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Relación entre el gen Apolipoteín E y el fenotipo hipérico en Maracaibo, Zulia State, Venezuela

Sociedad Latinoamericana de Hipertensión
Caracas, Organismo Internacional

Disponible en: http://www.redalyc.org/articulo.oa?id=170217040004
Apolipoprotein E gene polymorphism and hypercholesterolemic phenotype in Maracaibo, Zulia State, Venezuela

Abstract

ApoE gene polymorphisms have been associated with high plasma lipids levels and cardiovascular disease. The aim of this study was to determine allelic and genotypic frequencies and to evaluate the associations of polymorphisms with hypercholesterolemic phenotypes in a population of patients in Maracaibo, at Zulia State. 221 patients with ages between 9 and 78 years old attending the Endocrine-Metabolic Center at the University of Zulia, in Zulia, Venezuela were recruited. The lipids profile was determined by enzymatic methods. ApoE polymorphisms were determined by PCR-RFLP. 133 dislipidemic and 88 patients with normal lipids profile were evaluated. The higher proportion of patients corresponded to hypercolesterolemia isolated (46.61%), followed by hypercolesterolemia combined with hypertriglyceridemia and low levels of HDL-chol (21.8%). ApoE ε3 allele was the most frequent in the evaluated population (0.80), followed by hypercolesterolemia combined with hypertriglyceridemia and low levels of HDL-chol (21.8%). ApoE ε3 allele was the most frequent in the evaluated population (0.80), both in the control group (0.78) and the dyslipidemic group (0.82), followed by the ε4 allele (0.12) for both groups and the ε2 allele with values of 0.10 and 0.06, for control and dyslipidemic group, respectively. The ε2ε4 and ε3ε4 genotypes were the most frequent in the population, with values of 62.89% and 22.17%, respectively. The genotype frequencies were 57.95% and 66.17% for ε2ε3; 23.86% and 21.05% for ε3ε4 in nondyslipidémicos and dyslipidemic patient groups, respectively.

The ε2ε3 genotype was observed only at hypercholesterolemic patients. The homozygote ε2ε2 and heterozygote ε2ε3 genotypes were more frequent at the normal lipids profile group, consistent with diverse reports that indicate the association of the ε3 allele with elevated cholesterol levels and low cholesterol levels when the ε2 allele is present. ApoE polymorphism appears to be associated with variance in serum lipids levels in the population evaluated.

Key words: hypercholesterolemia, ApoE, polymorphisms, cardiovascular disease, lipids profile

Cardiovascular diseases (CVD) still are occupying the first place between morbidity and mortality causes worldwide. The most recognizable and better characterized cardiovascular risk factor is the chronic alteration of the serum lipids concentration, which depends not only of nutritional habits but also of lipoprotein synthesis and metabolism which at the same time are conditioned by several gene product activities.

There are several genes known to be involved in CVD, such as phenotypes of ApoE. The gene coding of apolipoprotein (apo) E has been one the most extensively characterized genetic polymorphisms, particularly for its effects on lipids profiles. In caucasian populations, the frequency of the ε2, ε3 and ε4 alleles are: 0.08, 0.77 and 0.15 respectively, whereas the ApoE allele frequency varies in other populations, but always predominating ε3. The polymorphisms in APOE are associated with variations at serum cholesterol levels, thus the individuals with
The e² allele have 10% lower cholesterol levels than the mean value, and e⁴ allele carriers exhibit cholesterol levels 10% higher than homocysteineous individuals for e³16,18. In general, e² allele lowers total cholesterol levels and e⁴ allele raises them. This effect has been reported in most populations8,10.

Although previous studies have reported the allelic distribution of the ApoE gene in some communities of Venezuela19, there does not exists data that allow evaluating the influence of particular ApoE genotypes on the serum lipids levels in our region. The aim of this study was to evaluate the allelic frequency of the apoE gene and to determine the relationship between apoE genotypes and hypercholesterolemic phenotypes in patients attending the Endocrine and Metabolic Diseases Research Center at the University of Zulia Venezuela, Maracaibo, Zulia State, Venezuela.

Type of Study: a cross-sectional and correlational study type was realized.

Population and Sample
In the present study 221 patients attending The Endocrine and Metabolic Diseases Research Center (EMRC) at the University of Zulia-Venezuela from February 2006 to May 2007 were recruited. 133 consecutive patients with diagnosis of hypercholesterolemia were included. Patients with diagnosis of type 2 diabetes mellitus were excluded of this study. The control group consisted of 88 non-dyslipidemics patients, clinically healthy. Both groups were randomly selected of the data base of the EMRC Metabolic Syndrome Project by using SPSS ver 15 for Windows. Participants were informed of the study’s purpuse and a written consent form was given to them to sign. The postulates of the declaration of Helsinki were followed in their revision of October, 2000.

Serum lipid Measurements
5 ml of blood without anticoagulant to assess the lipids profile were obtained from an antecubital vein. The patients underwent 10 hr-12 hr of overnight fasting for the determination of baseline serum lipids. The concentrations of total cholesterol (TC), HDL-cholesterol (HDL-chol) and triglycerides were determined by enzymatic methods using commercial reagents Human GmbH (Germany). Any alteration in serum lipid levels was confirmed, at least once, with another determination, in a period of two weeks following recommendations of the American Heart Association and American College of Cardiology (ACC and AHA)10. The risk category of TC ≥200 mg/dL and triglycerides ≥ 150 mg/dL concentrations were considered. LDL-cholesterol (LDL-chol) was calculated by Fridewald’s equation (when triglycerides did not exceed 400 mg/dL). Risk factor for this category was considered with values above 130 mg/dL.

ApoE genotyping by PCR-RFLP
DNA extraction was performed from leucocytes by salting-out technique21. To amplify a fragment of exon 4 of the ApoE gene, which contains the polymorphic region (244 bp) was prepared from a PCR mixture reaction with 50μl of final volume, containing 200mM Tris-HCl pH 8.0, 500 mM KCI, 1.5 mM MgCl2, 200μM of each deoxiribonucleotide (dATP, dCTP, dGTP and dTTP), 20 pmoles of each oligonucleotide, 7% dimethysulfoxide (DMSO), 1 unit of taq DNA polymerase (Promega) and 300ng of genomic DNA. The program consisted of initial denaturing for 5 minutes at 94°C, 35 cycles of 1 minute denaturing at 94°C, 1 minute at 60°C and 1 minute at 72°C, followed by a final extension at 72°C for 10 minutes.

For analysis RFLP (Restriction fragment length polymorphism), the PCR products were digested with 5 units Hhal (Promega) in buffer provided with the enzyme, for 4 hours at 37°C. The restriction fragments were separated on 10% polyacrylamide non-denaturing gels electrophoresis as described previously22. The gel was fixed with a mixture of ethanol-acetic acid and silver stained.

Statistical Analysis
Chi-squared test was used for comparison of observed and expected frequencies, assuming Hardy-Weinberg equilibrium (HWE) and the comparison of each variable with the allele frequencies and genotype distribution.

Analysis of variance (ANOVA) was performed to compare mean serum lipids concentration among APOE allele and genotype groups.

The Odds Ratio was calculated to estimate the relative risk with a confidence interval of 95% to test potential partnerships among genotypes of serum lipids patterns of both groups. A “p” value <0.05 was considered statistically significant. SPSS for Windows version 15 (SPSS, Chicago, IL, USA) was used in all analyses.
female and 61 (45.86%) males, aged between 9 and 78 years, with an average of 48 ± 21 years. The population of non dyslipidemic or control group included 51 (57.9%) female and 37 (42%) male, aged between 19 and 81 years, with an average of 41 ± 17 years old.

Mean serum triglyceride, total cholesterol, LDL-chol and HDL-chol were respectively 79.34 mg/dL; 155.84 mg/dl; 88.68 mg/dl and 43.69 mg/dl in dyslipidemic group (Table 1). The mean values for triglycerides, cholesterol and LDL-chol, were significantly higher in the dyslipidemmic group than in the control group (p <0.05), consistent with the definition and criteria for classifying these groups. HDL-chol levels were lower in the dyslipidemic group (42.71 ± 14) than in the control group (51.76 ± 12.23) (p = 0.032).

In the control group the mean of serum lipids were higher in men, mainly at the triglyceride levels, but no significant difference sex-specific was found (p> 0.05) (Table 1). The same trend was observed in the dyslipidemic group with triglycerides levels of 176.97 mg/dl and 150.16 mg/dl in men and women, respectively. The total cholesterol and LDL-chol concentrations did not differ significantly between sexes in this group. In the control group, the HDL-chol was equivalent in men and women, unlike the dyslipidemic group, wich HDL-chol mean lower in men (40.67 mg/dl) than in women (44.44 mg/dl) (p = 0.046) was observed.

Table 2: Frequency and percentage of isolated and combined hypercholesterolemia

<table>
<thead>
<tr>
<th>Lipids abnormalities</th>
<th>Frequency</th>
<th>Percentage %^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-c ↑</td>
<td>62</td>
<td>46.62%</td>
</tr>
<tr>
<td>LDL↑+TG↑+ HDL↓</td>
<td>29</td>
<td>21.80%</td>
</tr>
<tr>
<td>LDL ↑ + HDL↓</td>
<td>25</td>
<td>18.80%</td>
</tr>
<tr>
<td>LDL ↑ +TG ↑</td>
<td>17</td>
<td>12.78%</td>
</tr>
<tr>
<td>Total</td>
<td>133</td>
<td>100% (100)</td>
</tr>
</tbody>
</table>

^a Percentage expressed as a function of the total population with lipid abnormalities (n=133)

Allelic frequency was evaluated in patients with isolated hypercholesterolemia. As shown in Table 4, the allelic distribution observed was similar to that of e3 (0.78) dyslipidemic and control group, with the most frequent allele e4 (0.16) and less frequent the e2 allele (0.06). Although the e4 allele was the most frequent at the group hypercholesterolemic that group exhibiting other dyslipidemias and the control group, no significant differences were found among the groups tested (p> 0.05). (Tables 3 and 4).

Table 1: Serum lipids levels in control and dyslipidemic groups

<table>
<thead>
<tr>
<th>mg/dl</th>
<th>CONTROL GROUP</th>
<th>DYSLIPIDEMIC GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Women (n=51)</td>
<td>Men (n=37)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>79.34±</td>
<td>97.82±</td>
</tr>
<tr>
<td></td>
<td>30.46</td>
<td>24.38</td>
</tr>
<tr>
<td>CT</td>
<td>155.84±</td>
<td>162.18±</td>
</tr>
<tr>
<td></td>
<td>20.44</td>
<td>11</td>
</tr>
<tr>
<td>LDL-chol</td>
<td>88.68±</td>
<td>92.73±</td>
</tr>
<tr>
<td></td>
<td>21.95</td>
<td>22.61</td>
</tr>
<tr>
<td>HDL-chol</td>
<td>51.93±</td>
<td>51.09±</td>
</tr>
<tr>
<td></td>
<td>12.73</td>
<td>10.51</td>
</tr>
</tbody>
</table>

Data is given as mean ± standard deviation. E: Standard deviation. HDL: high density lipoprotein, LDL: low density lipoprotein.

The hypercholesterolemia was seen as a unique condition in 62 (46.61%) patients, instead of mixed dyslipidemia, the most frequent was increased LDL cholesterol combined with hypertriglyceridemia and lower levels of HDL cholesterol (21.8%) (Table 2). e4, e3 and e2 alleles frequencies were respectively 0.08, 0.80 and 0.12 for the total population assessed. The e3 allele was the most common, both, at the control group (0.78) and at the dyslipidemic group (0.82), followed by e4 allele 0.12 for both groups, and finally, by e2 allele with 0.10 and 0.06 for control and dyslipidic groups, respectively (Table 3). No differences in allelic frequencies of the ApoE gene between dyslipidemic and control groups (p> 0.05) was found, however, the e2 allele nearly doubled the frequency at the control group compared with the dyslipidemic group.

Table 3: Allelic frequency of Apoprotein E gene in control and dyslipidemic groups

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control Group</th>
<th>Dyslipidemic group</th>
<th>Total population</th>
</tr>
</thead>
<tbody>
<tr>
<td>e2</td>
<td>18</td>
<td>0.10</td>
<td>16</td>
</tr>
<tr>
<td>e3</td>
<td>137</td>
<td>0.78</td>
<td>217</td>
</tr>
<tr>
<td>e4</td>
<td>21</td>
<td>0.12</td>
<td>33</td>
</tr>
</tbody>
</table>

Total 176 1,00 266 1,00 442 1,00

Table 4: Allelic frequency of Apoprotein E gene in control and isolated hypercholesterolemia groups

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control patients</th>
<th>Isolated hypercholesterolemia patients</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>e2</td>
<td>18</td>
<td>0.10</td>
<td>7</td>
</tr>
<tr>
<td>e3</td>
<td>137</td>
<td>0.78</td>
<td>97</td>
</tr>
<tr>
<td>e4</td>
<td>21</td>
<td>0.12</td>
<td>20</td>
</tr>
</tbody>
</table>

Total 176 1,00 124 1,00 300 1,00
The allelic frequency was assessed in patients with isolated hypercholesterolemia. As shown in Table 4, the allelic distribution was similar to that observed in the group with combined hyperlipidemia and control group with $\varepsilon^3$ as the most frequent (0.78) allele and $\varepsilon^2$ as the less frequent allele. Although $\varepsilon^4$ allele was more frequent at the hypercholesterolemic group (0.16) than at the group exhibiting other dyslipidemias (0.12) and the control group (0.12), no significant differences were found among the groups assessed (p > 0.05)(Tables 3 and 4).

When genotype distribution was considered at the total population, genotypes $\varepsilon^3\varepsilon^3$ and $\varepsilon^2\varepsilon^4$ were the most frequent (63% and 22%) respectively. The frequency of these genotypes was 58% and 24% respectively for the control group and 66% and 21% in patients with lipids abnormalities (Table 5).

The $\varepsilon^2\varepsilon^4$ genotypes homozygous and heterozygous $\varepsilon^2\varepsilon^4$ were observed only at dyslipidemic patients (frequencies 1.5% and 0.75%), while homozigous $\varepsilon^2\varepsilon^2$ and heterozygous $\varepsilon^2\varepsilon^3$ isoforms were more frequent at the control group 2.27% and 16%, respectively. At the dyslipidemic group, the frequencies for these genotypes were 0.75% and 10% (Table 5). No significant differences were observed at the genotypic distribution between dyslipidemic and control groups (p > 0.05). (Table 5).

The genotypes homozygous $\varepsilon^4\varepsilon^4$ and heterozygous were observed only at dyslipidemic patients (frequencies 0.01 and 0.02), whereas homozigous $\varepsilon^2\varepsilon^2$ and heterozygous $\varepsilon^2\varepsilon^3$ were most frequent at the control group, with values of 0.02 and 0.16, respectively. No significant differences at the genotypic distribution between dyslipidemic and control groups were found (p > 0.05).

The genotype distribution observed at the population was similar to the expected values, so that the distribution of ApoE gene polymorphisms followed the Hardy-Weinberg equilibrium and no difference was observed between men and women.

The mean serum lipids concentration by each APOE genotype in control and dyslipidemic groups is presented in Table 6. At the control group, even though serum lipids levels were found in between the reference values, a higher concentration of cholesterol and LDL-chol was found in subjects with genotype $\varepsilon^2\varepsilon^3$ (160.84±19 and 94.10±22 respectively), compared with individuals carries of other genotypes, although the risk analysis with a confidence interval of 95%, showed a very weak Odds Ratio (OR = 0.781, 95% CI = 0.216-2.232).

When considering the total population, there was a more pronounced effect of the different isoforms on serum lipids, total serum cholesterol and LDL-chol concentrations were higher in individuals with and 4 allele ($\varepsilon^2\varepsilon^4$ and $\varepsilon^4\varepsilon^4$) OR=1.4, 95% CI = 0.696-2.846. These same genotypes carriers were associated with high levels of HDL-chol (OR = 1.73, 95% CI = 0.74-4.01), $\varepsilon^2$ allele was associated with low levels of triglycerides (OR = 1.82, 95% CI = 0.58-5.67), cholesterol (OR = 2.1, 95% CI = 0.12 12.5) and LDL-chol (OR = 1.57, 95% CI = 0.624-3.95).
In this study, the genotypic and allelic frequencies in patients with elevated levels of serum cholesterol and patients without lipid abnormalities were evaluated. The group of patients with hypercholesterolemia included hypercholesterolemia as unique condition or in combination with other lipids abnormalities.

No significant differences in serum lipids levels of men and women were found, however the average levels of triglyceride, total cholesterol and LDL-chol were lower in women than in men. It has been observed variability in serum lipids levels between the sexes in terms of body mass index, use of birth control pills and physical activity, but these parameters were not evaluated.

Predictably, it was noted that e4 was the allele predominant with a frequency of 0.80, followed by the allele e3 (0.12) and e2 (0.08), consistent with frequencies found in other populations worldwide (Table 7), which are located in the range of 0.028 to 0.09 for apoE e4; 0.77 to 0.87 for apoE e3 and 0.1 to 0.2 for ApoE e2. Among populations showing frequencies outside this range African Americans should be highlighted, because they exhibit a 0.131 frequency for the e3 allele and African with values of 0.31 for the e4 allele.

The frequencies for the three alleles in this report are very similar to the frequencies found in Europe, particularly in French and Spanish populations, who were part of the study Apo Europa, consistent with a significant contribution from the Spanish colonizers in our genetic background. The comparative analysis allows a clear distinction with Brazil, Mexico, United States and African populations whose data is available.

The genotype distribution observed in the population assessed, both in the control and at the hypercholesterolemic group was similar to the expected values, so that the distribution of ApoE gene polymorphisms remain in Hardy-Weinberg equilibrium and there were no differences in genotypic and allelic frequencies between control and hypercholesterolemic groups.

The e4e4 homozygous and e2e4 heterozygous genotypes were observed exclusively at hypercholesterolemic patients, while e4e2 homozygous and e2e2 heterozygous forms were more frequent at the control group.

At the control group, even though serum lipids levels were found in between the reference values, a higher concentration of cholesterol and LDL-chol in subjects with e4e3 genotype was found (160.84 ± 19 and 22 ± 94.10, respectively), compared with individuals with other genotypes, although the risk analysis with a confidence interval of 95%, showed a very weak Odds Ratio (OR = 0.781, 95% CI = 0.216 to 2.232).

When considering the total population, there was a more pronounced effect of the different isoforms on serum lipids; total cholesterol and LDL-chol with higher values for the genotypes with e4 allele (e4e4 and e4e3) (OR = 1.4, 95% CI = 0.696-2.846). An important finding is that individuals with genotype e4e4 exhibited higher levels of HDL-chol (OR = 1.73, 95% CI = 0.74-4.01) and an association, although weaker, with low concentrations of triglycerides (OR = 0.984, 95% CI = 0.96-1.01). In opposition, the presence of the e2 allele was associated with lower triglyceride levels (OR=1.82, 95% CI=0.58-5.67), cholesterol (OR=2.1, 95% CI=0.12-12.5) and LDL-chol (OR=1.57, 95% CI = 0624-3.95). These findings are consistent with other studies in which carriers of the e4 allele exhibited higher levels of cholesterol and LDL-chol, while the individuals with e2 allele are associated with lower cholesterol levels and LDL-chol.

The relationship between the e4 allele and the presence of ischemic heart disease has been strengthened in a number of studies both, in men and wo-men, which appears to be related to a predominance in these patients of a small and dense LDL, which are more prone to oxidation. It has been reported that individuals with the phenotype e4e4 have double the risk of myocardial infarction than subjects e3e3, and atherosclerotic extensive lesions were identified in thoracic aorta, abdominal and coronary arteries that have been identified in necrotic studies. Thus, a better understanding of the role of this common polymorphism in specific populations may allow the identification of individuals that have increased risk to develop such diseases.

Moreover, the increased frequency of allelle 2 at the control group, supports evidence from diverse studies, which takes as reference the e4 allele, if the allele 2 is present in the genotype, there are lower levels of total cholesterol and LDL-chol, whereas the presence of the 4 allele is associated with higher levels of cholesterol and LDL-chol.

It has been suggested that the levels of LDL-chol at e4 allele carriers are higher in women than in men. Other studies have reported that women with the e4 allele and men with the e2 allele had higher levels of triglycerides, but this study showed no differences sex-specific. So the differences between the levels of cholesterol and HDL-chol and polymorphic forms of the ApoE gene appear to vary among different populations.

Is remarkable that the e4e4 genotype was detected just in the hypercholesterolemic group, but not significant association was found, however this lack of association could be explained by the low frequency of this genotype in the population, so it is necessary to increase population studies to confirm these findings.

It has been suggested that elevated serum lipids levels in e4 allele carriers, even if the elevation is not very significant, it could contribute to a clinical progression.
less favorable than in individuals with other alleles, but other reports have demonstrated the absence of association between cholesterol levels and ε4 allele. The relationship between ApoE polymorphism and serum lipids levels is not very clear for several populations. It has been recognized that the gene-environment interactions may explain the discrepancies observed in diverse studies.

For example the association of the ApoE isoforms with serum lipid levels appears to be affected by smoking, alcohol consumption, body mass index, physical activity and diet rich in fats. Likewise, the interactions at molecular level between different gene products and some environmental assaults can contribute to the discrepancies. In this study, no data was collected on other possible risk factors that would allow to analyze other associations to detect gene-environment interactions.

References
