RAS Gene Polymorphisms and Renal Responsiveness to RAS Inhibition Therapy in Type 2 Diabetic Asian Indians

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Abstract

Objective: Inhibitors of renin angiotensin system (RAS), ACE inhibitors (ACEI) and angiotensin II receptor blockers (ARBs), are frequently used as renal-protective agents in type 2 diabetes (T2D). However, there is significant inter individual variability in response to these drugs. In the present study, we examined the role of genetic polymorphisms in ACE, AGT and AGTR1 genes, in modulating renoprotective response to ACEI and ARB therapy in north Indian T2DM subjects, with cases having diabetic nephropathy (DN) and controls without DN.

Method: 810 north Indian T2D patients treated with ACEI or ARB after diagnosis were followed up for 3 years. Percent changes in eGFR, urinary albumin excretion (UAE), serum creatinine at the end of 3 years of treatment were taken as points of renoprotective response.

Result: We observed that ACEI genotype and cumulative risk score of ≥ 1 was associated with better renoprotective response to ACEI in T2D, with normoalbuminuria (p<0.05). Whereas in T2D with micro/macrowavealbuminuria, DD genotype (ACE I/D) and a risk score of > 6 was associated with better renoprotective response to ARB (p<0.05).

Conclusion: Our results suggest that ACE I/D genotypes individually and in interaction with other RAS SNPs modulate renoprotective efficacy of ACEI and ARB in T2D patients, depending on the status of proteinuria.

Keywords: RAS gene polymorphisms; RAS inhibition therapy; Type 2 diabetes; Diabetic nephropathy

Introduction

Type 2 diabetes (T2D) is the most common cause of nephropathy, accounting for nearly 44% of renal failure cases [1,2]. In addition, in spite of adequate glycemic and blood pressure control, progression to renal failure in T2D patients is highly variable, indicating interplay between genetic and other predisposing factors in the development of the kidney disease in diabetic patients [3-5].

The renin–angiotensin system (RAS) has been strongly implicated in the pathogenesis of progressive renal diseases, including DN and inhibitors of RAS: Angiotensin converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARBs) are frequently used as renal-protective agents in T2D patients [6]. ACEI and ARB therapy is known to improve glomerular function, prevent proteinuria, and exerts beneficial effects on the progression of renal disease [7]. However, there is significant inter individual variability in responses to RAS inhibition by ACEI, or/and ARB. Studies over the past decade have shown that polymorphisms in RAS genes such as Angiotensin-converting enzyme (ACE), Angiotensinogen (AGT), and Angiotensin II type I receptor (AGTR1) may partly influence the observed inter individual variation, in response to RAS blockade [8-14]. It has been suggested that genetic variants, which have an association with the local tissue activity of RAS in diseased kidney, may also determine the responsiveness to ACE I/ARB [15,16]. The most common candidate gene variant proposed to date to influence response to RAS inhibition is an insertion/deletion (I/D) polymorphism in the ACE, which has been shown to influence the concentration of ACE, both in circulation and local tissue [7]. Several studies have examined association of ACE polymorphism, renoprotection and response to RAS inhibition therapy in T2D, but results have been conflicting, contrasting and less consistent [17]. For example, reduction of endpoints in NIDDM with the Angiotensin II antagonist losartan (RENAAL) study, comprising 1513 patients showed DD genotype to be associated with better response and higher risk reduction in end stage kidney disease (ESKD) [18], whereas a few studies with smaller sample size reported that response to ARB in T2D with renal disease was independent of ACE I/D polymorphism [12-14]. A post hoc analyses of a large prospective, randomized, double blind, placebo-controlled clinical trial of RAS inhibitor therapy in diabetic proteinuric renal disease, where end stage kidney disease (ESKD) was the primary outcome variable, reported that DD and ID genotypes were associated with greater reduction in risk of ESKD with RAS inhibition therapy, whereas no treatment effect was observed in the II genotype carriers [19].

Although gene polymorphisms for other components of RAS, such as AGT and the AGTR1, have been shown to be associated with development and progression of DN in T2D, however, their role in modulating response to RAS inhibition therapy is not well studied. Dragović et al. [20] showed that 1166 A/C AGTR1 polymorphism was associated with the renoprotective response to ARB therapy in type 1 diabetic patients. Narita et al. [21] observed that M235T and A (-20) C genotype of AGT could influence the therapeutic efficacy of a RAS blockade in immunoglobulin A nephropathy (IgAN). In a recent review, Konoshita [22] concluded that genetic variants of the RAS were not individually associated with antihypertensive effects by RAS blockade,

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but could act synergistically to modulate drug response. The goal of our study was to investigate the association of gene polymorphisms of ACE, AGT and AGTR1 genes, with renoprotective response to ACEI and ARB therapy in north Asian Indian T2D patients, with cases having DN and controls without DN. For this, we prospectively enrolled and genotyped T2D patients with and without nephropathy, on ACEI or ARB therapy from a large tertiary hospital of North India, and followed them up in renal clinic for 3 years for evaluating their renal responsiveness to therapy.

**Materials and Methods**

**Study population**

T2D patients of north Indian ethnicity attending the Endocrinology and Nephrology clinics were enrolled and followed up for a period of three years between January 2009 and March 2012. Their ethnicity was confirmed on the basis of language spoken, region of residence and ancestral history. All the patients were age, sex and ethnicity matched. No regional differences in disease prevalence or allele frequency were observed between recruitment sites. The inclusion criteria were as follow: age at onset of diabetes >35 years, T2D for more than or equal to 5 years, north Indian origin. Diabetes diagnosis was based upon WHO criteria with fasting plasma glucose ≥ 7.0 mmol/l. Moreover, since the patients had T2D for 5 years or more, they were well characterized T2D patients and were under chronic treatment. Based upon the inclusion criteria, a total of 940 patients of age >35 years with mean duration of diabetes (8.4 ± 2.0) years were screened, and 810 subjects were finally included in the study, after excluding patients with a history of glomerulonephritis, microscopic hematuria, or known history of obstructive uropathy, such as renal stone on ultrasound scan from the study. Patients who were intolerant to ACEI, ARB, pregnant and lactating women, and patients taking aspirin and other non-steroidal anti-inflammatory drugs (NSAIDS), were also excluded from the study. For each subject, detailed clinical history, biochemical and documentation of drug information were recorded. The study involved outpatient visits scheduled every 6 month. The study was approved by Post Graduate Institute of Medical Education and Research, Chandigarh, ethics committee. Written informed consent was taken from all subjects for genetic testing, and the genetic tests to be performed were pre-specified at the time of the study design.

T2D subjects were divided into two groups: Group 1 (n=490), consisted of patients without nephropathy (normoalbuminuria) and Group 2 (n=320) consisted of patients with nephropathy (microalbuminuria, and/or macroalbuminuria). Patients were considered normoalbuminuric when their urinary albumin excretion rate (UAE) was <30 mg/24 hours. Patients whose UAE increased into the range of 30-300 mg/24 hours were considered microalbuminuric. Patients having urinary albumin excretion rate greater than 300 mg/24 hours were considered macroalbuminuric. Patients received ACEI or ARB after the diagnosis and during their clinical course as per the decision of treating physician; patients who were on combined therapy, or were initially treated with ACEI and then switched to ARB, or vice versa were excluded from the study. In Group 1, 318 patients were treated with ACEI and 172 were treated with ARB. In Group 2, 190 patients were treated with ACEI and 130 were treated with ARB. None of the enrolled patients were on combination therapy with both ACEI and ARB. Patients taking aldosterone antagonists and renin inhibitors were also excluded from the study. Although, Group 1 patients were normoalbuminuric, they were prescribed ACEI/ARB as all the Group 1 patients were diabetic, and ACEI and ARB are used as antihypertensive agents, and for preventing kidney damage in people with hypertension or diabetes (Table 1).

**Clinical response points**

The change in eGFR, urinary albumin excretion rate (UAE) and serum creatinine levels at baseline and end of 36 months of active treatment period were taken as primary points of response to RAS inhibition therapy. UAE and serum creatinine were measured by Hemocue and Roche autoanalyzer, respectively. eGFR was calculated by Modification of Diet in Renal Disease (MDRD) formula. GFR (mL/min/1.73 m²)=175×(Scr)^-1.154×(Age)^-0.203×(0.742 if female).

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**Table 1:** Clinical characteristic of patients at baseline and after 36 months of ACEI/ARB therapy.
Genotyping

Genomic DNA was isolated from peripheral blood lymphocytes using proteinase K digestion and phenol chloroform method. AGT rs5050, AGT rs4762, AGT rs699 ACE rs4311, ACEI/D, ACE rs4343 and AGTR1 rs5186 SNPs were genotyped, as described by Ahluwalia et al. [15] (Supplementary Table 1). Positive and negative controls were used in each genotyping run, and 5% of randomly selected samples were re-genotyped by other lab personnel with 100% concordance. The genotypes were also confirmed by randomly sequencing some of the samples.

Risk score analysis

A Risk score from 0-7 was calculated for each patient, based on the presence of number of variant alleles of studied polymorphisms. Their association with change in eGFR, UAE, serum creatinine levels in response to ACEI and ARB therapy was analyzed in both the groups. Genetic risk score using seven different SNPs in theory could vary between 0 and 14 risk alleles; however, as we counted the number of carriers instead of alleles, so the risk score varies between 0 and 7.

Statistical analysis

The statistical tests were performed using the SPSS Inc., Chicago, IL version 11.0. We tested the genotype and allele frequencies for deviation from Hardy-Weinberg equilibrium (HWE) proportions, by using Hardy-Weinberg equilibrium calculator. Using a chi-square test, the deviation of genotype distribution from HWE was considered significant at P<0.05. Percentage increment or decrement are ratios and are considered as ordinal variables, so all statistical comparisons were performed with non-parametric test-the Kruskal-Wallis, and then using Bonferroni post-hoc test to indicate which group differs from the others. Wilcoxon t-test was used for the difference in delta values of means, before and after treatment. Power analysis was performed using Quanto (version 1.2; http://hydra.usc.edu/gxe). Positive associations observed between RAS genotype and ACEI and ARB blocker was adjusted for confounding factors using multivariate logistic regression, and this association persisted even after the influence of confounding factors, including hypertension, smoking, systolic blood pressure and triglyceride levels (P<0.05 for pair-wise comparison for the three polymorphisms of ACE and AGT genes). No significant LD was observed among either ACE or AGT variants (r² <0.05). The LD values were generated to look for association among the three polymorphisms of the ACE and AGT genes.

Genotype distribution of the polymorphisms examined in the study population is summarized in supplementary table 2. All the polymorphisms examined in the present study were in HWE in both the groups of patients. Baseline characteristics by genotype showed that patients with Group II had significantly higher initial baseline UAE and serum creatinine. LD values were generated to look for association among the three polymorphisms of the ACE and AGT genes. No significant LD was observed among either ACE or AGT variants (r² <0.05).

Results

Clinical characteristics of patients at baseline and after 36 months of ACEI/ARB therapy

A total of 810 patients completed the study. Diabetic patients with nephropathy (Group 2) were younger, had significantly longer duration of hypertension (HTN), had higher SBP, HbA1c and lower hemoglobin (Hb), as compared to diabetic patients without nephropathy (Group 1). Number of patients taking sulfonylurea (54.2% vs 35.1%, P=0.0001), metformin (85.2% vs 59.3%, P=0.0002) and pioglitazone (40.1% vs 21.5%, P<0.0001) were higher in Group I as compared to Group II. Use of insulin was common among patients with Group II as expected (31.2% vs 39.6%, P=0.002).

Change in eGFR, urinary albumin excretion rate (UAE), serum creatinine levels at baseline and the end of 36 months of active treatment within Group 1 and Group 2 patients

Both Group 1 and Group 2 patients within Groups showed significant delta eGFR, UAE and serum creatinine at the end of 36 months of active treatment of ACEI/ARB therapy (Table 2).

Association of polymorphisms with UAE, serum creatinine and eGFR

Genotype distribution of the polymorphisms examined in the study population is summarized in supplementary table 2. All the polymorphisms examined in the present study were in HWE in both the groups of patients. Baseline characteristics by genotype showed that patients with Group II had significantly higher initial baseline UAE and serum creatinine. LD values were generated to look for association among the three polymorphisms of the ACE and AGT genes. No significant LD was observed among either ACE or AGT variants (r² <0.05).

Percent change in eGFR, UAE, serum creatinine after ACEI and ARB treatment

Group 1 patients with II genotype (ACE I/D) showed greater percent decrease in UAE, serum creatinine and greater percent increase in eGFR (p=0.01), with ACEI after 36 months (Figure 1). No significant difference in percent change in UAE, serum creatinine and eGFR were observed among Group 1 patients, based on (ACE I/D) genotypes with ARB after 36 months of therapy (Figure 2). In Group 2, patients with DD genotype (ACE I/D) showed greater percent decrease in UAE, serum creatinine, and greater percent increase in eGFR (p=0.008) with ARB after 36 months (Figure 2). However, there was no significant difference in percent change in UAE, serum creatinine and eGFR in these patients, based on (ACE I/D) genotypes with ACEI after 36 months (Figure 1). None of the other studied gene polymorphisms of ACE, AGT or AGTIR showed any significant association with percent change in UAE, serum creatinine and eGFR in patients on either ACEI and ARB therapy in both the groups (Supplementary Table 3).

Effect of ACE and AGT gene haplotypes on clinical points

Group 1 patients with haplotype T-D-G (alleles of rs4311, I/D, and rs4343) showed significantly lower percent change in UAE, serum creatinine and eGFR with ACEI (p=0.009), as compared to other haplotypes (C-I-G and C-I-A) (Figure 3). However, no significant difference in response to ARB was observed based on ACE haplotypes in these patients (Figure 4).

Table 2: Change in eGFR, urinary albumin excretion rate (UAE), serum creatinine levels at baseline and the end of 36 months of active treatment.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
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<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>98.3 ± 19.8</td>
<td>128.1 ± 16.3</td>
</tr>
<tr>
<td>S.Creatinine (mg%)</td>
<td>0.9 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>UAE</td>
<td>70.1 ± 50.2</td>
<td>45.6 ± 42.3</td>
</tr>
</tbody>
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(p<0.05 is significant). The difference in delta values of means before and after treatment was done using Wilcoxon t-test. Group 1: type 2 diabetes without nephropathy. Group 2: type 2 diabetes with nephropathy.
percent change in UAE, serum creatinine and eGFR after treatment with ACEI, as compared to patients having 0 risk score (p=0.005). However, in Group 2 patients, no significant difference in response to ACEI was observed based on risk score (Figure 5).

**ARB therapy:** Group 2 patients with a risk score of > 6 showed maximum change in UAE, serum creatinine and eGFR after ARB treatment (p=0.01), whereas patients with zero risk score showed minimum change in UAE, serum creatinine and eGFR after ARB treatment. Group 2 patients with a risk score of > 2 showed significantly greater change in UAE, serum creatinine and eGFR after treatment with ARB, as compared to patients having no risk allele (p=0.009). However, in Group 1 patients, no significant difference in response to ARB was observed based on risk score (Figure 6).

**Discussion**

An inter-individual variation in response to ACEI or ARB therapies is often observed, and it has been suggested that this may be partly genetically determined.

Our results suggest that ACE I/D polymorphism was a significant modulator of response to ACEI and ARB therapy in our cohort of T2D patients, depending on their status of proteinuria. We observed that in normoalbuminuric T2D patients, renoprotective response to ACEI therapy was more effective in II genotype subjects. In contrast, in T2D patients with nephropathy and on ACEI, percent change in UAE, serum creatinine or eGFR was similar in all genotypes. Our results are consistent with findings from several studies in other ethnic groups, which also indicated that the anti-proteinuric response to ACEI was more favorable in patients with I allele [8-10]. However, our results differ from a study by Ha et al. [11], which showed that ACEI therapy decreased proteinuria more effectively in those with the DD, than in those with the II or ID genotype. However, the sample size was very small (n=83) in this study, and it had very short duration of follow-up (3 months).

We also observed that ACE I/D genotype based differences in renal response to ARB were more pronounced in T2D patients with
response to RAS blockade. For example, ACEI reduce glomerular capillary hydraulic pressure more effectively in patients with the II, than in those with the DD genotype. It has been suggested that in II carriers, decreased glucose-induced pre-glomerular vasodilatation and less severe hyperfiltration might amplify the long-term protective effect of ACEI therapy against development and progression of nephropathy [23,24,17]. ACE I/D is in non coding region, which makes it unlikely to be a functional variant, but several studies suggest that it may be in close linkage disequilibrium (LD), with a quantitative trait loci (QTL) controlling ACE levels [25,26]. Recent data also indicate that ACE expression may also be under epigenetic control [27].

We found no significant association of AGT and AGTR1 gene polymorphisms, with response to ACEI and ARB therapy in our cohort. This is in contrast to the observations by Narita et al. [21] and Konoshita [22], who reported that AGT haplotypes and AGTR1 A1166 C polymorphism could influence the therapeutic efficacy of a RAS blockade in nephropathy patients. This may be due to the different phenotypes of nephropathy patients, study design and duration of follow-up in these studies.

It has been suggested that haplotype approach to study common variations within relevant candidate genes is more likely to unravel any existing pharmacogenetic associations in multiple RAS genes, and their relation to ACE I and ARB treatment benefit. For example, Zhu et al. [28] showed an epistatic interaction between ACE variants (rs4311 T/C), and in (rs4343 A/G), in modulating blood pressure, and Su et al. [29] reported that two haplotypes of AGTR1 were associated with blood pressure reduction, in response to benazepril. Our results showed that ACE gene haplotypes were a significant modulator of response to ACEI and ARB therapy. SNPs associated with these specific haplotypes may be in linkage disequilibrium with SNPs that controls inter individual variation in response to ACEI or ARB therapies, depending on the status of proteinuria. SNPs associated with these specific haplotypes may be in linkage disequilibrium with some functional polymorphism that directly influence clinical efficacy of ACEI and ARB therapy, depending on the status of proteinuria.

We also analysed synergistic/additive effect of multiple SNPs in the nephropathy, as compared to patients with normoalbuminuria: DD and ID genotype carriers with proteinuria showed better beneficial response to nearly all endpoints, as compared to II genotype carriers. These results are consistent with those reported by Parving et al. [19], who also observed that D allele was associated with better response to angiotensin II blockade in T2D with overt nephropathy. However, Andersen et al. [13] and Haneda et al. [14] have reported no association between ACE I/D polymorphism, and reduction in treatment-induced proteinuria in T2D and overt nephropathy. These differences could be attributed to small sample size and ethnic differences between the studied cohorts. Thus, our results suggest that ACE genotypes may be a good marker for clinical efficacy of ACEI and ARB in DN. A plausible explanation for these observations may be that different ACE I/D genotypes may affect renal hemodynamics to a different extent, resulting in differential
RAS genes for pharmacogenetic associations, in relation to ACEI and ARB in T2D patients. For this, we calculated a risk score in each patient, based on the presence of risk alleles of the RAS gene polymorphisms genotyped in our study. We observed that response to ACEI /ARB was associated with the number of risk alleles carried by an individual patient; patients with no or 1 risk allele with normoalbuminuria showed greater reduction in UAE, serum creatinine, and better preservation of eGFR with ACEI therapy. In patients with nephropathy, response to ACEI was not influenced by the number of risk alleles carried by an individual patient. However, response to ARB was better in those with risk score of 6 or 7 in micro /macroalbuminuric patients. To date, there is no published data on association of synergistic/additive effect of multiple SNPs in the RAS genes, with antiproteinuric response to ACEI and ARB therapy. Thus, our is the first results suggesting that the risk score analysis may be a good marker for clinical efficacy of ACEI and ARB therapy in T2D patients, depending on the status of proteinuria.

There are several strengths and some limitations of our study: we had ethnically homogenous diabetic subjects who were enrolled from a single center, thus avoiding phenotyping errors and bias. No regional differences in disease prevalence or allele frequency were observed between the two recruitment sites (Department of Endocrinology and Department of Nephrology, Post Graduate Institute of Medical Education and Research, Chandigarh). Also, as patients were seen at a tertiary hospital, they were well phenotyped, and could be followed for a long time.

The sample size was predetermined for studied variants to have a minimum power of 85% (power ranged from 86%-94% for selected polymorphisms of RAS), at a small effect size (0.1) and alpha level (0.05). Positive associations observed between RAS genotype and ACEI and ARB blocker do not seem to be due to chance, as this association persisted even after the influence of confounding factors was corrected. A limitation of our study might be that instead of prescribing any ACEI genotypes individually, and in interaction with other RAS single-nucleotide polymorphisms may be a good marker for clinical efficacy of ACEI and ARB therapy in T2D patients, depending on the status of proteinuria.

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