Apolipoprotein E Polymorphism in an Iranian Hypercholesterolemic Population

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ABSTRACT

Apolipoprotein E (apo E) is a structural constituent of several serum lipoprotein classes. It plays an important role in lipid metabolism by acting as a ligand for low-density lipoprotein (LDL) and chylomicron remnant receptors. Three common alleles called ε2, ε3 and ε4 have been described, which code for three protein isoforms (E2, E3 and E4). The polymorphism is clinically significant, and it has therefore become very important in epidemiological studies attempting to obtain complete information about apo E allele frequencies in populations around the world. In the present study, two point mutations coding amino acid residues 112 and 158 were amplified using the polymerase chain reaction (PCR) from DNA extracted from 100 Iranian subjects. Apo E genotypes were determined by restriction fragment length polymorphism (RFLP) and allele frequencies were estimated by gene counting (ε2 = 0.01, ε3 = 0.88, ε4 = 0.11). The Hardy-Weinberg expectation of genotype distribution was calculated from the estimated allele frequencies and a χ²-test was used to test for equilibrium (χ² = 0.487, P>0.05). Student’s t-test indicated that subjects with the two most common apo E genotypes (E4/E4, E3/E3) were different from each other in means serum total cholesterol (t = 3.1, P<0.01). Allele frequencies of the present study performed on Iranian subjects were similar to those of Japanese, Korean (Asian groups) Mexican and African American populations, Iran. Biomed. J. 6 (4): 117-122, 2002

Keywords: Apo E, Polymorphism, PCR, Genetic Epidemiology, Lipids.

INTRODUCTION

Human apolipoprotein E (apo E), one of the least abundant plasma apoproteins, is a normal constituent of very low-density lipoproteins (VLDL), high-density lipoproteins (HDL), intermediate density lipoproteins (IDL), chylomicrons and chylomicron remnants. Apo E is synthesized in hepatic and extrahepatic tissues including brain. It plays an important role in lipid metabolism by acting as a ligand for the LDL receptor to mediate the uptake of apo E containing lipoproteins by cells [1] and apo E mediated lipoprotein assembly [2]. Human plasma apo E is a 299-amino acid protein (34 kDa), constituted by a single polypeptide chain [3]. The apo E gene is closely linked with the apo C-I and apo C-II genes and is located on chromosome 19, spans 3.6 KB, and includes 4 exons.

Apo E is polymorphic, with three common codominant alleles ε2, ε3, ε4) coding for three isoforms (E2, E3, E4), which differ by a single amino acid substitutions at residues 112 and/or 158 [4, 5]. The apo ε3 allele is considered to be the parental allele and amino acid substitutions at position 112 (Cys→Arg) and 158 (Arg→Cys) lead to the synthesis of the apo ε4 and apo ε2 allele products, respectively. In the general population, the apo E polymorphism has been shown to affect the plasma levels of total cholesterol, LDL-cholesterol (LDL-C), apo B and apo E [6].

As compared to the most common apo ε3 allele, apo ε2 allele is associated with lower total cholesterol, LDL-C, apo B and a higher apo E while the effect of the apo ε4 allele is to increase total cholesterol, LDL-C,
apo B and to lower apo C [1]. Apo E polymorphism is an important determinant of risk for the development of cardiovascular and Alzheimer’s diseases; the prevalence of the apo ε4 allele being increased in both diseases compared to control subjects [3]. It is not clear whether this effect is mediated through the apo E genotype on lipids or through other mechanisms [7]. Most patients with type III hyperlipidemia are homo-
yzous for the E2 isoform (112 Cys and 158 Cys) that binds to cellular receptors with reduced affinity [8]. In population studies, the E2 isoform was associated with decreased levels of cholesterol and betalipoproteins [9]. Recently, the methods were developed for apo E typing at the DNA level using genomic DNA for Southern-
blots with allele-specific oligonucleotide (ASO) probe that span positions 112 and 158 [10, 11]. The sensitivity of these methods were increased using the PCR to amplify apo E sequences for dot-bLOTS with ASO probes [12] single strand conformational polymor-
phism (SSCP) [13] and amplification refractory mutation system (ARMS) [14]. In addition, automated sequence analysis of PCR products has been used for typing common and rare apo E isoforms. Reports from various population groups have indicated the heterogeneity of apo E polymorphism [12]. In this report, we describe restriction isotyping the common apo E isoforms from an Iranian population.

MATERIALS AND METHODS

Clinical samples. DNA samples were taken from 100 Iranian hypercholesterolemic subjects from Shahid Shorideh Clinic in Tehran. Local ethic committee approval was obtained. An appropriate questionnaire was provided to the subjects in order to record their smoking habits and medical history. The subjects with cardiovascular diseases, diabetes mellitus, and taking hypolipidemic medication were not included in the study.

Preparation of DNA. Venous blood was drawn from fasting, sitting subjects between 08:30 and 09:30 h, and 1 mg Na₂EDTA/ml of blood was immediately added. Genomic DNA was extracted from peripheral blood by using a salting-out method [15].

DNA amplification. Leukocytic DNA was amplified for the region of the fourth exon of apo E gene, which encodes amino acid residues 112 and 158. Oligonucleotide primers F4 5’-ACAGAATTTC GCCCCGCTTGCATAC-3’ (nucleotides 3908-3 892) and F6 5’-TAAGCTTTGCCAGCGCTGCTCCA AGGA-3’ (nucleotides 3681-3699) were used as described before [12 16]. PCR assay was performed in a 50-
μl volume of 1 × PCR buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl, 1 mM MgCl₂; 0.01% Gelatin; 10% dimethyl sulfoxide), 1 μl of template DNA, 0.2 mM of dNTPs, 20 pmol (each) primer, and 2.5 units/μl Taq DNA polymerase (Perkin Elmer, Norwalk, CT). Reactions were run at 95°C for 5 min and 30 cycles at 94°C for 40 seconds, 64°C for 30 seconds and 72°C for 1 min and final extension of 72°C for 10 min [17, 18 19]. PCR products (40 μl) were digested with 10 unites of HhaI (New England Biolabs, Beverly, MA). Amplified products were not purified before restriction digestion [20]. The fragments were separated using 12% poly-
acrylamide gels. The gels were treated with ethidium bromide and DNA fragments were visualized by UV illumination. The HhaI generated fragments were compared with known size markers (Hinfl-digested pUC19 DNA) [17].

Statistical analysis. The frequencies of ε2, ε3 and ε4 alleles were estimated by gene counting, e.g., the
frequency of ε2 = (2 apo E2/E2 + apo E3/E2 + apo E4/E2)/Total number of alleles [21, 22]. The Hardy-
Weinberg expectation of genotype distribution was calculated from the estimated allele frequencies and a χ²-
test at P = 0.05 was used to test for equilibrium (χ² = 0.487, P>0.05). Because of the small sample size, subjects with the less common genotypes (E2/2, E2/4, E4/4) were not included in statistical analysis [5, 23].

RESULTS

All PCR amplifications showed an expected fragment size (244 bp) on agarose gel electro-phoresis (Fig. 1). Each genotype was determined by the specific combination of fragments generated from restriction endonuclease cut. Except for a 38-bp common fragment, other 16-bp and 18-bp common fragments were too small to detect. The codon change from cysteine residues (GTCG) to arginine residues (GCGC) makes HhaI cleavage
sites at amino residues position 112 and 158 on apo E alleles. The E2 isoform has cysteine at residues 112 and 158, so the ε2 allele had only two big fragments (91 bp and 83bp ). Whereas due to the substitution from arginine to cysteine residues at position 158 in E3, the 83-bp fragment was digested to 48-bp and 35-bp fragments in the ε3 allele. In addition to E3, E4 has arginine at position 112; therefore, the 91-bp fragment was digested to a 72-bp fragment in the ε4 allele (the 19-bp fragment was too small for detection). Therefore, each genotype generates unique combinations of Hhal fragment sizes. The E2/E2 sample contained 91 and 83 bp Hhal fragments reflecting the absence of sites at 112 Cys and 158 Cys. The E3/E3 sample also contained the 91-bp fragment (112 Cys), as well as 48- and 35-bp fragments from Hhal cleavage at 158 Arg site.

Also, the result presented here by student’s $t$-test indicated that subjects with the two most common apo E genotypes (E4/E4, E3/E3) have different serum total cholesterol levels ($t = 3.1, p<0.01$) (Table 2). All frequencies in the present study were similar to those of Japanese and Chinese population (Asian groups) [24] and American, Mexicans and Indians groups [25] (Table 3).

**Table 1.** Distribution of the apo E genotypes and allele frequencies in the study subjects.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Observed No.</th>
<th>Hardy-Weinberg expected No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4/E3</td>
<td>22</td>
<td>19.40</td>
</tr>
<tr>
<td>E3/E3</td>
<td>76</td>
<td>77.40</td>
</tr>
<tr>
<td>E3/E2</td>
<td>2</td>
<td>1.89</td>
</tr>
<tr>
<td>E2/E2</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>E4/E2</td>
<td>0</td>
<td>0.22</td>
</tr>
<tr>
<td>E4/E4</td>
<td>0</td>
<td>1.20</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Allele frequencies

ε2 0.01
ε3 0.88
ε4 0.11

$\chi^2$ (observed-expected) = 0.487; P(df = 2) = 0.7-0.8

**Table 2.** Comparison of mean total cholesterol levels according to the apo E genotypes.

<table>
<thead>
<tr>
<th>Apo E Genotype</th>
<th>N</th>
<th>Cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3/E3</td>
<td>76</td>
<td>220 ± 25 ($p&lt;0.05, t = 1.98$)</td>
</tr>
<tr>
<td>E4/E3</td>
<td>76</td>
<td>276 ± 27 ($p&lt;0.05, t = 2.08$)</td>
</tr>
<tr>
<td>E3/E2</td>
<td>76</td>
<td>260</td>
</tr>
</tbody>
</table>

$df = 964 \neq 3.1, P<0.01$

**DISCUSSION**

In the present report, the frequency of the common apo E isoforms and their influence on the total cholesterol levels in an Iranian population were determined. Recently, several reports have suggested that the apo E genotype influences the serum cholesterol level [25].
Table 3. Comparisons of apo E allele relative frequencies in several population studies.

<table>
<thead>
<tr>
<th>Populations</th>
<th>N</th>
<th>( \varepsilon^2 )</th>
<th>( \varepsilon^3 )</th>
<th>( \varepsilon^4 )</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland (Helsinki)</td>
<td>615</td>
<td>0.041</td>
<td>0.733</td>
<td>0.227</td>
<td>22</td>
</tr>
<tr>
<td>Norway (several regions)</td>
<td>239</td>
<td>0.090</td>
<td>0.795</td>
<td>0.115</td>
<td>22</td>
</tr>
<tr>
<td>Germany (Marburg &amp; Giessen)</td>
<td>1031</td>
<td>0.077</td>
<td>0.773</td>
<td>0.150</td>
<td>22</td>
</tr>
<tr>
<td>Switzerland (Geneva)</td>
<td>173</td>
<td>0.072</td>
<td>0.821</td>
<td>0.107</td>
<td>22</td>
</tr>
<tr>
<td>Japan (Tokyo)</td>
<td>197</td>
<td>0.038</td>
<td>0.843</td>
<td>0.112</td>
<td>22</td>
</tr>
<tr>
<td>Korea (Seoul)</td>
<td>145</td>
<td>0.020</td>
<td>0.870</td>
<td>0.110</td>
<td>26</td>
</tr>
<tr>
<td>Nigeria (Benin)</td>
<td>357</td>
<td>0.027</td>
<td>0.667</td>
<td>0.297</td>
<td>22</td>
</tr>
<tr>
<td>USA Blacks</td>
<td>194</td>
<td>0.034</td>
<td>0.706</td>
<td>0.260</td>
<td>22</td>
</tr>
<tr>
<td>Sudan (Khartoum)</td>
<td>103</td>
<td>0.083</td>
<td>0.626</td>
<td>0.291</td>
<td>22</td>
</tr>
<tr>
<td>Mexicans (Texas, USA)</td>
<td>963</td>
<td>0.039</td>
<td>0.859</td>
<td>0.102</td>
<td>22</td>
</tr>
<tr>
<td>Spain &amp; Morocco</td>
<td>660</td>
<td>0.041</td>
<td>0.855</td>
<td>0.104</td>
<td>27</td>
</tr>
</tbody>
</table>

Allele frequencies of the apo E polymorphism have been reported to vary among racial or ethnic groups. Allele frequencies reported in the present study were similar to those of Japanese, Mexican, and Korean [26] populations. The frequency of \( \varepsilon^4 \) appears to be 2-3 folds lower in Iranian subjects (Tehran; \( \varepsilon^4 = 0.11 \)) compared to Finlander (Helsinki; \( \varepsilon^4 = 0.227 \)), Nigerian (Benin; \( \varepsilon^4 = 0.297 \)), African Americans (\( \varepsilon^4 = 0.260 \)), and Sudanese populations (Khartoum; \( \varepsilon^4 = 0.291 \)) [22] and were similar to Korean (\( \varepsilon^4 = 0.11 \)) [26], Norwegian (\( \varepsilon^4 = 0.115 \)), German (Murburg; \( \varepsilon^4 = 0.119 \)), Swiss (Geneva; \( \varepsilon^4 = 0.107 \)), Japanese (Tokyo; \( \varepsilon^4 = 0.112 \)), Mexican (Texas, USA; \( \varepsilon^4 = 0.102 \)) [22], and Spanish and Moroccan population (\( \varepsilon^4 = 0.104 \)) [27]. The \( \varepsilon^4 \) allele frequency is high in African blacks, Polynesians, or New Guineans; in contrast, it has low frequency among Asian populations. In general, the \( \varepsilon^3 \) allele frequency of Asian groups, including the present data, is higher than that of Caucasian (Canada, USA, New Zealand) and blacks (Nigeria, USA blacks, Sudan) [22, 26].

As the possible explanation for the differences of the apo E polymorphism among populations, it may be due to genetic drift by a founder effect or a selective mechanism. Another possibility is that these differences can be influenced by different prevalence rates of cardiovascular disease, the analytical methods of the apo E polymorphism, and the selection bias of samples. Variation of the apo E gene locus is known to affect total cholesterol levels, although the results have not always been concordant among populations. In general, the average effect of the \( \varepsilon^4 \) allele is to increase significantly the levels of total cholesterol in a healthy population. In this study, the \( \varepsilon^4 \) allele had similar values to mean total cholesterol levels (Table 2) [26].

Utermann [28] has postulated that the apo E allele effects on cholesterol are dependent on environment, e.g. nutrition. That is, a significant effect of the \( \varepsilon^4 \) allele is present in populations with high fat intake and high total cholesterol, but not in those with low total cholesterol. However, the effects of the \( \varepsilon^4 \) allele on cholesterol levels did not differ significantly among the populations. Other examples were seen in Nigerian black and Koi San (Bushman) populations [29, 34]. These two populations had an exceptionally high frequency of \( \varepsilon^4 \) allele despite their low levels of plasma cholesterol. In conclusion, HhaI polymorphism analysis by PCR in this study is a rapid and convenient strategy that can be used directly on genomic DNA. It is also a useful tool for genetic or population study.

REFERENCES


