Studies on Association of ACE Insertion/Deletion Polymorphism in Smokeless Tobacco Induced Renal Failures in Costal Belt of Andhra Pradesh, India

P. Sravana Kumar, P. Jaganmohan and M. Subbarao

1Acharya Nagarjuna University Post Graduate Centre, Nuzvid, Krishna District, A.P., India-521201
2Harrison Institute of Biotechnology, Shrimp Care Unit, Ramamurthy Nagar, Nellore, A.P., India-524003

Abstract: The present study has been carried out at selective districts of Andhra Pradesh to evaluate the relation between renal failure and ST induced renal damage in means of angiotensin I converting enzyme (ACE) polymorphism. The contribution of insertion/deletion (I/D) polymorphism of the gene encoding ACE has been investigated and the deletantype is documented to be a risk factor in the development of this disease. All the subjects, identified as DD, were reconfirmed with an insertionspecific primer. There was no significant difference in the distribution of DD, ID and II genotypes between ST induced renal failure and normal healthy subjects. The findings of the present study suggested that the ACE I/D polymorphism is not associated with renal failures due to ST usage and intake of nicotine within the selected regional population.

Key words: ACE · Polymorphism · Nicotine · Smokeless Tobacco

INTRODUCTION

Although the tobacco has dangerous effect on human health, it has been used throughout the world [1, 2]. Nicotine is used in different forms including smoking and smokeless tobacco [1]. Most of the educated and uneducated young peoples are addicted to Gutka powder in India. For the preparation of Gutka nicotine tobacco is used. Tobacco leaves are powdered and mixed with lime [3]. This mixture is packed in 9 g/packet and sold in local groceries. This simply readily available powder is placed between lower labile mucosa and gingival for about 5-10 min and then spit out. The mixture is used (7-10 times per day) 2-3 g for each time. The immediate availability and the low price give rise to high consumption of chewing tobacco. The effect of smokeless tobacco on different biochemical and hematological profiles were studied [4, 5], studies on its effect on renal functions have been reported by Sravana Kumar et al.[6]. In this connection it is very much essential to see the role and possible relation of ACE-1 gene insertion/deletion polymorphism to renal damage in ST users as the ACE is as a part of The renin-angiotensin aldosterone system (RAAS) has a key role in both cardiovascular and renal pathophysiology [7, 8].

Angiotensin II (angII), the most important biological active product, is synthesized via a pathway that involves several precursor peptides and enzymes, some of them regulated by separate genes. The main known action of angII is its potency to constrict vascular smooth muscle cells and to stimulate fluid and sodium retention, by directly acting on tubular cells and through stimulating aldosterone release. However, more recently other potential actions of angII have been elucidated. Several studies have revealed that angII in vitro promotes vascular smooth muscle, glomerular mesangial and renal tubular cell growth [8]. Therefore, it is clear that genetic polymorphisms of the RAAS have gained interest in the search for genetic factors that might influence the progression of chronic renal failure and the response to treatment to renoprotective regimens.

Angiotensin I converting enzyme (ACE) is a zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells. By stimulation of renin, angiotensinogen is converted to angiotensin I. ACE then converts angiotensin I to angiotensin II, the main active product of the RAAS. The human ACE gene is located on chromosome 17 and includes 26 exons. The coding sequence codes for a 1306 amino-acid protein, including a signal peptide. The gene product, ACE, is composed of 2 homologous domains with 2 active sites. The main aim
of the study is to study the association of insertion/deletion polymorphism of ACE gene-1 in development of renal failures due to smokeless tobacco usage.

**MATERIALS AND METHODS**

Around six hundred ST consumers (n=600) were taken from three districts (200 per district) for the analysis among the three selected coastal districts with different age groups (ranging 16-40 years) who are consuming Gutka 10-12 g/day for a period of 4-5 years. Controls also found in the same selected area those who did not take any smokeless powder (n=260). They were provided with explanations for all experimental procedures and informed consent was obtained before the beginning of the study. Blood and urine samples were collected from the subjects and preceded for the biochemical and molecular analysis.

**Estimation of Serum and Urine Nicotine and Related Content:** The nicotine and its other analytes have been analyzed in the serum and urine of both controls and test samples using a combination of solid phase extraction with liquid-chromatography-tandem mass spectroscopy. Qualitative identification and quantitation are accomplished by comparison of the specimen components to a standard curve of each analyte addressed in the assay [9].

**Estimation of Random Blood Glucose:** Blood glucose measured routinely using an ‘One Touch Ultra’ blood glucose meter. Regular calibration and quality control were by means of samples sent to the local hospital biochemistry laboratory. Normal random blood sugar level should be less than 200mg/dl.

**Estimation of Serum Creatinine:** Blood was collected into the sterile micro tubes and stand at room temperature to clot. Centrifuge the tube at 3000 rpm for separation of blood clot with the serum. The supernatant was collected and analyzed for the presence of creatinine. Serum creatinine levels were all assayed with the rate-Jaffe reaction on a Hitachi 747 autoanalyzer (Roche Diagnostics Corp., Indianapolis, Indiana). This assay was calibrated daily with a Cfas calibrator (Roche Diagnostics Corp.) by using the uncompensated method during the study period.

**Determination of ACE Genotypes:** The D and I alleles were identified on the basis of polymerase chain reaction (PCR) amplification of the respective fragments from intron 16 of the ACE gene and size fractionation and visualization by electrophoresis. DNA was extracted from peripheral leukocytes with standard techniques. PCR was performed with 20 pmoles of each primer: sense oligo 5’CTGGAGACCACCTCCCATCCTTTCT3’ and anti-sense oligo: 5’GATGTTGCCATCATCATTGTCAGAT3’. In a final volume of 25 µl, containing 1.5 mM MgCl₂, 50 mM KC, 10 mM Tris-HCl pH 8.3, 0.2 mM of each dNTP and 1.25 unit of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). The DNA was amplified for 30 cycles with denaturating at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min (DNA Thermal Cycler 480, Perkin Elmer-Cetus) [10, 11]. PCR products were electrophoresed in 2% agarose-gel with 5 µg ethidium bromide per milliliter. The amplification products of the D and I alleles were identified by 300-nm ultraviolet trans-illumination as distinct bands (D allele: 191 bp; I allele: 478 bp) Because the D allele in heterozygous samples is preferentially amplified, each sample found to have the DD genotype was subjected to a second independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (hace 5a, 5’TGCCAGCCTCAGCCCTCAATCACT3’; hace 5c, 5’TCGCCAGCCTCAGCCCTCAATCACTA3’), with identical PCR conditions except for an annealing temperature of 67°C. The reaction yieleds 335-bp amlicon only in the presence of an I allele and no product in samples homozygous for DD [12, 13].

**Statistical Analysis:** Statistical analysis was carried out using SPSS for windows 10.0 software (SPSS Inc., Chicago, IL, USA) and Microsoft Excel. Values were reported as mean ± standard deviation. SD was not more than 10%. The difference between groups was compared by Pairwise Multiple Comparison Procedures (Duncan's Method). p value <0.001 was considered statistically significant.

**RESULTS AND DISCUSSION**

The analysis of serum and urinary nicotine analytes have revealed that all the three analytes were found to be increased than the controls to greater extent both in serum and urine samples of the ST users than the controls (Table 1).

Blood glucose level (fasting and post prandial) was assayed with the help of one pick glucometer. These results showed that the values are not significant (p>0.001) and there was not much change when compared to that of control value. Mean values of both fasting and post prandial glucose levels are within the normal range.
Table 1: Analysis of serum nicotine and its metabolites

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Control (Serum)</th>
<th>Test (Serum)</th>
<th>Control (Urine)</th>
<th>Test (Urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine (ng/ml)</td>
<td>2.7</td>
<td>39.4</td>
<td>3.1</td>
<td>3730</td>
</tr>
<tr>
<td>Cotinine (ng/ml)</td>
<td>1.9</td>
<td>403</td>
<td>2.2</td>
<td>5024</td>
</tr>
<tr>
<td>OH-Cotinine (ng/ml)</td>
<td>2</td>
<td>153</td>
<td>2.7</td>
<td>7789</td>
</tr>
</tbody>
</table>

Table 2: Distribution of the genotype and allele frequencies in the study groups for the angiotensin converting enzyme (ACE) I/D polymorphism

<table>
<thead>
<tr>
<th>Population (n)</th>
<th>DD</th>
<th>ID</th>
<th>II</th>
<th>D allele</th>
<th>I allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=240)</td>
<td>10 (16.6%)</td>
<td>33 (55.0%)</td>
<td>17 (28.3%)</td>
<td>0.441</td>
<td>0.559</td>
</tr>
<tr>
<td>Test (n=240)</td>
<td>16 (17.7%)</td>
<td>48 (53.3%)</td>
<td>26 (28.8%)</td>
<td>0.44</td>
<td>0.56</td>
</tr>
</tbody>
</table>

$\chi^2$ based on allele frequency [degrees of freedom (df) = 1], (ST usersVs Controls) = 0.00025

Fig. 1: Comparison of Blood glucose levels and Blood pressure in controls and ST users

Fig. 2: Comparison of serum creatinine levels in controls and ST users

Both in control and test samples, which indicates that the selected population is not having prior history of diabetes or hypertension (Figure 1). Control subjects showed the creatinine content of 1.43 mg/dl, whereas the ST consumers showed a significant ($p<0.05$) increase of 2.75 mg/dl, which shows a drastic increase in the serum creatinine value and the loss of renal function (Figure 2).

For this study a total of 90 people were selected for the DNA analysis, since it is a cost effective issue. The DNA samples from 90 ST mediated nephropathy and 60 normal healthy controls were amplified for I/D polymorphism in the ACE gene and analyzed. Figure 3 represents the PCR products of 190 and 490 bp indicating the presence of deletion (DD) and insertion (II) genotype, respectively. The preferential amplification of the D allele and inefficiency of the amplification of I allele may result in the mistyping of ID heterozygotes as DD homozygotes. Therefore, in order to increase the specificity of DD genotyping, all samples, identified as DD after initial amplification were reconfirmed with an insertion-specific primer pair, as mentioned in material and method section.
Fig. 3: Agarose gel electrophoresis stained with ethidium bromide, showing the initial amplification for ACE I/D polymorphism. Lane L represents the 100 bp ladder. The I allele genotype was identified by the presence of single 490 bp product (Lanes 3, 4, 5 and 7). The D allele genotype was identified by the presence of a single 190 bp product (Lanes 1, 2 and 6). The DD homozygotes were reconfirmed with insertion specific primer pair to avoid mistyping as ID heterozygotes.

Fig. 4: Agarose gel electrophoresis of PCR products, using insertion specific primer pair, of individuals labeled as DD homozygotes following initial amplification. Absence of a product in the lanes 4 confirms the presence of DD genotype. Heterozygous individuals (ID genotypes) were confirmed by the presence of a single 275 bp product (Lanes 1, 2, 3, 5 and 6). Lane L represents the 100 bp ladder.

The presence of insertion sequence was revealed by the amplification of a 275 bp fragment, while DD homozygotes failed to amplify due to the lack of annealing site (Figure 4).

Table 2 shows the distribution of ACE genotypes in ST mediated nephropathy patients and normal controls. The frequency of D allele and DD genotype was only marginally higher in ST affected patients as compared to the normal controls. The observed and expected genotypic frequencies were in Hardy-Weinberg Equilibrium.

In recent years a vast amount of data has been published on the association between the insertion/deletion (I/D) polymorphism of the gene coding for angiotensin- converting enzyme and renal disease. It has become clear that the polymorphism does not affect the prevalence of renal disease. However, data on the association with progression of renal disease and therapy response are still contradictory. Moreover, sufficient data on the physiological significance of this polymorphism are still lacking. This contribution provides an overview of the available studies and the potential pitfalls in interpreting the data. We also discuss the putative mechanisms for the association between the DD genotype and progression of renal disease and suggest directions for the future that might be employed to further clarify the role in renal pathophysiology.

Renal failure is an outcome of complex pathophysiological process resulting from multiple etiologies with contribution from both genetic and environmental factors. A large variation abounds in the frequencies of ACE I/D polymorphism in different ethnic groups. It is evident from table 2 that the D allele frequency of our controls was intermediate to most reported Caucasian [14-18] Asian [19-23] populations. However, two Caucasian [24, 25] and an Asian [21] population are reported to have comparable allele frequencies. The failure to find statistically significant differences in the distribution of ACE gene I/D genotypes.
and their allele frequencies between the ST mediated nephropathy patients and the controls suggest that this polymorphism is not a risk factor for the development of renal failure in the studied population. These observations find support in the work of Tamaki et al. [21] and Ergen et al. [23]. In conclusion, our study suggests that the ACE I/D polymorphism is not associated with advanced form of renal failures due to ST intake within the selected regional population.

REFERENCES


