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## Angiotensin-converting enzyme insertion/deletion genotype is associated with premature coronary artery disease in Indian population

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**The insertion/deletion polymorphism of angiotensin-converting enzyme (ACE) gene has been implicated in the pathogenesis of cardiovascular diseases. However, the precise association between them remains unclear. The objective of the present study was to find the influence of angiotensin-converting enzyme (insertion/deletion) polymorphism on cardiovascular diseases. We genotyped the *Alu* insertion/deletion of the ACE gene in 434 patients and 500 control subjects. Information on severity of vessel disease, clinical status, age, smoking and alcohol status, lipid profile and body mass index was also collected. The present study indicated a significant association of coronary artery diseases (CADs) with *Alu* ACE insertion/deletion polymorphism, located in the intron 16 region of the ACE gene. Thus, we identified a clinical subgroup of CAD patients, characterized with triple vessel defect and most often associated with diabetes.**

ANGIOTENSIN-converting enzyme (EC 3.4.15.1; dipeptidyl carboxypeptidase) is a zinc metallopeptidase which cleaves the C-terminal dipeptide (His–Leu) from angiotensin 1 and generates a vasoconstrictor<sup>1</sup>, angiotensin II. Through protease activity it also inactivates bradykinin, which is a potent vasodilator. Due to its role in the renin-angiotensin-aldosterone system, human vascular tone and blood salt/water balance have been maintained. The gene for angiotensin converting enzyme (ACE) has been mapped to chromosome 17q23 and comprises 26 exons and 25 introns<sup>2,3</sup>. The activity of ACE was strongly influenced by a quantitative trait locus which is in linkage disequilibrium with the *Alu* insertion/deletion (I/D) marker<sup>4–7</sup> in intron 16. A relationship between D-allele dose and enzymatic levels was established for both circulating and cellular ACE<sup>8–13</sup>. Numerous studies reported association of D-allele with cardiovascular diseases<sup>14–17</sup>, in addition to the diseases like non-insulin-dependent diabetes mellitus, nephropathy<sup>18–20</sup>, knee osteoarthritis<sup>21</sup> and breast cancer<sup>22,23</sup>. However, this association was not observed in all the studies<sup>24–29</sup>. Thus, there has been a considerable controversy over the association of ACE (I/D) polymorphism and disease status. Also, the influence of the

Received 2 August 2004; revised accepted 16 November 2004

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ACE (I/D) polymorphism on cardiovascular diseases still remains unresolved. Beyond this, polymorphism as a predictor of risk and usage of target-specific pharmacologic agents in the form of ACE inhibitors are clinically important. Therefore, we conducted a retrospective study to address this question among coronary artery disease (CAD) patients who were diagnosed by angiogram.

Among eight hundred and fifty-six patients who attended for routine coronary angiography at our adult cardiology inpatient department, 434 cases (males 381; females 53) below the age group of 45 years, were included in this study. Patients with valvular, cardiomyopathy and other major parallel diseases and those with age >45 years were excluded from the study in order to have a more homogenous group of patients. Informed consent was obtained from all participants after explaining the objectives and details of the study according to the approval of the medical ethics committee of the hospital. The following details were obtained from the Medical Records Department; clinical details (ischaemic heart attack, angina pectoris, myocardial infarction, coronary artery bypass, coronary angioplasty, stenting, peripheral arterial disease, renal failure and history of other surgical interventions), cigarette smoking (code 1 = newer, 2 = presently), alcohol intake (1 = newer, 2 = presently), hypertension (1 = controllable, 2 = uncontrollable and 0 = absence), diabetes (1 = IDDM, 2 = NIDDM and 0 = absence), hypercholesterolaemia and hypertriglyceridaemia. Body mass index was calculated as  $\text{weight/height}^2$  ( $\text{kg/m}^2$ ). Blood pressure was measured by mercury sphygmomanometer using Korotkoff phase, according to the World Health Organization guidelines. A total of 500 control subjects (males 464; females 36) were genotyped for *Alu* ACE locus. All the control subjects were healthy and below 45 years. They were mainly from among the staff and students who volunteered to participate in the study and also from among unrelated blood donors attending the blood bank attached to the hospital. The inclusion criteria were negative family history for cardiovascular disease, myocardial infarction, stroke and absence of hypertriglyceridaemia, hypercholesterolaemia, hyperlipidaemia and diabetes. Angiography was not performed on any of the control subjects to rule out coronary artery diseases, as it is not ethical.

Angiography was carried out using SIEMENS COROSKOP TOP digital system and evaluated by the senior cardiologists attached with the hospital. The severity of disease was classified as single, double or triple CAD as defined by the presence of  $\geq 50\%$  stenosis in major arteries. The patients were classified as follows: single vessel disease = code 1; double = code 2; triple = code 3;  $< 50\%$  stenosis = code 4, and normal vessels = code 5.

Two millilitre peripheral blood was obtained in EDTA vacutainer and DNA was isolated either by salting-out procedure<sup>30</sup> or using a commercial kit (ABgene, UK). I/D polymorphism of the ACE gene was detected using the polymerase chain reaction according to the method suggested by

Rigat *et al.*<sup>7</sup>. The sequences of the sense and the antisense primers were  ${}^5\text{CTG GAG ACC ACT CCC ATC CTT TCT}{}^3$  and  ${}^5\text{GAT GTG GCC ATC ACA TTC GTC AGA}{}^3$  respectively. Reactions were performed in a final volume of 20  $\mu\text{l}$  containing 100–150 ng genomic DNA, 50  $\mu\text{mol}$  of each dNTP, 0.5  $\mu\text{mol}$  of each primer, 2.0 mmol  $\text{MgCl}_2$  and 0.5 units of Supratherm Taq DNA polymerase. The thermo cycling procedure was carried out using Gene Amp 9700 (Applied Biosystems, USA) for 35 cycles with denaturation at 94°C for 2 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. The initial denaturation and final extension were carried out at 94 and 72°C respectively, for 5 min. The PCR products were electrophoresed on 2% agarose gel and DNA was visualized by ethidium bromide staining using Gel doc system (Ultra Lum Inc, Japan).

The circulating ACE concentration was quantitated using Sigma Diagnostic kit (Catalog Number: 305-10) on the basis of the hydrolysis of furyl acryloyl phenylalanyl glycyl glycine (FAPGG), a synthetic substrate. The rate of decrease in absorbance at 340 nm is directly proportional to ACE activity in the sample. The total cholesterol, triglycerides, HDL and urea (Randox Laboratory Ltd, UK) and glucose (Roche/Hitachi, Germany) were estimated using HITACHI 902 automatic analyser.

Results are expressed as mean  $\pm$  SD. Student's *t* test was used to compare the means. Consistency of the genotype frequencies with the Hardy–Weinberg equilibrium was tested by chi-square analysis. Odds ratios for genotypes and alleles of *Alu* ACE gene were computed using SPSS package 10.0 version.

Risk factors among patients and control subjects are shown in Table 1. Among the 434 patients who had  $\geq 50\%$  stenosis in one, two or three vessels, 381 (88%) were men. The mean age of patients and control subjects was  $40 \pm 5$  and  $35 \pm 9$  years respectively. The patients' group presented significantly higher mean levels of body mass index, total cholesterol, triglycerides and glucose than control subjects. There was a significant difference between the two groups in the mean systolic blood pressure, but not in diastolic blood pressure and high density lipoprotein. Though patients and control groups have significant difference in most of the baseline characteristics, there is no significant variation in severity of the disease.

Overall, 45.1% of the patients and 10.3% of the control subjects were current smokers, 13% of the patients and 0.2% of the control group were obese; and 2% of the patients and 0.5% of the control were asthmatic. Patients were more commonly reported with the following clinical characteristics: angina (87%), dyspnoea (32%), palpitation (7%), syncope (4%) and myocardial infarction (MI) (34%). About 41% of the patients had undergone Coronary Artery Bypass Grafting (CABG) and about 23% had Percutaneous Transluminal Coronary Angioplasty (PTCA) (Table 2). The mean ejection fraction among the patients was  $54 \pm 11\%$ . About 41% of the patients had family history of CAD. Other clinical features include alcoholism (25%), hypertension

**Table 1.** Baseline characteristics of patients with coronary artery disease and control subjects

Variable	Control N = 500	SVD N = 176	DVD N = 93	TVD N = 165	Total N = 434	C vs SVD P value	C vs DVD P value	C vs TVD P value	SVD vs DVD P value	SVD vs TVD P value	DVD vs TVD P value	C vs T P value
Age (yrs)	35 ± 9	39 ± 5	41 ± 4	41 ± 4	40 ± 5	0.000	0.000	0.000	0.12	0.000	NS	0.000
BMI (kg/m <sup>2</sup> )	22 ± 3	25.6 ± 4.5	25.4 ± 3.1	25.3 ± 3.0	25.4 ± 3	0.000	0.000	0.000	0.78	NS	NS	0.000
SBP (mm Hg)	126 ± 8	128 ± 16	130 ± 21	132 ± 20	130 ± 18	NS	0.000	0.001	NS	0.05	NS	0.000
DBP (mm Hg)	82 ± 4	82 ± 11	82 ± 10	84 ± 13	83 ± 11	NS	NS	0.002	NS	NS	NS	NS
TCL (mg/dl)	16 ± 20	182 ± 41	172 ± 35	184 ± 49	180 ± 43	0.000	0.003	0.000	NS	NS	0.05	0.000
TGL (mg/dl)	117 ± 19	194 ± 98	185 ± 98	186 ± 109	89 ± 102	0.000	0.000	0.000	NS	NS	NS	0.000
HDL (mg/dl)	39 ± 5	42 ± 16	41 ± 33	40 ± 6	42 ± 18	0.007	0.5	0.039	NS	NS	NS	NS
Glu (mg/dl)	95 ± 8	126 ± 48	135 ± 65	140 ± 75	135 ± 61	0.000	0.000	0.000	NS	NS	NS	0.000

C, Control; T, Total; N, Number of cases; BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; TCL, Total cholesterol; TGL, Triglycerides; HDL, High Density Lipoprotein; Glu, Glucose; NS, Not significant; SVD, Single vessel disease; DVD, Double vessel disease; TVD, Triple vessel disease.

**Table 2.** Clinical characteristics of patients with coronary artery disease

Variable	Patients (N = 434)	Control (N = 500)
EF (%)	54 ± 11	NA
Angina (%)	87	0
MI (%)	34	0
CABG (%)	41	0
PTCA (%)	23	0
Dyspnoea (%)	32	0
Palpitation (%)	7	0
Syncope (%)	4	0
Asthma (%)	2	0.5
Familial CAD (%)	41	0
Obesity (%)	13	0.2
Smoking (%)	45.1	10.3
Alcohol (%)	25	8
Hypertension (%)	45	0
Diabetes (%)	38	0
Nitrates (%)	14	0
Statins (%)	26	0
Coronary artery disease		
SVD (%)	40.6	0
DVD (%)	21.4	0
TVD (%)	38	0

EF, Ejection fraction; NA, Not assessed.

**Table 3.** Risk factors and severity of coronary artery disease

Risk factor	SVD (N = 176) (%)	DVD (N = 93) (%)	TVD (N = 165) (%)
No risk factor	22.2	9.7	14.5
Smoking	28.4	19.4	10.9
Diabetes	8.0	7.5	15.2
Hypertension	13.6	15.1	12.7
Smoking + hypertension	11.9	14.0	10.3
Diabetes + smoking	6.8	7.5	9.1
Diabetes + hypertension	6.3	15.1	20.0
Smoking + diabetes + hypertension	2.8	11.8	7.3

**Table 4.** *Alu* ACE genotype and allele frequencies in control and patients with coronary artery diseases with variable severity

Subject	N	II	ID	DD	c <sup>2</sup>	I	D	c <sup>2</sup>
Control	500	152	246	102	<0.001	0.55	0.45	<0.001
Patient	434	76	220	138		0.43	0.57	

II, ID and DD *Alu* ACE insertion (I)/deletion (D) genotypes.

**Table 5.** *Alu* ACE genotype and allele frequencies in control (N = 500) and patients with coronary artery diseases with variable severity

Genotype/allele	N	c <sup>2</sup>	P value	OR	95% CI
All patients vs control					
DD vs ID		6.710	0.009	1.51	1.45–1.99
DD vs II		27.516	0.000	2.71	1.93–3.77
ID vs II	434	12.053	0.000	1.79	1.34–2.39
DD + ID vs II		20.916	0.000	2.06	1.57–2.71
DD vs ID + II		15.806	0.000	1.819	1.40–2.36
D vs I		27.41	0.000	1.63	1.39–1.91
SVD vs control					
DD vs ID		6.422	0.011	0.543	0.35–0.83
DD vs II	176	1.226	0.268	1.39	0.815–2.35
ID vs II		17.17	0.000	2.56	1.71–3.86
D vs I		2.058	0.151	1.19	0.97–1.48
DVD vs control					
DD vs ID		0.807	0.369	1.29	0.77–2.16
DD vs II	93	0.589	0.443	1.27	0.72–2.21
ID vs II		0.004	0.952	0.984	0.616–1.59
D vs I		0.514	0.474	1.12	0.85–1.48
TVD vs control					
DD vs ID		42.549	0.000	3.72	2.59–5.34
DD vs II	165	51.891	0.000	6.56	4.02–10.85
ID vs II		4.169	0.041	1.76	1.08–2.93
D vs I		65.122	0.000	2.94	2.33–3.71

DD, ID and II, *Alu* ACE I/D genotypes; D and I, Alleles; N, Number of patients; OR, Odds ratio; CI, Confidence interval.

**Table 6.** Circulating angiotensin converting enzyme concentration of the patients and their matched controls

Case	Genotype	N	Mean units/l	Range	Standard deviation	Significance
Patients (N = 63)	II	16	27.97	26.90–29.70	0.7752	0
	ID	25	34.49	32.10–37.80	1.7770	
	DD	22	51.59	48.70–53.70	1.4136	
Controls (N = 37)	II	7	27.64	26.90–29.20	0.8101	0
	ID	10	35.15	32.20–38.70	2.3101	
	DD	20	51.18	49.50–53.10	1.0491	

ANOVA:  $P < 0.001$  for both groups.

(45%) and diabetes (38%). Among the total 434 patients, 176 had single vessel defect (SVD), 93 had double vessel disease (DVD) and 165 had triple vessel disease (TVD).

About 17% of the patients had none of the risk factors. Patients with no risk factor are more frequent in SVD group (about 22%) and least frequent in DVD group (about 10%). In case of TVD, about 15% had no risk factor (Table 3). Smoking is the more frequently observed risk factor in SVD (about 28%) compared to TVD group of patients (~11%). On the other hand, diabetes is more common among TVD group of patients compared to SVD or DVD groups, among whom this risk factor is equally frequent (Table 3).

The genotype and allele frequencies of the *Alu* ACE locus in patients and control groups are given in Table 4. The genotype and allele frequencies in both the groups differ significantly ( $P < 0.001$ ).

The ODD's ratios were computed between the genotypes DD vs ID, DD vs II, ID vs II, DD + ID vs II, DD vs ID + II (Table 5). All the values are significantly greater than one. The ODD's ratio is highest for DD vs II (2.71; 95% CI: 1.93–3.77) and for DD + ID vs II (2.06; 95% CI: 1.57–2.71). Individuals with D-allele appear to be at greater risk compared to those with I-allele (1.63; 95% CI: 1.39–1.91).

ODD's ratios were computed in subsets of the data that vary by the severity of the disease (SVD, DVD and TVD). The values are not significant for the DVD group ( $P > 0.37$ ). In the case of SVD, only two comparisons, DD vs ID and ID vs II, are significant ( $P < 0.05$ ). The two crucial comparisons, DD vs II and D vs I, are not significant ( $P > 0.15$ ). On the other hand, Odds ratios are highly significant for the TVD groups for all comparisons ( $P < 0.05$ ). The risk to CAD patients of this group appears to be quantitative. It means that individuals who are homozygous for the D-allele are at greatest risk (OR: 6.56; 95% CI: 4.02–10.85), followed by heterozygous individuals (OR: 1.76; 95% CI: 1.08–2.93) compared to those of II individuals. In general, the D-allele has greater risk compared to the I-allele (2.94; 95% CI: 2.33–3.71).

Quantitative values of circulating ACE activity are available for a limited number of subjects. Mean values were computed based on the genotype of *Alu* ACE (Table 6). The mean values for DD, ID and II genotypes among control

subjects were:  $51.18 \pm 1.05$ ,  $35.15 \pm 2.31$  and  $27.64 \pm 0.81$  respectively, and among patients for the genotypes were:  $51.59 \pm 1.41$ ,  $34.49 \pm 1.78$  and  $27.97 \pm 0.78$  respectively. Irrespective of the group, the mean values of the three genotypes differ significantly. The circulating ACE was found to increase with the D-allele, quantitatively.

The present study indicated a significant association of CADs with the *Alu* ACE I/D polymorphism, located in the intron 16 region. This association is significant only among patients with TVD rather than with SVD or DVD. Thus, our results identified a clinical subgroup of CAD patients, characterized with TVD and most often associated with diabetes. Individuals with D-allele of *Alu* ACE locus are more susceptible to this clinical disorder. Moreover, susceptibility is dependent on the number of D-alleles that one would have. This means that individuals with DD genotype are most susceptible followed by ID and II genotypes. The frequency of I-allele among populations of South Asia is higher compared to Koreans<sup>31</sup>, Chinese<sup>32,33</sup>, in Japanese<sup>34–36</sup> and in people from the Indian subcontinent<sup>37–39</sup>. Despite this, the frequency of D-allele among CAD patients (the present study) is higher compared to those who do not have CAD. Although the prevalence of diabetes and impaired glucose metabolism is higher in Africans and South Asians, increased frequency of CADs are observed only in South Asians but not in Africans, Caribbeans or in Europeans<sup>40,41</sup>. This raises the question of genetic susceptibility due to *Alu* ACE locus. *Alu* ACE locus has been shown to be in strong linkage disequilibrium with the major gene locus controlling plasma ACE<sup>8</sup> and accounts for half of the variance of circulating ACE<sup>6</sup>. The ACE-DD genotype is associated with higher tissue and plasma ACE, whereas the ACE-II genotype is associated with lower ACE activity.

The higher level of circulating ACE will produce corresponding higher levels of angiotensin II. It stimulates  $Ca^{2+}$ , aldosterone pathways<sup>42</sup> and vascular endothelial growth factors<sup>43</sup>, and the infusion of more cholesterol in the coronary vessel to cause pathophysiological condition.

The endothelial injury can be induced by increased superoxide anion production in arteries by angiotensin II (Ang II) through  $AT_1$  receptor-dependent mechanism<sup>44</sup>. Ang II also influences the architecture and integrity of the vascular endothelial wall by modulating cell growth increas-

ing inflammation and by regulating extracellular matrix composition. Alterations of these highly regulated signalling pathways lead to structural and junctional abnormalities that underlie the vascular pathological process such as hypertension and atherosclerosis<sup>45-47</sup>.

In the present study, the D-allele of *Alu* ACE is strongly associated with triple vessel disease. Increased Ang II levels in turn augment circulating catecholamines, which decrease insulin sensitivity. This explains why most of the triple vessel patients are diabetic. Studies show that ACE inhibitor therapy is associated with improved insulin sensitivity<sup>48</sup>, and in fact even low dose infusion of Ang II increased insulin sensitivity<sup>49</sup>.

Our study provides evidence for the strong association of *Alu* ACE D-allele with CAD among young Indians. The study also identifies a clinical subgroup triple vessel disease with a strong predisposition to diabetes. Thus, *Alu* ACE genotyping seems to be useful for predicting the risk among Indian patients below 45 years age having CAD.

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ACKNOWLEDGEMENTS. We thank the patients and all control individuals for their cooperation. We also thank Dr Pramod Jaiswal, Department of Cardiology for comments on the manuscript. A.R. is supported by a research grant from DBT, New Delhi.

Received 18 August 2004; revised accepted 16 November 2004

## Encapsulation of pineapple micro shoots in alginate beads for temporary storage

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**Micro shoots developed from the meristematic basal portion of the innermost leaves of pineapple [*Ananas comosus* (L.) Merr] were encapsulated in alginate beads for short-term storage as a prelude to the transformation programme. Among the four temperature regimes for storage, beads stored at 8°C showed maximum percentage of shoot proliferation when placed again in MS medium; the rate of 'conversion' was satisfactory even beyond 45 days of incubation. The genetic fidelity of the pineapple plants growing out after storage in encapsulated form was ascertained by RAPD and ISSR techniques.**

PINEAPPLE [*Ananas comosus* (L.) Merr] is one of the most economically important tropical fruit crops<sup>1</sup>. From an Indian perspective, the agro-climatic conditions of West Bengal and Tripura are conducive for pineapple cultivation. Consequently, West Bengal is a major producer of pineapple in India (<http://www.ieport.com/2003-2004/forms/App-15.doc>). Its production is gaining momentum with the advancement of the packaging industry and canning facilities. Pineapple plants are conventionally propagated vegetatively from crowns ('tops') and slips (side shoots arising in older leaf axils) (<http://www.uga.edu/fruit/pinapple.htm>); and there is always a demand for superior planting materials. This commercially important plant material naturally calls for development of suitable protocols for production of sufficient number of uniform planting materials from 'elite' pineapple varieties through biotechnological intervention.

Among the biotechnological approaches, micropropagation is one of the most viable techniques and reports of somatic embryogenesis<sup>2</sup>, development of micropropagation protocol through shoot-tip culture<sup>3</sup> and mass propagation of pineapple using a temporary immersion bioreactor technique (TIB)<sup>4</sup> are available. Furthermore, the TIB technique has been successfully implemented to produce large number of transgenic pineapple plants<sup>5</sup>.

In order to develop a suitable regeneration system for use in the pineapple transformation programme, we encountered

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