Journal of Korean Medical Science
Vol. 8, No. 4, 262-266 August 1993

Apolipoprotein E Genotypes of Normal and Hyperlipidemic Subjects

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Apolipoprotein E (apo E) plays a role in the regulation of the lipid metabolism of humans. Apo E, 229 amino acid polypeptide, is classified into three major isoform (E2, E3, E4) according to the differences of amino acid in position 112 and 158. In the normal population apo E3 isoform is most prevalent and apo E2 or E4 is frequently associated with hyperlipoproteinemia.

To find out the frequency of apo E isoform distribution in the Korean population, apo E genotyping was performed. After amplification of apoE gene by polymerase chain reaction (PCR), restriction isotyping was done by cleavage with restriction enzyme Hha I and polyacrylamide gel electrophoresis.

The apo E allele frequency in 73 normal subjects was 4.8% for E2, 84.9% for E3 and 10.3% for E4. In diabetic patient with hyperlipoproteinemia, the frequency of apo E allele was 6.3% for E2, 81.0% for E3 and 12.7% for E4. There was no significant difference in apo E isoform distribution between diabetics and normal populations. But in patients with cardiovascular disease with hyperlipidemia, the apo E4 allele frequency was significantly higher than normal (20.0% vs 10.3%, $p<0.005$). Apo E3 was the most common isoform in normal and diabetic subjects and apo E2 isoform was rather low frequency compared to Caucasians. This pattern is similar to the Japanese population but somewhat different from other populations.

From the data of a high association of apo E4 allele and cardiovascular disease with hypercholesterolemia, apo E isoform may be one of the determinants of hyperlipoproteinemia. The PCR method may be useful in apo E genotyping.

Key Words: *Apolipoprotein E, Polymerase chain reaction, Hyperlipoproteinemia*

INTRODUCTION

Apolipoprotein E (apo E) is one of the main proteins that makes up the molecules of serum lipoproteins such as very low density lipoprotein (VLDL), chylomicrons, chylomicron remnants, intermediate density lipoprotein (IDL) and some high density lipoproteins (HDL). It has been known that apo E had a role in the maintenance of the stability of the lipoprotein structure and in the control of its metabolism (Mahley et al., 1984, 1988).

Apo E serves as a ligand to apo B/E receptor and it mediates transport of the various lipids such as cholesterol into cytoplasm. Apo E is a glycoprotein of molecular weight 34.2kilodaltons (Kd) which is composed of 299 amino acids, and it is synthesized in various organs such as the liver, spleen and kidneys (Blue et al., 1983). The length of human apo E gene is 3.7kilobases with 4 exons and it is located in chromosome 19 (Oilaisen et al., 1982)

According to different amino acids at 112 and 158 position, apo E molecule has its gemetic polymorphisms designatea as apo E2, E3, or E4 (Zannis et al., 1981, 1982). The alterations of gene structure determine the polymorphisms of apo E. Polymorphisms are thought to be a major deciding factor for developing hyper-

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lipidemia such as familial type III hyperlipoproteinemia and can cause atherosclerosis by problems in the cholesterol transport mechanism (Davignon et al., 1988). Recently the frequency of cerebral apoplexy and acute myocardial infarction has been increasing as complications of atherosclerosis. The role of apo E gene polymorphisms in the development of hyperlipidemia and atherosclerosis is not well known.

Therefore, this study was designed to reveal the frequency of apo E genotypes in the normal population and their role in the occurrence of atherosclerotic disorders and hyperlipidemia.

Recently polymerase chain reaction (PCR) method was introduced to the exact genotyping for the apo E gene (Hixon et al., 1990), but complete evaluation of this method in the hyperlipidemic population was rarely reported. In this study, we tried to detect common types of apo E gene polymorphism using PCR and restriction patterns as efficient tools for predictive diagnosis of hyperlipidemic complications.

SUBJECTS & METHODS

Subjects:

Blood samples were obtained from 73 normal people who visited to Kyung Hee university hospital for routine check ups. They had no past or family history of metabolic diseases and their laboratory data were normal. In the hyperlipidemic group, 79 patients were diabetics, 35 were cerebral strokes, and 9 were myocardial infarction.

Method:

1) To obtain genomid DNA, peripheral leucocyte DNA was extracted as previously described (Kim et al., 1989). Ten ml of the peripheral blood was taken from patients with a heparinized syringe. RBC lysis buffer (1.55M NH4CLI, 1 M Tris in dH20) was added to blood samples up to 50 ml, and left for 30 minutes. The supernatant was thrown away after centrifugation of 3,500 rpm for 15 minutes, and the white blood cell (WBC) pellet was resuspended with 1 ml of lysis buffer. After brief centrifugation, DNA was extracted with the same amounts of phenol/chloroform/isoamylalcohol (25:24:1) mixture. The supernatant was collected and extracted DNA was precipitated with absolute ethanol (EtOH), and centrifuged at 4°C for 20 minutes. DNA pellet was washed with 70% EtOH three times, then redissolved in 1 x TE buffer (pH 8.0). DNA samples were stored at 4°C before PCR amplification.

2) PCR amplification of apo E gene was performed by thermal cycler (Perkin-Elmer 9600, Roche Molecular Systems Inc., NJ, USA) using oligonucleotide primer F4 (AAATTCGCCCGGCTGGTACAG) and F6 (TAAGCTTGGCACGGCTGTCCAAGA) as described before (Hixon et al,. 1990). The oligonucleotide primers were synthesized by chemical method using DNA synthesizer (Applied Biosystem).

Each PCR amplification reagent contained 1 μg of DNA, 5 units of Taq polymerase, and 1 μl of 0.25μM dNTP, 10% dimethysulfoxide (DMSO) and added water to 100 μl. Thirty cycles were programed for DNA amplification with steps of denaturation (95°C for 1 minute), annealing (60°C for 1 minute), and extension (72°C for 2 minutes).

3) For determining restriction isotyping of apo E gene, we digested PCR products with Hha I restriction enzyme and digested DNAs were electrophoresed on 8% polyacrylamide gel containing urea for 3 hours at 45 mA. The gel was stained with ethidium bromide (0.1mg/ml) for 15 minutes and we took pictures of the DNA bands using a UV transilluminator.

RESULTS

PCR products were 244 bp in size on electrophoresis with 1.5% agarose gel. Apo E genotyping relied on cleavage at Hha I sites to distinguish E2, E3, and E4 sequences. We could determine six apo E genotypes from Hha I degestion. (Fig. 1)

According to Hha I fragment size, six apo E genotypes were determined as shown in Fig. 1. The cut sites were shown at 6 places in E4, 5 in E3 and 4 in E2. The genotypes were decided based on the different restriction fragment sizes digested with Hha I restriction enzyme. Apo E2 was composed of 91, 83 base pairs, apo E3 was 91, 48, 35 base pairs, and apo E4 was 72, 48, 35 base pairs respectively (Fig. 2).

The polymorphic gene frequency of apo E in the normal population was 9.6% for E2/E3, 72.6% for E3/E3, 15.1% for E3/E4, 2.71% for E4/E4. As shown in table 1, the allelic frequencies of apo E gene were 4.8% in E2, 84.9% in E3 and 10.3% in E4.

In patients with cerebral apoplexy and acute myocardial infarction, polymorphic gene frequencies of apo E were 11.4% in E2/E3, 79.5% in E3/E3, and 9.1% in E3/E4. In diabetic patients the polymorphic apo E gene frequencies were 12.7% in E2/E3, 64.6% in E3/E3, 20.2% in E3/E4, and 2.5% in E4/E4. In cardiovascular diseases, the polymorphic apo E gene frequencies of E2/E3 and E3/E4 showed slightly higher than those of the normal population, but it was not

statistically significant. In diabetic patients the E3/E4 gene frequency wqs higher than that of thenormal group ($P<0.025$, a $X^2=10.8$).

Allelic frequencies of apo E4 in diabetic group were 12.7%, which is higher than those of the normal group. (Table 1). Although apo E gene frequencies in cardiovascular disease group were not different from that of normal group, their allelic frequencies according to plasma cholesterol were different between the two groups.

According to plasma cholesterol levels, it didn't show any difference between groups of below 200 and of 201-240mg/dl, but groups of above 241mg/dl showed a higher E3/E4 genotype ($P=0.005$, $X^2=36.9$) as well as higher E4 allelic frequencies ($P<0.005$, $X^2=14.7$) among these groups. (Table 2)

DISCUSSION

Polymorphism of apo E genotypes is divided into 3 isoforms of E2, E3, E4 depending on net charge of amino acids on isolectric focusing. They are produced as the 3 alleles locate on a gene (Zannis et al., 1982). The three isoforms are classified by changes of their amino acid components of cysteine and arginine (Zannis et al., 1981). In isoform E2, arginine is substituted by cysteine at position 112 that reflects change in its corresponding codon CGC into

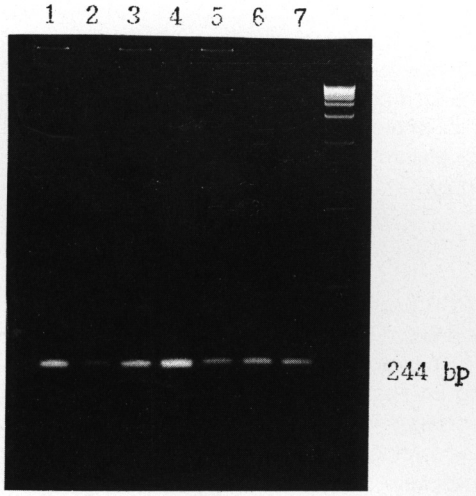

Fig. 1. Agarose gel electrophoresis. Lane 1-7, PCR products (244 bp).

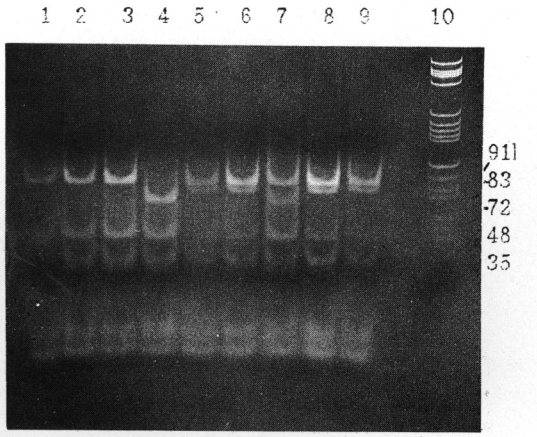

Fig. 2. 8% polyacrylamide gel electrophoresis of Hha I fragments after PCR amplification. lane 1-3: E3/E3 (91bp). lane 4: E4/E4 (72bp). lane 5-6, 8-9: E3/E2 (91.83bp). lane 7:E4/E3 (91, 72bp). lane 10: size marker.

Table 1. Apo E genotype and allele frequency

	genotype frequency (%)					allele frequency (%)		
	E2/E2	E2/E3	E3/E3	E3/E4	E4/E4	E2	E3	E4
Normal (n=73)	0	9.6	72.6	15.1	2.7	4.8	84.9	10.3
Diabetes (n=79)	0	12.7	64.6	20.2	2.5	6.3	81.0	12.7
Cardiovascular disease (n=44)	0	11.4	79.5	9.1	0	5.7	89.8	4.5

Table 2. Apo E genotype and allele frequency according to serum cholesterol in cardiovascular disease patients.

cholesterol	genotype frequency (%)					allele frequency (%)		
(mg/dl)	E2/E2	E2/E3	E3/E3	E3/E4	E4/E4	E2	E3	E4
<200	0	7.6	88.6	3.8	0	3.8	94.2	2.0
201-240	0	15.4	76.9	7.7	0	7.7	88.5	3.8
241<	0	0	60.0	40.0*	0	0	80.0	20.0*

*$p<0.05$

TGC (Weisgraber et al., 1981). The isoform E4 produces by changes the codon TGC into CGC at its position 158, results in change of cysteine to arginine.

Apo E polymorphism is about to be important since it is known to be a contributable factor for type III hyperlipoproteiniemia by biochemical defect with abnormal apo E (Zannis et al., 1980). Apo E isoform plays an important role in the regulation of human plasma lipoprotein concentration. Apo E2 allele could be found in dyslipoproteinemia characterized by increased cholesterol content of VLDL. (Weisgraber et al., 1982). Such dyslipoproteinemia could be explained by apo E2 binding defect on lipoprotein receptors and decreased catabolism produce accumulation of abnormal lipoproteins.

The binding domain of apo E to receptor is known to located near amino acid 140-160 residues. If one of these amino acid residues changes into an other aminoacid, binding capacity of apo E to its receptors will be decreased to 48-98%, which causes type III hyperlipoproteinemia (Mahley 1988). However, there has been no study of the exact role of apo E2/E2 homozygote patients with hyperlipidaemia in developing atherosclerosis later on.

Meanwhile, E4 allele is frequently observed in patients with high plasma cholesterol level (Uterman et al., 1984, Eto et al., 1986). There is a report for association of hyperlipoproteinemia with increased frequencies of apo E4 allele in diabetic patients (Eto et al., 1986).

The apo E3/E4 homozygote were the most frequent genotypes in normal and hyperlipidemic patients as previously reported in other populations (Black et al., 1990). Allelic frequencies of apo E2 gene were low in this study as compared to other study in Caucasians but similar frequencies were observed in Japanese studies. These data suggested that racial differencies were existed in apo E gene polymorphism.

Various types of apo E gene polymorphism were associated with hyperlipidemia and metabolic disorders. In this study, the frequencies of apo E2/E3, E3/E4 were high in hyperlipidemic cardiovascular disease patients. These results strongly support the view that there is some inter-relation between apo E2, E4 and the development of hypercholesterolemia. The exact mechanism of increased plasma cholesterol in apo E4 is unclear, but two possible considerations could be suggested. The first is stimulated catabolism of apo E4 containing VLDL versus increment of LDL cholesterol (Gregg et al., 1982). The second mechanism is due to the linkage disequilibrium between allele where the LDL receptor gene is located and the allele where the apo E gene is located (Ehnholm et al., 1986). The LDL receptor genes are highly polymorphic and possibility of multiple mutation occurrence is relatively frequent. Therefore, it is possible that apo E4 allele links to allele that mutated LDL receptor gene produce decreased binding to LDL at peripheral tissues.

Apo E4, unlike apo E2, plays an important role in hypercholesterolaemia through abnormal lipoprotein function. There was a report that the patients with coronary heart disease showed high frequency of E4 allele (Cumming et al., 1984). However, another report stated that apo E4 allele was not associated with coronary heart disease (Utermann et al., 1984).

In this study, we found that apo E4 gene frequency was not different between the cardiovascular disease and normal groups. Compared to patients with hypercholesterolaemia, cardiovascular disease patients showed a significantly high frequency of apo E4. apo E4 allele may be a strong candidate gene for the development of hypercholesterolaemia and play an important role in various atherosclerotic complications.

To find out the risk factors of cardiovascular diseases in patients with hypercholesterolaemia, apo E polymorphism study would be useful. We would suggest a larger population is needed for further study.

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