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Apolipoprotein E (Apo E) Gene Polymorphism in the Bangladeshi Population and its Comparison with Other Asian Populations

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Apolipoprotein (Apo E) gene polymorphism was determined in 53 unrelated Bangladeshi individual by using restriction enzyme isoform genotyping. This gene is polymorphic, with three common alleles coding for three major isoforms ε2, ε3 and ε4, resulting in six genotypes (ε2/ε2, ε3/ε2, ε4/ε2, ε3/ε3, ε4/ε3 and ε4/ε4). These isoforms differ from each other by a single amino acid substitution. Apo E functions as ligands of various receptors and determines the metabolic fate of lipoproteins. The genetic variations at the Apo E locus could thus be a major determinant of the inter-individual variation in susceptibility to coronary artery disease. The allele frequencies for Apo ε2, ε3 and ε4 alleles were found to be 0.05, 0.80 and 0.15, respectively in Bangladeshi population. The allele frequencies found in this study were compared with other Asian populations using Chi-square (χ^2) test and cluster analysis. The Bangladeshi population shows close relationship with Japanese population living in Tokyo, Sendai and Indian populations living in Singapore. This study found ε3 to be the predominant isoform but the relative proportions of the three isoforms have shown variation among populations.

Key words: Apo E, polymorphism, allele, genotype, restriction isotyping

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INTRODUCTION

Apolipoprotein E is a member of the apolipoprotein gene family, a group of genes that serve a variety of functions related to lipoprotein metabolism. The Apo E gene is located on chromosome 19q13.2 consisting four exons and three introns spanning 3,597 nucleotides and produce a 299 amino acid polypeptide (Scott *et al.*, 1985; Rall *et al.*, 1982). The structural gene is polymorphic with three common alleles, ε2, ε3 and ε4, producing three isoforms of the protein, known as E2, E3 and E4. These isoforms differ in amino acid sequence at position 112 and 158. Apo E3 contains cysteine at 112 and arginine at 158. Apo E2 has cysteine at both positions and E4 has arginine at both sites (Weisgraber *et al.*, 1981). It is synthesized primarily in the liver, but other organs and tissues also synthesize ApoE, including brain, lung, spleen, kidneys, gonads, adrenals and macrophages (Mahley, 1988).

Apo E is a constituent of chylomicron remnants, Very Low Density Lipoproteins (VLDL) and High Density Lipoproteins (HDL) (Davington and Petkar, 1998). It plays a central role in the normal catabolism of triglyceride rich lipoproteins and is probably involved in reverse cholesterol transport. Apo E due to the presence of arginine at position 112, binds preferentially to triglyceride rich lipoproteins such as VLDL. In contrast, Apo E2 and E3, which contain two and one cysteine residue, respectively bind preferentially to HDL. Since lipoproteins play central role in the development of atherosclerotic Cardiovascular Disease (CVD) in humans, this give rise to the hypothesis that mutations in the genes coding for any of the apolipoproteins may result in impaired clearance of liproteins. It has been found that Apo E polymorphism has functional effect on lipoprotein metabolism.

Several studies have shown that the E4 isoform is associated with higher and E2 with lower levels of total and LDL cholesterol (Boerwinkle *et al.*, 1989). Differences in relative frequency of Apo E alleles among different populations have also led to increased interest in rapid typing of Apo E isoforms for population studies. In this study, restriction isotyping of human Apo E by gene amplification was conducted in a sample of Bangladeshi population. The Apo E genotype and gene frequencies were determined and compared with available studies on Apo E polymorphism with other Asian populations.

MATERIALS AND METHODS

Study subjects: Total of 53 randomly selected individuals of different ages (21-44 years), were included in this study. The study conducted in the Department of Biochemistry and Molecular Biology, Dhaka University and the study subjects were recruited from Dhaka Metropolitan City area. Written informed consent was obtained from the study participants and local ethical review committee approved the design of the study.

Collection of blood sample: Approximately 5 mL of peripheral venous blood was collected in a screw cap tube containing 20% K_3-EDTA. The specimen was capped and transported to the laboratory under cold condition. It was then stored at -20°C if not assayed immediately.

Amplification of Apo E sequences: Genomic DNA was isolated from whole blood according to the published protocols for extracting DNA (Gustincich *et al.*, 1991). To determine the Apo E genotype, amplification of the apolipoprotein E target sequences was performed by polymerse chain reaction (PCR) in a thermal cycler 9600 (Applied Biosystems, USA) using oligonucleotide primers. The sequence of the primers was P1: (5'-ACA GAA TTC GCC CCG GCC TGG TAC AC-3') and P2: (5'-TAA GCT TGG CAC GGC TGTC CAA GGA-3') (Emi *et al.*, 1988). In addition to the buffer and nucleotide, each amplification reaction contained 1 μg of leukocyte DNA, 1 pmol μL^{-1} of each primer, 10% dimethyl sulfoxide (DMSO) and 0.025 units μL^{-1} of *Taq* polymerase (Sigma) in a final volume of 30 μL. Each reaction mixture was heated at 95°C for 5 min for denaturation and subjected to 30 cycles of amplification by primer annealing (60°C for 1 min), extension (70°C for 2 min) and denaturation (95°C for 1 min). This primer pair amplified a 314 bp region of the DNA that spans both Apo E polymorphism sites (Hixson and Vernier, 1990).

Restriction isotyping of Apo E sequences: After amplification, the 244 bp PCR product was digested with 5 units of restriction enzyme *Hha*I, isolated from *Haemophilus haemolyticus* (Invitrogen, Life Technologies) for 3 h at 37°C. This process did not require purification of PCR products or addition of specific buffer components for *Hha*I digestion. Incomplete digestion was not a significant problem, most likely due to large amounts of *Hha*I enzyme relative to the small amount of amplified sequences in each reaction (5 units *Hha*I/300 ng DNA). Each reaction mixture was loaded onto 8% non-denaturing polyacrylamide gel electrophoresis (20×20 cm) for about 5 h under constant current (80 volt). The enzyme digested PCR product was visualized by UV illumination after Ethidium Bromide staining (0.2 mg L^{-1}).

Determination of genotype: The amplified 244 bp PCR product (Fig. 1.) has six *Hha*I cleavage site (GCGC). Each genotype possessed unique combination of *Hha*I fragment sizes. Therefore, ε4/ε4 homozygote contained 72, 48, 35 and 19 bp fragment as well as three other 16, 18 and 38 bp fragments, indicating the presence of *Hha*I restriction site at codon 112 (Arg) and 158 (Arg). The ε3/ε3 homozygote pattern was composed of 91, 48 and 35 bp fragments along with the shared 16, 18 and 38 bp fragments, reflecting the absence of the *Hha*I restriction site at codon 112 (Cys). The ε2/ε2 homozygote contained 91 and 83 bp fragments along with the three shared fragments mentioned above, indicating the absence of the *Hha*I restriction site at both codon 112 (Cys) and 158 (Cys) (Hülya *et al*., 2001). With the exception of 38 bp shared fragment, other shared fragments (16 and 18 bp) and the 19 bp fragment were not detected after electrophoresis due to their smaller size.

Statistical analysis: Gene frequencies were calculated using gene-counting method for both the sexes among Bangladeshi population. The difference in Apo E genotype frequency distribution between the Bangladeshi sample and other Asian populations were determined by Chi-square (χ^2) test using SPSS Ver. 10.0 for Windows. A cluster analysis was performed by single linkage minimum distance method (Sokal and Sneath, 1963) to suggest the clusters of different Asian populations. To perform cluster analysis, Euclidian distances based on ε2 and ε4 gene frequencies for 17 populations (including the present study sample) were estimated with the help of same SPSS package.

RESULTS AND DISCUSSION

The present study was undertaken in an attempt to evaluate the Apo E gene polymorphism in Bangladeshi population. A total of 53 randomly selected individuals including male and females were recruited in this study. Genomic DNA was isolated from blood leukocytes and subjected to PCR using specific primers for Apo E gene (Emi *et al*., 1988). The PCR product was then digested with restriction enzyme *Hha*I. A polymorphism in Apo E gene causes a shift in the *Hha*I restriction site. The digested products were then separated on 8% non-denaturating polyacrylamide gel. The resulting polymorphism causes a shift in the banding pattern. The genotype frequency was determined from the banding positions and allele number was determined by gene counting.

It was found that genotype ε3/3 was highest in the overall population as well as in the males and females

Table 1: Observed genotype and allele frequency of Apo E gene in Bangladeshi population

Study subjects	Genotype frequency			Allele frequency		
	ε 3/2	ε 3/3	ε 4/3	ε 2	ε 3	ε 4
Male (n = 38)	0.07	0.57	0.34	0.04	0.79	0.17
Female (n = 15)	0.13	0.67	0.20	0.07	0.83	0.10
Total (n = 53)	0.09	0.60	0.30	0.04	0.80	0.15

(Table 1), which was almost similar with the available data developed by the North American, Europe and Asian studies (Eichner *et al*., 2002). Genotype ε4/3 was next to ε3/3 and followed the same pattern. This study found a total absence of the genotype ε4/4, ε4/2 and ε2/2, respectively. According to this study there was no significant difference in genotype frequency between male and females.

The Apo E allele frequencies were ε2 = 0.05, ε3 = 0.80 and ε4 = 0.15, respectively (Table 2). It was observed that most of the Japanese populations, two populations from India and also the Bangladeshi population were characterized by the low frequency of Apo ε2 allele compared to the populations from China. However, the frequency of ε4 allele was higher in most of the Japanese population. This ε4 allele is also found in a significantly higher frequency (0.15) in Bangladeshi population, Indian living in Singapore (Hallman *et al*., 1991) and the population living in Niigata, Japan.

The majority of the Asian populations from Japan, India, Indian people living in Singapore and Bangladeshi populations showed an insignificant difference with the present study and most of the Chinese populations showed significant difference. The population of China (CH2), Singapore (SIN, MAS) and Chinese people living in Montreal (CH4) have shown comparatively higher χ^2 value which may be due to the greater frequency of the Apo ε2 allele in those population (Table 2).

In the present study the Apo E gene polymorphism was compared with 16 other Asian populations for which the data on Apo E polymorphism were available. The dendrogram was constructed based on ε2 and ε4 allele frequencies to suggest the cluster of different Asian population (Fig. 2).

The dendrogram depicts two clusters and were named, D_1 and D_2 (Fig. 2). The cluster D_1 includes ten populations. The D_1 has two sub cluster, named as D_{1A} and $D_{1B.}$ D_{1A} sub cluster includes five populations (JA4, JA6, JA7, IND and CH3). D_{1B} sub cluster has also five populations (BAN, JA2, JA5, JA8 and SIN_S). The cluster D_2 has seven populations, three from china (CH1, CH2 and CH4), two from Japan (JA1 and JA3), one from Malaysia (MAS) and Singapore (SIN). In cluster D_2 the two Chinese populations (CH2 and CH4) are closely related.

Table 2: Apo E allele frequency among the Asian populations and its comparison with Bangladeshi population

Population studied	N	Allele frequency ε2	ε3	ε4	χ^2 (df = 2)	References	Definition of the study population	Code
Indians	220	0.05	0.86	0.086	3.94	Venkataramana *et al.* (2001)	Unrelated individual from two populations of India	IND
Indians (Singapore)	142	0.04	0.82	0.13	0.4	Hallman *et al.* (1991)	Unrelated blood bank donor who are Indian immigrants	IND (S)
Chinese (Singapore)	190	0.09	0.83	0.07	7.98*	Hallman *et al.* (1991)	unrelated blood bank donor who are Chinese immigrants	CHI
Chinese (Beijing)	507	0.12	0.80	0.07	18.0**	Bao-Sheng *et al.* (1990)	Randomly selected population	CH2
Chinese (Beijing)	95	0.05	0.88	0.06	3.24*	Wang *et al.* (19870	Healthy volunteers	CH3
Chinese (Montreal)	101	0.11	0.82	0.06	9.0	Wang *et al.* (1987)	Chinese immigrants living in Montreal	CH4
Malaysian (Singapore)	118	0.11	0.76	0.12	4.24	Hallman *et al.* (1991)	Malaysian immigrants living in Singapore	MAS
Singapore	188	0.12	0.78	0.06	7.61*	Utermann (1987)	Unrelated individuals of Chinese Indians and Malays	SIN
Japanese (Kyushu and Fukuoka)	319	0.08	0.84	0.06	9.17*	Hallman *et al.* (1991)	Unrelated medical professional student	JA1
Japanese (Osaka)	208	0.06	0.82	0.10	2.37	Yamamura *et al.* (1990)	CHD case-control study	JA2
Japanese (Niigata)	129	0.09	0.78	0.12	2.69	Miida (1990)	CHD case-control study	JA3
Japanese (Kumamoto)	188	0.03	0.87	0.09	3.52	Kobori *et al.* (1988)	Hyperlipidemic case-control study	JA4
Japanese (Sendai)	107	0.06	0.82	0.17	0.09	Sano *et al.* (1988)	Hyperlipidemic case-control study	JA5
Japanese	305	0.03	0.86	0.10	2.60	Horita *et al.* (1993)	Randomly selected civil worker	JA6
Japanese (Hiroshima and Nagasaki)	110	0.02	0.89	0.08	4.86	Asakawa *et al.* (1985)	Healthy volunteers	JA7
Japanese (Tokyo)	197	0.03	0.84	0.11	0.64	Tsuchiya *et al.* (1985)	Healthy outpatients	JA8
Bangladeshi	53	0.05	0.80	0.15	-	Present study	Healthy volunteers	BAN

Note: p values shown in column 6 are <0.05 (*) and <0.01 (**)

The Bangladeshi populations showed close relationship with Japanese population living in Tokyo, Sendai and Indian populations living in Singapore due to closer allele frequencies. Although, coronary artery disease is a major cause of death from all over the world, south Asians have been found to have the highest mortality rates due to coronary artery disease amongst all ethnic groups so far studied (Dhawan and Patkar, 1998). The conventional risk factors of the disease, namely high cholesterol, smoking and high blood pressure do not explain all the differences in mortality due to this disease. People of these regions do tend to suffer from diabetes, higher insulin levels, abdominal obesity, low high density lipoprotein levels, higher triglycerides levels, lower levels of physical exercise and higher lipoprotein (a) levels. While the exact cause of this increased rate of mortality due to coronary artery disease has yet to established. It appears that both genetic and environmental factors play a role (Kathy *et al.*, 2001). When the contribution of Apo E polymorphism to the incidence of cardiovascular disease across populations of the world was considered, most of the available data are suggestive of a beneficial influence of the ε2 allele on cardiovascular disease, while ε4 allele appears to predispose to cardiovascular disease (Davington, 1993).

The studies on Caucasian populations have showed that the Apo E polymorphism and particularly the population frequency of ε4, may contribute to the interpopulation variability in CHD mortality rates (Gerdes *et al.*, 2000; Sing and Moll, 1989). The frequency of the ε4 allele tends to be higher in populations with higher CHD mortality rates e.g., the Finns (Ehnholm *et al.*, 1986) and lower in those with lower rates (e..g., Japanese, Chinese). Since the frequency of ε4 allele has been a contributory factor for the difference in CHD mortality rates in Caucasians (e.g., Finns) the allelic variation at Apo E locus may provide an opportunity to understand the cause of this CHD mortality in Bangladeshi people.

The frequency of ε4 alleles in the present study was (0.15) comparable with Japanese living in Sendai (0.17) or Indians living in Singapore (0.13) and who are mostly inhabitant of the urban area, Dhaka city. In a study conducted in Indian rural area (Venkatramana *et al.*, 2001), the prevalence of ε4 allele was lower (0.08) than the urban

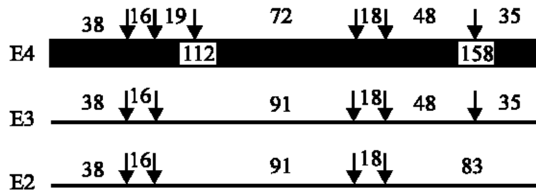

Fig. 1: *Hha*I cleavage maps for each isoform. Downward arrows show *Hha*I restriction sites. E4 is shown as filled box containing codon 112 and 158. The ε4/ε4 homozygote contains 72, 48, 35 and 19 bp as well as three other shared 16, 18 and 38 bp fragments, indicating the presence of *Hha*I restriction site at codon 112 (Arg) and 158 (Arg). The ε3/ε3 homozygote pattern is composed of 91, 48 and 35 bp fragments along with the shared 16, 18 and 38 bp fragments, reflecting the absence of the *Hha*I restriction site at codon 112 (Cys). The ε2/ε2 homozygote contains 91 and 83 bp fragments along with the three shared fragments mentioned above, indicating the absence of the *Hha*I restriction site at both codon 112 (Cys) and 158 (Cys)

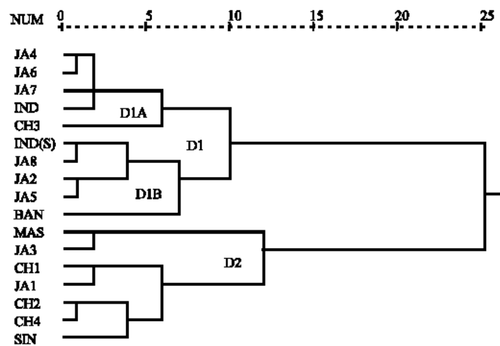

Fig. 2: Dendogram showing the cluster of 17 Asian Population based on Apo ε2 and ε4 allele frequency

areas (0.13). Since the present study samples were taken from urban area, the high frequency of ε4 allele in the present study sample might be one of the reasons. Also, Apo E is recognized by all members of the constantly growing LDL receptor gene family. LDL receptor binds avidly Apo E probably, due to interaction of four Apo E molecule with four LDL receptors. The interaction of LDL receptor with Apo E is essential for normal lipoprotein metabolism. It is possible that the receptor binding activity of ε4 is modulated by lipid composition (Weisgraber, 1994) and expressed only with additional environmental (for example, diet) factors.

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