ONLINE RESOURCES

Polymorphism of angiotensin converting enzyme (insertion/deletion) and endothelial nitric oxide synthase (intron 4ab) genes in a population from northeast India

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Introduction

A number of genes are implicated in the pathogenesis of cardiovascular disease including hypertension (Jeng *et al.* 1997). The gene encoding angiotensin converting enzyme (ACE) of renin-angiotensin system (RAS) regulating fluid and electrolyte balance, and blood pressure (Malik *et al.* 1997) contains a number of polymorphic variants that can be of potential use in genetic analysis of populations (Rieder *et al.* 1999). The insertion/deletion (Ins/Del) polymorphism present in intron 16, in particular, has been extensively investigated and involves the insertion (Ins) or deletion (Del) of a 287-bp *Alu* repeat sequence near the 3′ end of intron 16 (Rigat *et al.* 1990).

The enzyme endothelial nitric oxide synthase (eNOS) modulates endothelial synthesis of nitric oxide (NO) and acts as a potent vasodilator. The gene for this enzyme has three distinct nitric oxide synthase (NOS) isoforms in mammalian cells: neuronal (nNOS, type I), inducible (iNOS, type II) and endothelial (eNOS, type III). The eNOS gene is polymorphic, and a functional 27 bp variable tandem repeat (VNTR) in intron 4 (intron 4a/b) of eNOS gene has been extensively investigated (Patkar *et al.* 2009). Such polymorphism may result in impairment of NO bioavailability and endothelial dysfunction and play a pivotal role in the pathogenesis of hypertension, heart failure and coronary artery disease (Tamemoto *et al.* 2008).

Prevalence of polymorphic markers of these genes varies across ethnic groups (Agarwal *et al.* 2004; Patkar *et al.* 2009) and determines genetic susceptibility to various forms of cardiovascular disease. Northeast India is inhabited by people of diverse population groups with a variable prevalence of hypertension (Hazarika *et al.* 2004) and therefore studies of the polymorphisms in genes implicated in the pathogenesis of hypertension is necessary. Such information is absent in the population from northeast India. The present study was an attempt to explore such information.

Materials and methods

Study site and subjects

The present study is a community-based cross-sectional study carried out in Assam and Mizoram which included three population groups viz. Assamese, tea garden workers (TGW) from Assam, and Mizo from Mizoram. Selection of population group was based on variable prevalence of hypertension in these groups (Hazarika *et al.* 2004) and study site was selected based on operational feasibility. For TGW, Assamese and Mizo, subjects were recruited from the selected villages, tea gardens and municipal blocks respectively. Temporary health check up clinic was organized in the study sites and subjects were invited to participate in our study. Subjects attending the clinic and providing informed consent were included in our study. A total of 812 (tea garden workers, 527; Assamese, 153; Mizo, 132) healthy unrelated individuals of either sex of the age group 18–72 years were recruited. Before initiation of the study, ethical approval for the study was obtained from institutional ethical committee of Regional Medical Research Centre, NE Region (ICMR), Dibrugarh, Assam.

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Collection of blood samples

Approximately 5 mL of peripheral venous blood from each subject was collected in a screw cap tube containing EDTA. The specimen was transported to the laboratory on ice and stored at −70°C till DNA extraction.

DNA extraction

DNA was extracted from whole blood by the conventional phenol–chloroform method. DNA purity and quantity were assessed by absorbance values in spectrophotometer and checked by 0.5% agarose gel electrophoresis.

Determination of ACE Ins/Del polymorphism

The Ins/Del polymorphism in intron 16 of the *ACE* gene was detected by the polymerase chain reaction (PCR) using the forward primer: 5′-GCC CTG CAG GTG TCT GCA GCATGT-3′ and reverse primer: 5′-GGA TGG CTC TCC CCG CCT TGT CTC-3′ to amplify the D and I alleles, which would result in the amplification of 319 bp and 597 bp products, respectively. PCR amplification used 20.0 μL reaction volumes and consisted of 10 pmol of each primer (Sigma, St Louis, USA), 10 mM Tris-HCL pH 9.0 (Bangalore Genei, Bangalore, India), 10 mM dNTPs, 1 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), and 50–100 ng of genomic DNA. The amplification cycle was performed on a Gene Amp. PCR System 9700: version 3.08 thermal cycler (Applied Biosystems, Carlsbad, USA) following standard protocol with slight modifications (Raynolds *et al.* 1993). Amplification of the PCR products was detected on 2% agarose gel containing ethidium bromide. The ACE I/D polymorphism consists of the presence (insertion, *I*) or absence (deletion, *D*) of 287-bp *Alu* repeat in intron 16 resulting in three genotypes: insertion homozygous (Ins/Ins), insertion-deletion (Ins/Del) heterozygous and deletion homozygous (Del/Del). Thus, after electrophoresis, each DNA sample revealed one of the three possible patterns: a 597-bp band (Ins/Ins genotype), a 319-bp band (Del/Del genotype) or both 597-bp and 319-bp bands (Ins/Del genotype) (figure 1). Each sample found to have the Del/Del genotype was subjected to a second PCR amplification with insertion-specific primers (5′-TGG GAC CAC AGC GCC CGC CAC TAC-3′ and 5′-TCG CCA GCC CTC CCA TGC CCA TAA-3′) to avoid Del/Del mistyping (Shanmugam *et al.* 1993).

Determination of eNOS 4ab polymorphism

The eNOS 4ab polymorphism was determined by PCR using forward primer: 5′-AGG CCC TAT GGT AGT GCC TTT-3′ and reverse primer 5′-TCT CTT AGT GCT GTG GTC AC-3′ to amplify the *A* and *B* alleles, which will result in the amplification of 393 bp and 420 bp products, respectively. PCR consisted of 10 pmol of each primer (Sigma, St Louis, USA), 10 mM Tris-HCL pH 9.0 (Bangalore Genei, Bangalore, India), 10 mM dNTPs (Bangalore Genei, Bangalore, India), 1 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) and 50–100 ng of genomic DNA following standardized protocol with slight modifications (Wang *et al.* 1996). Amplification of PCR products was confirmed by 3.0% agarose gel. The eNOS a/b polymorphism results in three genotypes viz., aa homozygous (AA), bb homozygous (BB) and ab heterozygous (AB) (figure 2).

Statistical analysis

Statistical analysis was performed using SPSS version 13.0 (SPSS, Chicago, USA). Specific genotype frequencies of the population were compared by chi-square test. Allele frequencies were deduced from genotype frequencies. The Hardy–Weinberg equilibrium (HWE) was examined using Epi-info 2002 (CDC, Atlanta, USA). A P value ≤ 0.05 was considered to be statistically significant.

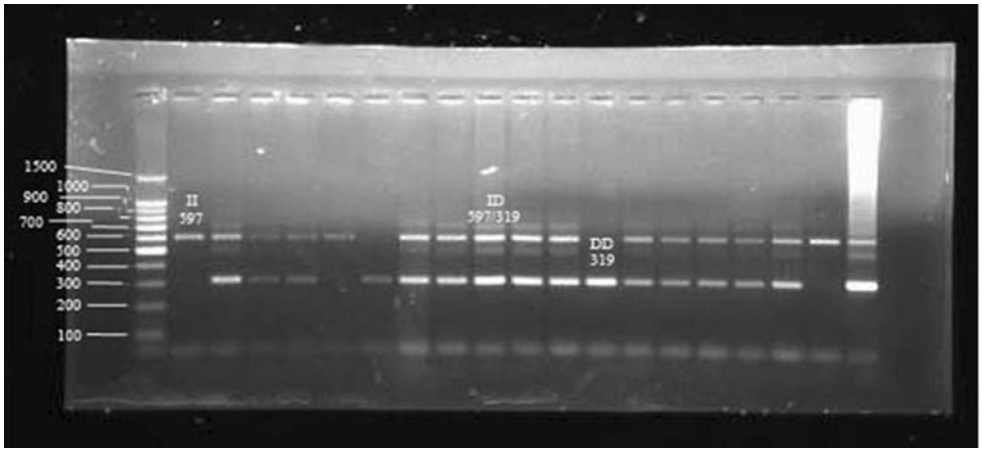

Figure 1. Agarose gel photograph of angiotensin-converting enzyme gene insertion/deletion (*I/D*) polymorphism.

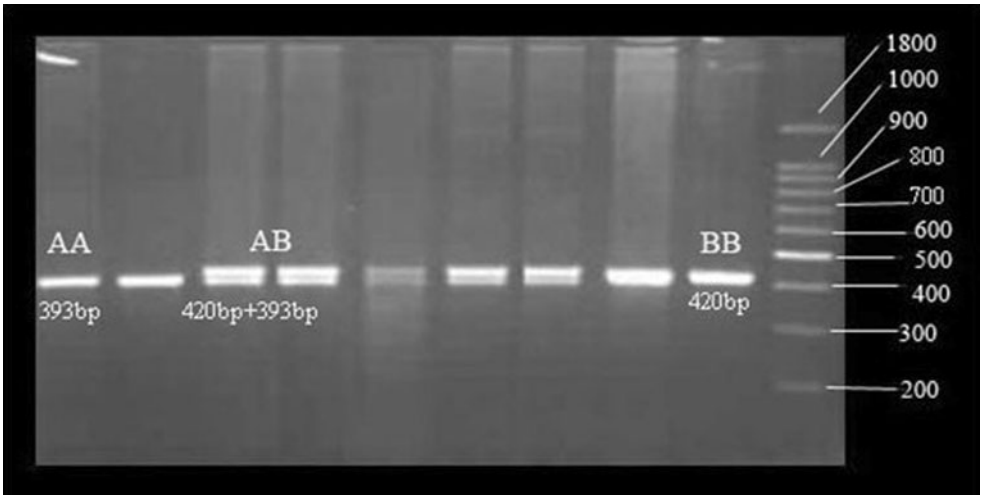

Figure 2. Agarose gel photograph of the 27 bp variable tandem repeat (VNTR) polymorphism in intron 4 of endothelial nitric oxide synthase (eNOS) gene.

Results

In the present study, a total of 812 subjects and of either sex (male, 329; female, 483), aged between 18 and 72 years from three communities (TGW, 527; Assamese, 153; Mizo, 132) of northeast India were included. Mean age of the communities did not reveal any significant difference. Consumption of alcohol by the study communities was found to be similar but habit of smoking was significantly higher in case of Assamese subjects. Body mass index (BMI) was significantly higher in case of Mizo subjects (table 1).

PCR analysis was carried out in 812 subjects to study polymorphism of ACE Ins/Del and eNOS intron 4ab genes. The frequencies of genotypes of the two polymorphisms in each group were in HWE.

The 287-bp insertion/deletion polymorphism located in intron 16 of *ACE* gene was detected by PCR analysis in the three ethnic groups. The insertion allele of 597 bp was designated as 'Ins' and the deletion allele of 319 bp was designated as 'Del'. Thus, each DNA sample revealed one of the three possible patterns after electrophoresis: a 597-bp band (Ins/Ins genotype), a 319-bp band (Del/Del genotype) or both 597-bp and 319-bp bands (Ins/Del genotype). Similarly, the 27 bp variable number tandem repeats (VNTR) in intron 4 of eNOS gene was detected by PCR analysis. The larger allele of 420 bp designated as '*b*' has five tandem repeats and the smaller allele of 393 bp designated as '*a*' has four repeats. Thus, the eNOS 4*a*/*b* polymorphism results in three genotypes viz., *aa* homozygous (393 bp), *bb* homozygous (420 bp) and *ab* heterozygous (both 393 bp and 420 bp).

Insertion allele of *ACE* gene was predominantly found in our study communities. The genotype Ins/Ins (*II*) was predominant (50.0%) in Assamese subjects but genotype Ins/Del (*ID*) was predominantly found in case of TGW and Mizo subjects (46.0% and 53.0%, respectively). Prevalence of Del/Del (*DD*) genotype was lower in all the communities. Such community specific distribution of ACE genotypes revealed statistically significant difference ($P < 0.05$) (table 2).

Presence of *b* allele of eNOS gene was predominant in three population groups with a higher prevalence of eNOS *bb* genotype than the others. Of all the communities, prevalence of this genotype was found to be the highest among the Mizo population (80.0%) which was followed by the Assamese population (74.0%) and tea garden workers (69.0%). The interesting finding in our study was that eNOS *aa* genotype was virtually absent in Assamese population. Our results demonstrated a statistically significant difference in the distribution of eNOS-4 genotype between the study groups (table 2).

Table 1. Demographic characteristics of the study subjects.

Parameter	All subjects (n = 812)	Tea garden worker (n = 527)	Assamese (n = 153)	Mizo (n = 132)
Age, yrs (mean ± S.D.)	39.9 ± 12.02	39.9 ± 12.07	40.7 ± 11.8	39.2 ± 12.1
Gender, male *N* (%)	329 (40.5%)	232 (44.0%)	58 (37.9%)	39 (29.5%)
Alcohol consumption, yes *N* (%)	384 (47.3%)	253 (48.0%)	73 (47.7%)	58 (43.9%)
Smoking habit, yes *N* (%)	207 (25.5%)	143 (27.1%)	47 (30.7%)	17 (12.9%)
Body mass index (mean ± S.D.)	18.7 ± 3.3	18.6 ± 3.4	18.7 ± 3.7	19.3 ± 1.7

Table 2. ACE and eNOS 4 genotypes and allele distribution among three different ethnic groups of northeastern India.

Community	ACE genotype				ACE alleles		eNOS 4 genotypes				eNOS 4 alleles	
	II	*ID*	*DD*	*P*	*I*	*D*	*aa*	*ab*	*bb*	*P*	*a*	*b*
Tea garden community ($n = 527$)	217 (0.41)	241 (0.46)	69 (0.13)		0.64	0.36	18 (0.03)	145 (0.28)	364 (0.69)		0.17	0.83
Indigenous Assamese community ($n = 153$)	77 (0.50)	55 (0.36)	21 (0.14)	0.04*	0.68	0.32	0 (0.0)	40 (0.26)	113 (0.74)	0.000*	0.13	0.87
Mizo community ($n = 132$)	51 (0.39)	70 (0.53)	11 (0.08)		0.65	0.35	8 (0.06)	18 (0.14)	106 (0.80)		0.13	0.87

*Statistically significant (P value < 0.05); numbers in parentheses denote genotype frequency.

Discussion

Study of genotypic distribution of two potential candidate genes associated with cardiovascular disease in the three specific population groups from northeast India is a first report of its kind. The finding of our study is important because population groups of our study had a significant variation in the prevalence of hypertension. We observed a higher frequency of '*I*' allele in our study groups and the finding is in agreement with distributions found in Asiatic populations (Zee *et al.* 1992) and studies conducted by Pasha *et al.* (2002) but differs from the Americans, Caucasians and Europeans who had a higher frequency of ACE deletion allele (Johanning *et al.* 2005). Frequency of Del/Del genotype was lower in our study subjects and the finding was in conformity with earlier study conducted in Mexican population (Barley *et al.* 1996).

The striking preponderance of the '*b*' allele observed in our study was in conformity with earlier studies conducted in Japanese and UK populations (Fowkes *et al.* 2000; Hwang *et al.* 2002). However, our findings were different from the study conducted in Australian (Wang *et al.* 1996).

A large number of studies had been conducted to find out association between candidate gene polymorphism and cardiovascular diseases. Association studies of *ACE* gene polymorphism (Morshed *et al.* 2002) revealed significant association of *DD* genotype with hypertension. In the contrary, study conducted by Zee *et al.* (1992) found association of *I* allele with cardiovascular diseases. Similar to *ACE* genotypes polymorphism of eNOS gene was also found to be associated with cardiovascular diseases (Salimi *et al.* 2006).

Our study revealed a significant difference in the distribution of polymorphic variants of ACE Ins/Del and eNOS-4ab genes across the population groups. Our findings may provide a genetic background of our population for undertaking future case–control study and also to understand genetic polymorphism in the development of cardiovascular diseases. Our findings may be also useful in recognizing the role and mechanisms contributing to the development cardiovascular diseases.

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