship between genotype and reduced fasting blood sugar in Iranian depressed patients is presented in Table 2. As shown GG carriers of ACE A2350G had significantly lower FBS

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Primer sequence (5′-3′)</th>
<th>Location</th>
<th>Restriction enzyme digestion</th>
<th>Allele</th>
<th>DNA fragment size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE I/D</td>
<td>F-CTG GAG ACC ACT CCC ATC CTT TCT</td>
<td>Intron 16</td>
<td>none</td>
<td>I</td>
<td>490</td>
<td>(Rigat et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>R- GAT GTG GCC ATE ACA TTT GTC AGA T</td>
<td></td>
<td></td>
<td>D</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td>A-240T</td>
<td>F- TCG GGC TGG GAA GAT CGA GC R- GAG AAA GGG CCT CCT CTC TCT</td>
<td>5′UTR</td>
<td>XbaI at 37 OC/24 h</td>
<td>A</td>
<td>137</td>
<td>(Hsieh et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T</td>
<td>114+23</td>
<td></td>
</tr>
<tr>
<td>A2350G</td>
<td>F-CTG ACG AAT GTG ATG GCC GC</td>
<td>Intron 17</td>
<td>BstUI at 60 OC/24 h</td>
<td>A</td>
<td>122</td>
<td>(Iqbal et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>R-TTG ATG AGT TCC ACG TAT TTC G</td>
<td></td>
<td></td>
<td>G</td>
<td>100+22</td>
<td></td>
</tr>
<tr>
<td>M235T</td>
<td>F-CAG GGT GCT GTCCAC ACT GGA CCC C</td>
<td>Exon 2 (+704)</td>
<td>PflI at 37 OC/24 h</td>
<td>M</td>
<td>165</td>
<td>(Russ et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>R-CCG TTT GTG CAG GGC CTG CTCT C</td>
<td></td>
<td></td>
<td>T</td>
<td>140+25</td>
<td></td>
</tr>
<tr>
<td>A1166C</td>
<td>F-ATA ATG TAA GCT CAT CCA CC</td>
<td>3′ UTR</td>
<td>Ddel at 37 OC/24 h</td>
<td>A</td>
<td>367</td>
<td>(Takami et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>R-GAG ATT GCA TTT CTG TCA GT</td>
<td></td>
<td></td>
<td>C</td>
<td>224+143</td>
<td></td>
</tr>
<tr>
<td>C3123A</td>
<td>F-GGA TTC AGA TTT CTC TTT GAA</td>
<td>chromosome X</td>
<td>Alul at 37 OC/24 h</td>
<td>C</td>
<td>321</td>
<td>(Katsuya et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>R-GCA TAG GAG TAT GAT TTA TCT C</td>
<td></td>
<td></td>
<td>A</td>
<td>214+107</td>
<td></td>
</tr>
</tbody>
</table>
levels after fluoxetine treatment (P=0.043). No significant association was found between FBS levels after a course of treatment with fluoxetine and other studied variants.

### 4. Discussion

Along with environmental factors that have substantial contribution to diabetic morbidity and mortality, genetic background seems to play a vital role as well. Data from epidemiological, twin and family studies suggest that genetic susceptibility play a major role in the development and progression of T2DM (38).

The pro-inflammatory & pro-oxidant characteristics of ACE and the role of inflammation in prediction of future risk of diabetes may suggest the effect of RAS polymorphisms in diabetes. Furthermore it is reported that ACE gene polymorphisms may influence the metabolism of lipids and lipoproteins in diabetic patients (39-41) still some studies do not confirm these associations (42, 43).

Inhibition of ACE and Ang II receptors have been shown to ease microcirculation of skeletal muscles, providing better blood circulation leading to higher secretion of insulin (44). On the other hand, given that fluoxetine decreases serum Ang II, it may be assumed that depressed patients carrying certain variants of RAS, associated with higher serum ACE, may have significantly reduced FBS levels after taking fluoxetine.

To the best of our knowledge, this is the first report regarding the association of RAS gene polymorphisms and FBS changes after a course of treatment with fluoxetine in depressed patients.

The most widely studied gene polymorphism of the RAS pathway, with respect to association with depression and T2DM has been ACE I/D (29, 30, 45, 46). The association between the D allele of the mentioned polymorphism and diabetes has been repeatedly reported (29, 30, 47). Regarding association with depression, except one study in a Japanese population (48), no association has been observed between this variant and depression in other studies (46, 49-51). In Japanese, Indian and Bahraini populations, association between DD genotype and an increase in FBS has been reported (29, 52, 53). On the other hand, evidences indicate that carriers of DD genotypes of ACE I/D had lower levels of blood insulin (54) which may lead to higher FBS levels. Considering the relationship between the D allele of ACE I/D and higher incidence of diabetes it may be postulated that elevated serum ACE activity may lead to higher FBS levels.

The novel finding of our study was the association between GG genotype of ACE A2350G and significant reduction in FBS levels after a course of treatment with fluoxetine. In our previous study (55), a strong association was observed between the mentioned genotype and depression. Likewise, depressed patients carrying the same variant (GG genotype) showed significantly higher serum ACE levels. Considering these findings, all together, this may be assumed that use of fluoxetine in GG carriers significantly reduced the FBS levels by inhibiting serum ACE activity.

Considering A-240T polymorphism and higher serum ACE levels (56), we also analyzed the relationship between the mentioned variant and FBS levels after treating with fluoxetine. The FBS level was not influenced by this variant in our

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Depressed patients (n=100)</th>
<th>Pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE I/D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>16 (16%)</td>
<td>0.128</td>
</tr>
<tr>
<td>ID</td>
<td>42 (42%)</td>
<td>0.231</td>
</tr>
<tr>
<td>ACE A-240T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>10 (10%)</td>
<td>0.623</td>
</tr>
<tr>
<td>AT</td>
<td>49 (49%)</td>
<td>0.650</td>
</tr>
<tr>
<td>ACE A2350G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>13 (13%)</td>
<td>0.043</td>
</tr>
<tr>
<td>AG</td>
<td>33 (33%)</td>
<td>0.333</td>
</tr>
<tr>
<td>M235T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>7 (7%)</td>
<td>0.955</td>
</tr>
<tr>
<td>MT</td>
<td>69 (69%)</td>
<td>0.658</td>
</tr>
<tr>
<td>A1166C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>58 (58%)</td>
<td>0.311</td>
</tr>
<tr>
<td>AC</td>
<td>30 (30%)</td>
<td>0.751</td>
</tr>
<tr>
<td>C3123A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>58 (58%)</td>
<td>0.506</td>
</tr>
<tr>
<td>CA</td>
<td>42 (42%)</td>
<td>0.473</td>
</tr>
</tbody>
</table>
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studied group. Regarding lack of association between this polymorphism and serum ACE levels in our previous study (55) this finding may be rationalized.

It is noteworthy that information on the genetic pattern of various polymorphisms of RAS not only assists in screening individuals more at risk of encountering illnesses such as depression and diabetes, but may also affect the clinical response to treatment. Yet the foregoing theory may need verification in prospective studies in the future and also in different ethnic groups.

Conflict of Interest
None declared.

5. References
18. Tuck ML, Bounoua F, Esami P, Nyby MD, Eggensa P, Corry DB. Insulin stimulates endogenous angiotensin II production via a mitogen-activated protein kinase pathway in


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