DISTRIBUTION OF APOLIPOPROTEIN E GENE POLYMORPHISM IN STUDENTS AND IN HIGH-EDUCATED ELDERLY FROM SERBIA

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Apolipoprotein E (ApoE) play important role in lipid metabolism and in processes of remodeling and reparation in central nervous system. Three common ApoE isoforms, ApoE2, ApoE3 and ApoE4, show strong genetic determination by $\varepsilon_2$, $\varepsilon_3$, and $\varepsilon_4$ allele. In human genome gene encoding Apolipoprotein E (APOE) is located on chromosome 19, and $\varepsilon_2/\varepsilon_3/\varepsilon_4$ haplotype system is defined by 2 non-synonymous single nucleotide polymorphisms (SNPs) in the APOE exon 4. The frequency of the three APOE alleles and corresponding genotypes varies across human populations, with possible clinical implications. At least, variable distribution of $\varepsilon_4$ allele may contribute to the regional risk of cardiovascular and Alzheimer’s diseases. Allele-frequency comparisons between younger and older populations suggest an effect of APOE on mortality, but these data are not consistently confirmed. In the present study we have analyzed the distribution of APOE gene polymorphism in a group of University students and retained University professors living in Serbia. After DNA extraction from peripheral blood samples, the APOE genotype was determined by polymerase chain reaction (PCR) followed with HhaI restriction digestion. We found no statistically significant difference in alleles and genotypes distribution between younger and elder group of participants. Also, there was no significant difference compared to APOE data previously obtained in YUSAD cohort of healthy school children (15 y of age) from different regions of Serbia. In both of our groups, as well as in YUSAD cohort, frequency of APOE $\varepsilon_4$ allele was <10%. The observed frequencies are lower than in neighboring countries, but similar with Spanish data and some Asian populations. Our results do not support important role of APOE $\varepsilon_4$ in the morbidity and mortality in Serbian population, but gene-environmental-social interactions should be considered.

Key words: apolipoprotein E, gene polymorphism, population study, Serbia, $\varepsilon_4$ allele

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INTRODUCTION

Apolipoprotein E (ApoE) E has a crucial role in the metabolism of plasma lipoproteins, as well as in lipid synthesis and in reparative and remodelling processes in central nervous system. As one of the major protein constituents of several lipoprotein classes, ApoE serves as a ligand for the low density lipoprotein (LDL) receptor and LDL receptor-related protein (LRP) and enables lipids removal from plasma. In brain, ApoE is involved in endogenous cholesterol and amyloid protein metabolism as well as in synaptic plasticity during reparation and remodelling. Three common ApoE isoforms, ApoE2, ApoE3 and ApoE4, show strong genetic determination by ε2, ε3, and ε4 allele forms, respectively (SVOBODOVA et al., 2007; YU et al., 2007).

The gene encoding ApoE (APOE gene) in human genome maps on the long arm of chromosome 19. The ε2/ε3/ε4 haplotype system is defined by 2 non-synonymous single nucleotide polymorphisms (SNPs) in APOE exon 4. Both SNPs are C/T substitutions changing arginine to cysteine in ApoE at amino acid position 112 and 158, respectively. The allelic compositions of the haplotypes are TT for ε2, TC for ε3, and CC for ε4, with corresponding protein isoforms Cys,Cys for ApoE2, Cys,Arg for ApoE3 and Arg,Arg for ApoE4 (YU et al., 2007). The most frequent allele in human populations is ε3, with overall prevalence of 70–80%.

Allele ε4 is associated with increased plasma total and LDL cholesterol and ApoB levels, and it is designed as risk factor for cardiovascular diseases (CVD). Usually, allele ε2 is associated with lower total and LDL cholesterol levels, but its relation to the risk of atherosclerosis is controversial (NOVAKOVIĆ et al., 2010; SVOBODOVA et al., 2007). Regarding ApoE role in central nervous system, ε4 allele is well known genetic risk factor for Alzheimer’s disease, confirmed in genome wide association studies (GWAS) (GHARESOURAN et al., 2013; BELBIN et al., 2011; YU et al., 2007). Also, there are number of reports of APOE ε4 association with affective and cognitive disorders, subarachnoid hemorrhage etc. (KAUSHAL et al., 2007).

The frequency of the three APOE alleles and corresponding genotypes varies across human populations, with possible clinical implications. At least, variable distribution of ε4 allele may contribute to the regional risk of cardiovascular and Alzheimer’s diseases. Allele-frequency comparisons between younger and older populations suggest an effect of APOE on mortality, but these data are not consistently confirmed (SCHUPF et al., 2013; ROSVALL et al., 2009). Serbian population shows high prevalence of CVD, which are still major cause of death in the country (DAMNJANOVIĆ et al., 2011). On the other hand, population age-structure is not convenient, with high prevalence of aging-related neurodegenerative disorders, such as Alzheimer’s disease. Under these circumstances, the evaluation of APOE allele distribution in different age-related groups from Serbia could be interesting from genetic, as well as from epidemiological and clinical viewpoints. We therefore studied the APOE gene polymorphism in a group of University students and retained University professors living in Serbia.

MATERIALS AND METHODS

Apo E genotype was examined in two groups of subjects from Serbian population: a group of University students (N=548, 188 males, 366 females, age 21+/-2y) and a group of retained University professors (N=94, all men, age 83+/-3y). The young participants were recruited from University of Belgrade, and they arrived from different parts of Serbia. The senior participants were part of Serbian cohort in Seven Countries Study (ALONSO et al., 2009). For
molecular genetic analysis, 5ml peripheral blood samples were collected and DNA was extracted using salting-out method.

The APOE genotype was determined by polymerase chain reaction (PCR) followed with HhaI restriction digestion of PCR products and electrophoresis of restriction fragments on polyacrylamide gels (Hixton and Vernier, 1990). Each of the genotype forms was distinguished by a unique combination of HhaI fragment sizes that enabled unambiguous typing of all homozygotic and heterozygotic combinations. HhaI cleaves at GCGC encoding 112arg (ε4) and 158arg (ε3, ε4), but does not cut at GTGC encoding 112cys (ε2, ε3) and 158cys (ε2). PCR was conducted in a thermal cycler ABI 2700 (Applied Biosystems, USA). The oligonucleotide primers used for PCR amplification were P1 (5’-TCCAAGGAGCTGCAGCGCGCA-3’) and P2 (5’-ACAGAATTCCGCCCCGCTGGTACACTGC-3’). The amplification mixture contained 2 mM MgCl2, 1U of DreamTaq DNA Polymerase (Fermentas, Germany), 0.2 mM of each dNTP (Promega, Madison, WI), 0.8 µM of each primer and 300ng DNA in a final volume of 25 µl. The PCR conditions were: initial denaturation at 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing and elongation at 67.5°C for 1 min 30 s. A final extension step at 72°C for 10 min was done. The amplification generated a DNA fragment of 227 bp. After amplification, 20 µl of the PCR product were directly digested with 12 units of the restriction endonuclease HhaI (Promega) at 37°C overnight. Gene fragments were separated using 10 % vertical polyacrylamide gel electrophoresis and detected by ethidium bromide staining under ultraviolet illumination, using an appropriate DNA size marker.

The allelic and genotypic frequencies of APOE were estimated by counting alleles and genotypes and calculating sample proportions; the statistical significance of differences of frequencies between groups was compared by χ2 test.

RESULTS AND DISCUSSION

The frequencies of APOE genotypes are presented in Table 1. The observed distributions were compared by χ2 test and no statistical significance was found (p = 0.829). (Table 1)

<table>
<thead>
<tr>
<th>APOE genotypes</th>
<th>students M&amp;F</th>
<th>%</th>
<th>M</th>
<th>%</th>
<th>F</th>
<th>%</th>
<th>M</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε2/ε2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ε2/ε3</td>
<td>55</td>
<td>10.1</td>
<td>13</td>
<td>7.1</td>
<td>42</td>
<td>11.5</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>ε3/ε3</td>
<td>426</td>
<td>77.7</td>
<td>148</td>
<td>81.3</td>
<td>278</td>
<td>76.0</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>ε2/ε4</td>
<td>6</td>
<td>1.1</td>
<td>1</td>
<td>0.5</td>
<td>5</td>
<td>1.4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>ε3/ε4</td>
<td>57</td>
<td>10.4</td>
<td>20</td>
<td>11.1</td>
<td>37</td>
<td>10.0</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>ε4/ε4</td>
<td>4</td>
<td>0.7</td>
<td>0</td>
<td>0.0</td>
<td>4</td>
<td>1.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>total</td>
<td>548</td>
<td>100.0</td>
<td>182</td>
<td>100.0</td>
<td>366</td>
<td>100.0</td>
<td>94</td>
<td>100</td>
</tr>
</tbody>
</table>

(M = males, F = females). χ² = 0.374, p = 0.829
Table 2 shows the allelic frequencies of APOE in present study and in several European countries; the frequency of the risk APOE ε4 allele in Serbian population is the lowest among the listed countries. Data for YUSAD study (Yugoslav study of precursors of atherosclerosis in school children) are from cohort of more than 500 school children (15y) living in different regions of Serbia (DAMNJANOVIĆ et al., 2011). (Table 2)

<table>
<thead>
<tr>
<th>Population</th>
<th>ε2</th>
<th>ε3</th>
<th>ε4</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serbia - students</td>
<td>0.056</td>
<td>0.879</td>
<td>0.065</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Serbia - professors</td>
<td>0.0797</td>
<td>0.8457</td>
<td>0.0744</td>
<td>5.449</td>
<td>0.076</td>
</tr>
<tr>
<td>Serbia – YUSAD</td>
<td>0.067</td>
<td>0.863</td>
<td>0.070</td>
<td>1.316</td>
<td>0.518</td>
</tr>
<tr>
<td>Croatia</td>
<td>0.069</td>
<td>0.787</td>
<td>0.144</td>
<td>36.294</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Finland</td>
<td>0.064</td>
<td>0.807</td>
<td>0.129</td>
<td>24.721</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>France</td>
<td>0.087</td>
<td>0.782</td>
<td>0.131</td>
<td>34.609</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Germany</td>
<td>0.082</td>
<td>0.782</td>
<td>0.136</td>
<td>35.643</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hungary</td>
<td>0.065</td>
<td>0.808</td>
<td>0.127</td>
<td>23.678</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Spain</td>
<td>0.075</td>
<td>0.849</td>
<td>0.075</td>
<td>3.99</td>
<td>0.136</td>
</tr>
<tr>
<td>Turkey</td>
<td>0.061</td>
<td>0.860</td>
<td>0.079</td>
<td>1.782</td>
<td>0.410</td>
</tr>
<tr>
<td>USA</td>
<td>0.072</td>
<td>0.786</td>
<td>0.140</td>
<td>34.632</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

(students, professors = our results; other = from DAMNJANOVIĆ et al., 2011)

We determined APOE genotypes in total number of 642 subjects from Serbia, belonging to two age-related groups: University students and retained University professors. We found no statistically significant difference in alleles and genotypes distribution between younger and elder group of participants (p=0.076 and 0.829 for alleles and genotypes, respectively). Also, there was no significant difference (p=0.518) compared to APOE data previously obtained in cohort of YUSAD study, which comprises more than 500 healthy school children (15 y of age) from different regions of Serbia (DAMNJANOVIĆ et al., 2011).

In both of our groups, as well as in YUSAD cohort, frequency of APOE ε4 allele was <10% (6.5%, 7.44% and 7.0%, in students, professors and school children, respectively). The observed frequencies are significantly lower than in neighboring countries (Croatia, Hungary p<0.0001), but similar with Spanish data (p=0.136) and some Asian populations (Turkey p=0.410).

It is well documented that the distribution of APOE alleles varies across populations (DAMNJANOVIĆ et al., 2011; MAKSIMOVIĆ et al., 2009; SVOBODOVA et al., 2007). In general, the
European populations traditionally have higher APOE ε4 frequency than Asians. The cause for this regional variability is still not clear. Allele ε4 is assumed as an ancestral form, but due to mechanisms of negative selection its frequency has been decreased, and ε3 allele has become most frequent in contemporary populations. Notably, the frequency of ε4 appears to be higher in northern regions of Europe than in southern regions, thus following the incidence of CVD. In Asia, a similar trend has not been described (SVOBODA et al., 2007).

Low APOE ε4 allele frequency in Serbia is not consistent with high prevalence of CVD in the country. However, analyses of lipid profiles in YUSAD study showed significantly higher mean level of total cholesterol and in ε4 carriers, even in childhood (DAMNJANOVIC et al., 2011).

Because of no significant difference in APOE alleles and genotypes distribution in a group age ≤25y and in seniors, we could not confirm the role of APOE in mortality as well as in longevity in Serbian population. However, this conclusion should be accepted cautiously, because of demographic profile of our advanced-age participants. Our senior group comprised male participants with highest education (PhD degree at least). Several recent studies from different parts of world: Italy (MARENGONI et al., 2011), Sweden (WANG et al., 2012; ROSVALL et al., 2009), Canada (MENG and D’ARCY, 2013), USA (PETERSEN et al., 2010), showed that education level and gender, as well as leisure activities, diet, could modify ε4 allele related risks. Generally, lower education level is associated with higher dementia risk in ε4 allele carriers, but some investigators did not confirm this influence (CHEN et al., 2010). The ε4 allele in APOE gene accompanied with the linked G allele in rs2075650 of TOMM40 gene have been associated with increased mortality and the ε2 allele with decreased mortality in advanced age, although inconsistently (SCHUPF et al., 2013). Schupf’s group findings support the hypothesis that both reduction in the frequency of the ε4 allele and increase in the frequency of the ε2 allele contribute to longevity (SCHUPF et al., 2013).

Similarly, Rosvall’s group reported increased mortality-risk of 22% among elderly with the ε4 allele, whereas a 28% decreased mortality-risk was detected in those with the ε2 allele compared to those with the ε3ε3 genotype (ROSVALL et al., 2009). Adjustment for severe vascular events did not change the observed risks. In this investigation dementia accounted for the majority of the increased mortality-risk associated with the ε4 allele, but the protective effect of the ε2 allele remained. Also, both effects of the ε4 allele and the ε2 allele were strongly modified by gender. A 49% elevated risk for death in men was related to the ε4 allele, and a 36% decreased mortality-risk was found in women with the ε2 allele. These findings suggest different roles for the APOE alleles in survival by gender in old age.

Again, some studies showed only weak or even lack of these associations (VAN GERVERN et al., 2012; CHEN et al., 2010; WELSH-BOHMER et al., 2009), and our investigation supports these observations. In order to solve such controversies, very recent studies suggest that single SNP approaches may be inadequate to identify genetic risks. An alternative approach is the use of multilocus genotype patterns (MLGPs) that combine SNPs at different susceptibility genes (BARRAL et al., 2012). Genome wide association studies (GWAS) also confirmed modest effect of single SNPs in complex traits, such as CVD or longevity. On the other hand, huge amount of data obtained by GWASs encourages MLGPs strategy. In addition, gene-environmental-social interactions during human life are very important and may be another part of “missing heritability” explanation (NOVAKOVIĆ et al., 2013, GRÜNBLATT et al., 2009).
ACKNOWLEDGEMENT

This work is supported by Serbian Ministry of education, science and technology development, Grant No. ON175091

Received June 20th, 2013
Accepted October 05th, 2013

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I. NOVAKOVIC et al: APOE GENE POLYMORPHISM IN SERBIA

871


DISTRIBUCIJA PLOMORFIZMA GENA ZA APOLIPOPROTEIN E KOD STUDENATA I PENZIONISANIH UNIVERZITETSKIH PROFESORA U SRBIJI

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Primljeno 20. VI 2013.
Odobreno 05. X. 2013.