Association of ACE, AGT and AT1R gene polymorphisms with severity of Coronary Artery Disease

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Abstract: Coronary Artery Disease (CAD) is one of the most frequent causes of morbidity and mortality. A both environmental and genetic factor contributes to the development of CAD. Renin angiotensin system which regulates blood pressure plays an important role in pathogenesis of CAD. Angiotensin converting enzyme (ACE) and angiotensin II type 1 receptor (AT1R) are the major components of Renin Angiotensin System (RAS).

Objective of the study is to examine whether these RAS component gene polymorphisms have any impact on severity of CAD.

A total of 150 CAD cases mostly acute myocardial infarction (78%) and unstable angina (22%) were included in the study. Nearly age and sex matched 150 healthy controls were taken for comparison. The severity of disease was classified as Single Vessel Disease (SVD), Double Vessel Disease (DVD) and Triple Vessel Disease (TVD). Gene polymorphisms were studied by PCR-RFLP method.

The mean age of the patients was 54.6 ± 11.4 years. Most common risk factor for CAD was hypertension (48.6%). Among the total 150 CAD cases, 54.6% had Triple Vessel Disease. TVD was most frequently associated with DD of ACE (P<0.001) and TT of AGT genotype (P=0.016) when compared to DVD and SVD. Risk genotypes of ACE and AGT might influence the severity of disease. So genotype may predict prognosis and outcome of coronary artery disease.

Key words: Coronary Artery Disease, severity, Renin Angiotensin System, Gene polymorphism

I. Introduction

The term Coronary Artery Disease (CAD) or Ischaemic Heart Disease (IHD) defines a disease spectrum of diverse aetiology with common factor being an imbalance between myocardial oxygen supply and demand 1.

Coronary artery disease is the most common cause of death. The WHO estimated that in 2002, 12.6% of deaths worldwide were from CAD 2. Coronary artery disease is the leading cause of death in the developed countries, but third to AIDS and lower respiratory tract infection in developing countries 3.

In India also Coronary Artery Disease (CAD) is the leading cause of death 4. Although a relatively new epidemic in India, it has quickly become a major health issue with death due to CAD expected to double by 2015 5,6. According to WHO report 2002, CVD are projected to be the largest cause of death and disability by 2020, with 2.6 million Indians predicted to die due to Coronary artery disease predominantly with Myocardial Infarction (MI). Mortality estimates due to CAD vary widely with State, ranging from 10% in Meghalaya to 49% in Punjab, Punjab (49%), Goa (42%), Tamil Nadu (36%) and Andhra Pradesh (31%) have the highest CAD related mortality estimates 7. CAD affects Indians at a younger age (in their 30s and 40s) compared to western countries.

CAD in Asian Indians is known to be severe, extensive and malignant 8, 9. This gives information regarding the accelerated atherosclerotic process that begins early in life.

Enas and Senthilkumar, reported high rate of CAD in Asian Indians in USA which is accompanied by low rate of conventional risk factors 10. The same was also revealed by CADI (coronary artery disease in Indians) study. So the high prevalence of CAD among Asian Indians despite low incidence of risk factors suggests an important role of genetic risk factors.

There are established conventional risk factors like age, male sex, family history, hypertension, diabetes mellitus, smoking, dyslipidemia and obesity for CAD. Together with environmental factors, the involvement of multiple genes is also responsible for coronary artery disease 11.

Subjects having systolic blood pressure ≥140 mmHg and / or diastolic ≥90 mmHg or current use of antihypertensive medication are considered as hypertensives. Fasting blood glucose level of 126mg/dl or greater
are considered as diabetes. Serum total cholesterol level more than 200 mg/dl are hyperlipidemic. Obesity is defined as a body mass index (BMI) (weight in kg divided by the square of height in meters) of $\geq 30 \text{ kg/m}^2$ and overweight as 25-29.9 kg/m$^2$.

**Cad And Genes**

There are various candidate genes have been studied for the pathophysiology and clinical symptoms for coronary atherosclerosis 12, 13. The renin-angiotensin system (RAS) or the renin–angiotensin–aldosterone system (RAAS) has an important role in the pathogenesis of atherosclerosis. This enzyme cascade is a hormone system that regulates blood pressure, fluid and electrolyte balance 14.

The main polymorphic gene component of RAS are angiotensinogen (AGT), angiotensin converting enzyme (ACE) and angiotensin II type 1 receptor (AT1R) gene 15.

The severity depends on the number of vessels involved and the degree of stenosis evaluated by coronary angiogram. Patients were classified according to the number of significant stenotic vessels (vessels with more than 50% narrowing ) as Single Vessel Disease (SVD), Double Vessel Disease (DVD) and Triple Vessel Disease (TVD). The progression and extent of disease is extremely variable and do not depend on the conventional risk factors.

**Objectives Of Study**

The aim of the present study is to see the association of RAS gene polymorphisms with the severity of CAD in patients of north coastal Andhra Pradesh. India.

**II. Materials And Methods**

**Study Population**

A total of 150 CAD patients (unstable angina and acute myocardial infarction) and 150 healthy controls were included in the present study. Patients attending in-patient and out-patient departments of cardiology and cardiothoracic surgery unit of Care Hospital, The institute of medical sciences, Visakhapatnam, Andhra Pradesh (India) were included in the study after obtaining their informed consent. The study is approved by the Institutional Ethics Committee for research on human volunteers of Andhra University, Visakhapatnam. Patients were mainly from north coastal Andhra Pradesh which constitutes three districts (Visakhapatnam, Vizianagaram and Srikakulam). Normal healthy controls were also collected from the same region with informed consent. The period of study was from 2007-2011.

**Sample Collection**

With prior informed consent, 6 ml of peripheral blood was collected from 150 CAD cases and controls. Four ml of blood was collected into sterile centrifuge tubes containing 100 µl of 15% EDTA solution. Two ml of blood was allowed to clot for serum separation. Collected EDTA whole blood samples were transported to Department of Human Genetics, Andhra University, and Visakhapatnam for further analysis. Serum parameters like blood sugar and lipid profile were tested in Care Hospital Laboratory, maintaining internal (BIORAD QC sera) and external quality control (with CMC Vellore). Coronary angiogram was done in all cases to know the number of blocks in vessel and the severity of disease.

**Dna Isolation**

Total DNA was extracted using non-enzymatic method described by Lahiri and Nurnberger, (1991) 16.

**Genotyping Of The Ace Gene I/D ( Rs 4340 ) Polymorphism**

ACE gene was amplified by polymerase chain reaction (PCR) according to the method suggested by (Stone king et al, 1997) with primers from sigma laboratories 17.

Forward primer : 5'-CTGGAGACCACCTCCCATCTTTCT-3'
Reverse primer : 5'-GATGTGGCCATCACATTCGTCAGAT-3'

The amplified PCR product was separated on 2% agarose gel. The PCR products were of 490 bp for allele I and 190 bp for allele D.

**Genotyping Of The Agt M235t ( rs 699 ) Gene Polymorphism**

AGT M235T genotype was determined by PCR amplification followed by digestion with restriction enzyme Tthl III (fermentas) according to the described method (Russ et al, 1993) 18. Primers are procured from sigma laboratories as follows

Forward primer : 5'-CCGTTCGAGGCCTGGGCTCT-3'
Reverse primer : 5'-CAGGGTGCTGTCACACATCGGCCC-3'
Digested products were run by 2% agarose gel electrophoresis. The products were of 165 bp for allele M and 141 bp for allele T.

**Genotyping Of The At1r A1166c ( rs 5186 ) Gene Polymorphism**

For the analysis of the A/C polymorphism of the AT1R gene PCR protocol (Hingorani et al, 1995) was used taking primer sequence from sigma laboratories were as follows

Forward primer : 5'ATAATGTAAGCTCATCCACCCAAGAAG-3'
Reverse primer : 5'TCTCCTTCAATTCTGAAAAGTACTTAA-3'

PCR product was further digested by restriction enzyme (BspTI-Afl II) from fermentas. Digested products were run by 2% agarose gel electrophoresis. The AT1R allele were visualised as fragments of 166 bp (A) and 139 bp (C).

**Statistical Analysis**

Results are expressed as percentage and mean ± SD. Hardy-Weinberg law of equilibrium was tested for the ACE, AGT and AT1R gene polymorphism in CAD patients. Genotype distribution between cases and controls were compared by using chi-square and odds ratio (OR) with 95% confidence interval (CI). It was done by using SPSS package 16.0 version. Comparison of genotype was made between triple vessel disease with double and single vessel disease separately using two by two epidemiological calculator. For all cases P < 0.05 was considered as significant.

### III. Result

We genotyped 150 angiographically diagnosed CAD patients. Age and sex distribution of analyzed groups are shown in (Table I). Maximum numbers of patients were of 51-60 years with a M: F Ratio of 121:29. The mean age of the patients was 54.6 +/- 11.4. Risk factor profile of CAD patients (Table II) revealed hypertension in 48.6%, diabetes mellitus in 33.3%, dyslipidemia in 27.3%, and smoking in 26.0% and obesity in 6.0%.

Out of 150 cases, 54.6% were TVD, 34.0% were SVD and 11.3% of cases were DVD. According to clinical presentation 117 were of acute myocardial infarction cases and 33 cases were unstable angina. In-hospital death was there in 5.3% of cases (Table III).

Genotype distributions of total CAD cases irrespective of vessel disease were compared with normal healthy controls in Table IV. Frequency of ACE DD was 4.5 times higher (p= 0.03) and AGT TT was 12.61 times higher (p=0.0003) in cases when compared to controls.

About 22.6% of the patients had no risk factors. Cases with or without risk factors were compared among themselves according to the severity of disease (Table V). The percentage of Triple vessel disease was found almost similar in both the groups.

**Genotype distribution and allele frequencies:**

Genotypes at all genes were in the Hardy-Weinberg equilibrium. The genotypes and allele frequencies of the Alu ACE locus in patients and different groups of vessel diseases are given in Table VI. Chi-square (χ²) and p value were computed in subsets of the data that vary by the severity of the disease (SVD, DVD & TVD). Genotype and allele frequencies comparison was made between TVD vs DVD and TVD vs SVD. DD genotype and D-allele were significantly more frequent in the subgroup of TVD in comparison to DVD and SVD. This result suggests the presence of an association between ACE I/D gene polymorphism and the severity of coronary artery disease.

Comparison of AGT M235T genotypes were made between TVD with DVD and SVD separately (Table VII). Chi-square (χ²) test showed that TT genotype and T allele had a higher frequency in TVD in comparison to DVD and SVD which was statistically significant.

A genotype and allele frequency of AT1R A1166C polymorphism based on number of affected coronary vessels has been shown in table VIII. There is no CC genotype found in CAD patients which is a risk genotype. CAD patients having SVD have mostly AA genotype (96.1%) which is a less severe genotype. But C-allele had borderline significance for TVD in comparison to SVD.

### IV. Discussion:

The present study indicated a significant association of CAD with the Alu ACE I/D polymorphism, located in the intron 16 region. This association is highly significant among patient with TVD rather than with DVD or SVD. Individual with D-allele of Alu ACE locus are more susceptible to this clinical disorder. Individuals with DD genotype are more susceptible followed by ID and II genotype. In India very few authors reported association of DD genotype and D-allele with CAD. Globally association of DD genotype with
CAD has been also reported\textsuperscript{23, 24, 25, 26, 27}. A large body of literature suggests D-allele association with myocardial infarction\textsuperscript{28, 29, 30, 31, 32}.

Asians Indians are more susceptible to CAD has been reported\textsuperscript{10}. This raises the question of genetic susceptibility due to the ACE locus. Carriers of DD genotype have elevated serum as well as cardiac ACE activity and that exposed to higher angiotensin II level than II and ID genotype\textsuperscript{33, 34}. Angiotensin II is involved in the modulation of vascular tone and proliferation of smooth muscle cells\textsuperscript{22}, stimulates ca\textsuperscript{24}, aldosterone pathway\textsuperscript{35} and vascular endothelial growth factors\textsuperscript{36} and deposition of more cholesterol in the coronary arteries to cause atherosclerotic plaque and hypertension.

Our study shows DD genotype and D-allele is strongly associated with subgroup of CAD patients having TVD. This study is in accordance with study done by Emmanuel et al\textsuperscript{21}, Mendonca et al and Hibi et al also reported association of DD genotype with extent and severity of coronary artery disease\textsuperscript{37, 38}.

Looking beyond the classical risk factors for CAD many investigators have recently pursued the study of genetic factors in the renin angiotensin system to predict the development and severity of CAD. Genetic polymorphism in the angiotensinogen gene M235T increases the plasma concentration of angiotensinogen\textsuperscript{39}. High allele frequencies of AGT TT genotype were observed in Asians\textsuperscript{40} and blacks\textsuperscript{41}.

Angiotensinogen concentration is a limiting factor for angiotensin II generation\textsuperscript{42}. Thus its increased amounts may increase Angiotensin II levels many fold, potentially promoting atherosclerosis\textsuperscript{43} or modulating the severity of CAD\textsuperscript{44}. Our result shows that the genotype TT and T-allele may influence the severity of CAD as it is strongly associated with TVD through the generation of numerous and critical atherosclerotic lesion. Jeunemaitre et al\textsuperscript{45} reported an association of the T-allele with the extent of coronary lesions. Pereira et al\textsuperscript{46} and Meheri et al\textsuperscript{47} showed that the presence of the TT genotype and T-allele was associated with the severity of CAD independent of other cardiovascular risk factors. No significant association was found by Gardeman et al\textsuperscript{48} between AGT T homozygosity and the severity of the disease in caucasians.

The AT1R receptor is a major component of the renin-angiotensin system. The AT1R receptor mediates most of the classical and biological functions of angiotensin II\textsuperscript{49}. It was hypothesized that the adverse effect of the 1166C allele is due to the increased responsiveness to angiotensin II\textsuperscript{50}.

In our study there is very less number of CAD having 1166C allele. No study from India was done regarding AT1R gene polymorphism and severity of CAD. Pulhareddy et al\textsuperscript{51} from India reported that CC genotype is not a risk factor for MI in South Indian population. Globally many studies reported that CC genotype is not associated with MI\textsuperscript{52, 53, 54}. Some studies reported association of CC homozygote genotype with MI and CHD\textsuperscript{30, 35, 55, 56, 57}.

In our study population among the RAS gene risk genotype of ACE and AGT polymorphism are associated strongly with severity of coronary artery disease whereas AT1R gene found to be almost normal.

V. Conclusion

Thus Alu ACE genotyping seems to be useful for predicting the risk of severity among CAD patients. AGT M235T polymorphism is also associated with CAD risk and influence the severity of CAD. AT1R gene polymorphism did not show any association significantly with severity. However due to our limited sample size our findings require confirmation in larger cohorts. Clinical translation of this study may help in management and predicting prognosis of CAD patients.

References

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Association of ACE, AGT and AT1R gene polymorphisms with severity of Coronary Artery Disease


<table>
<thead>
<tr>
<th>Table I.</th>
<th>Age and sex distribution of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>CAD cases</td>
</tr>
<tr>
<td>Up to 40</td>
<td>18</td>
</tr>
<tr>
<td>41-50</td>
<td>36</td>
</tr>
<tr>
<td>51-60</td>
<td>50</td>
</tr>
<tr>
<td>61-70</td>
<td>36</td>
</tr>
<tr>
<td>71-80</td>
<td>9</td>
</tr>
<tr>
<td>More than 80</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
</tr>
</tbody>
</table>

CAD- Coronary Artery Disease

<table>
<thead>
<tr>
<th>Table II.</th>
<th>Risk factor analysis in CAD Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk factors</td>
<td>Number (150)</td>
</tr>
<tr>
<td>Hypertension (HTN)</td>
<td>73</td>
</tr>
<tr>
<td>Diabetes mellitus (DM)</td>
<td>50</td>
</tr>
<tr>
<td>Dyslipidemia (Tc &gt;200 mg/dl)</td>
<td>41</td>
</tr>
<tr>
<td>Smoking (SM)</td>
<td>39</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>24</td>
</tr>
<tr>
<td>Obese</td>
<td>10</td>
</tr>
<tr>
<td>Overweight</td>
<td>67</td>
</tr>
<tr>
<td>No risk factors</td>
<td>34</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Table III.</th>
<th>Demographic and clinical data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td>CAD cases</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.6±11.4</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>121/29</td>
</tr>
<tr>
<td>SVD</td>
<td>51(34.0%)</td>
</tr>
<tr>
<td>DVD</td>
<td>17(11.3%)</td>
</tr>
<tr>
<td>TVD</td>
<td>82(54.6%)</td>
</tr>
<tr>
<td>AMI</td>
<td>117(78%)</td>
</tr>
<tr>
<td>Unstable angina</td>
<td>33(22%)</td>
</tr>
<tr>
<td>Morbidity</td>
<td>37(24.66%)</td>
</tr>
<tr>
<td>Mortality</td>
<td>8(5.3%)</td>
</tr>
</tbody>
</table>

BMI-Body mass index, RBS-Random blood sugar, SVD- Single vessel disease, DVD-Double vessel disease, TVD-Triple vessel disease, AMI-Acute myocardial infarction

<table>
<thead>
<tr>
<th>Table IV.</th>
<th>Frequency distribution of ACE, AGT and ARIR gene in cases and controls</th>
</tr>
</thead>
</table>

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### Association of ACE, AGT and AT1R gene polymorphisms with severity of Coronary Artery Disease

#### Table V. Severity in Cases with and without risk factors

<table>
<thead>
<tr>
<th>Vessel disease</th>
<th>Cases without any risk factors</th>
<th>Cases with risk factors</th>
<th>$\chi^2$</th>
<th>P value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVD</td>
<td>14(41.2%)</td>
<td>37(31.9%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD</td>
<td>01(2.9%)</td>
<td>16(13.8%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVD</td>
<td>19(55.9%)</td>
<td>63(54.3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SVD = single vessel disease, DVD = double vessel disease, TVD = triple vessel disease

#### Table VI. Genotype and allele frequencies of Alu ACE I/D polymorphism in the CAD patients based on the number of affected coronary vessels.

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>TVD N=82</th>
<th>DVD N=17</th>
<th>$\chi^2$</th>
<th>P value</th>
<th>SVD N=51</th>
<th>$\chi^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD</td>
<td>43(52.4%)</td>
<td>1(5.8%)</td>
<td>15.82</td>
<td>0.0003</td>
<td>7(13.7%)</td>
<td>43.33</td>
<td>0.000</td>
</tr>
<tr>
<td>ID</td>
<td>28(34.1%)</td>
<td>8(47.05%)</td>
<td></td>
<td></td>
<td>9(17.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>11(13.4%)</td>
<td>8(47.05%)</td>
<td></td>
<td></td>
<td>35(68.6%)</td>
<td></td>
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</tr>
</tbody>
</table>

#### Table VII. Genotype and allele frequencies of AGT/ M235T polymorphism in the CAD patients based on the number of affected coronary vessels.

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>TVD N=82</th>
<th>DVD N=17</th>
<th>$\chi^2$</th>
<th>P value</th>
<th>SVD N=51</th>
<th>$\chi^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>14(17.07%)</td>
<td>4(23.5%)</td>
<td>8.20</td>
<td>0.016</td>
<td>17(33.3%)</td>
<td>5.84</td>
<td>0.05</td>
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<tr>
<td>MT</td>
<td>28(34.1%)</td>
<td>11(64.7%)</td>
<td></td>
<td></td>
<td>18(35.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>40(48.7%)</td>
<td>2(11.7%)</td>
<td></td>
<td></td>
<td>16(31.3%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Additional Text

- **Table V. Severity in Cases with and without risk factors**
- **Table VI. Genotype and allele frequencies of Alu ACE I/D polymorphism in the CAD patients based on** the number of affected coronary vessels.
- **Table VII. Genotype and allele frequencies of AGT/ M235T polymorphism in the CAD patients based on** the number of affected coronary vessels.
Table VIII. Genotype and allele frequencies of AT1R / A1166C polymorphism in the CAD patients based on the number of affected coronary vessels.

CAD – coronary artery disease, SVD- single vessel disease, DVD- double vessel disease, TVD- triple vessel disease.

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>TVD N=82</th>
<th>DVD N=17</th>
<th>χ²</th>
<th>P value</th>
<th>SVD N=51</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>69(84.1%)</td>
<td>14(82.3%)</td>
<td>0.03</td>
<td>0.85</td>
<td>49(96.1%)</td>
<td>4.47</td>
<td>0.034</td>
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<tr>
<td>AC</td>
<td>13(15.8%)</td>
<td>3(17.6%)</td>
<td></td>
<td></td>
<td>2(3.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele frequencies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.92</td>
<td>0.91</td>
<td>0.03</td>
<td>0.86</td>
<td>0.98</td>
<td>5.93</td>
<td>0.014</td>
</tr>
<tr>
<td>C</td>
<td>0.08</td>
<td>0.09</td>
<td></td>
<td></td>
<td>0.02</td>
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