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Determination OF Apolipoprotein (E) Genotypes By PCR And Relation To Plasma Lipid In Coronary Heart Disease Patients

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ABSTRACT

Coronary artery disease (CAD) is the most prevalent type of cardiovascular disease (CVD) which, according to the American Heart Association accounts for 35% of all deaths in U.S.A. Apolipoprotein E (ApoE) is an apoprotein found in the chylomicrons and IDLs that binds to a specific receptor on liver cells and peripheral cells and it is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. This study was carried out on 35 subjects divided into two groups. First: 25 CAD patients, the patients ages were ranged between 43 to 71 years. Second: 10 control were normal, the control ages were ranged between 29-54 years. All the patients and control were selected from Cairo Medical Center, cardiology department.

All subjects were submitted to the following:
1. Full history taking.
2. General clinical examination.
3. Coronary angiography (transfemoral catheterization).
4. Laboratory investigation:
   • Assessment of apolipoprotein E polymorphism by PCR and restriction enzymes.
   • Measurement of serum cholesterol.
   • Measurement of serum triacylglycerol.
   • Measurement of serum low density lipoprotein cholesterol (LDLc).
   • Measurement of serum high density lipoprotein cholesterol (HDLc).
   • Measurement of serum cardiac enzymes and troponine.

INTRODUCTION

Coronary artery disease (CAD) also called coronary heart disease (CHD) is defined as a condition in which a plaque builds up inside the coronary arteries and this plaque narrows the arteries and reduces blood flow to the heart muscle (NHLPI, 2009).

Coronary artery disease (CAD) is the most prevalent type of cardiovascular disease (CVD) which, according to the American Heart Association accounts for 35% of all deaths in U.S.A (Corwin, 2008).
Apolipoprotein E (ApoE) is an apoprotein found in the chylomicrons and IDLs that binds to a specific receptor on liver cells and peripheral cells and it is essential for the normal catabolism of triglyceride-rich lipoprotein constituents (Entrez Gene, 2009).

The ApoE gene (ApoE) is mapped to chromosome 19 in a cluster with apolipoprotein C1 and apolipoprotein C2, ApoE consists of four exons and three introns, totaling 3597 base pairs (Hoek et al., 2008).

The genetic polymorphism of ApoE results from the existence of three common co-dominant alleles (E2, E3, and E4) that code for three apolipoprotein isoforms (E2, E3, and E4) resulting in six common genotypes (E2E2, E2E3, E2E4, E3E3, E3E4, and E4E4) (Tascilar et al., 2009).

These isoforms differ from each other only by single amino acid substitution at position 112 and 158, but have profound physiological consequences (Entrez Gene, 2009).

The most frequent isoform E3 contains a cystein at residue 112 and an arginine at residue 158. E2 and E4 differ from the E3 isoform in the fact that the E2 isoform contains a cystein at residue 158 and the E4 contains an arginine at residue 112 (Chaaba et al., 2008).

Plasma lipids are ApoE isofrom dependent and it has been reported in association with atherosclerotic vascular disease (Graner et al., 2008; Tascilar et al., 2009).

Lipoproteins play a central role in development of atherosclerotic vascular disease, so, with their ability to affect lipid level, the ApoE polymorphism could be one of the factors influencing development of atherosclerosis (Chaaba et al., 2008).

The ApoE gene is one of the candidate genes for the risk of CHD, the E4 allele of the ApoE gene is maladaptive in the developed world leading to increased risk of CHD, consequently, the derived E3 allele is adaptive in the modern environment (Ding and Kullo, 2009).

The ApoE4 allele was found to be associated with high LDLc and CAD in type 2 diabetes (Chaaba et al., 2008).

**AIM OF THE WORK**

The aim of this work is to determine the relation between apolipoprotein E polymorphism with the lipid profile in atherosclerotic coronary artery diseases.

**SUBJECTS and METHODS**

**Subjects:**

This study was carried out on 35 subjects: 25 coronary artery disease patients (8 females and 17 males) who angiographically had stenosis in at least one of the major coronary vessels and 10 subjects as control group consisted of (6 females and 4 males) who underwent angiography procedures and were normal. All The patients and control were selected from Cairo Medical Center, Cardiology Department, The patient's ages were ranged between 43 to 71 years. The controls ages were ranged between 29 to 54 years. The excluding criteria for enrollment into the study included familial hypercholesterolemia, cancer, renal disease, and any other chronic illnesses. The genotypes were grouped in three groups: E2 group (E2E2, E2E3), E3 group (E3E3) and E4 group (E2E4, E3E4, E4E4) (Table 1).

Table 1: Subjects included 25 patients and 10 matched apparently healthy controls:

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>E2E2</th>
<th>E2E3</th>
<th>E2E4</th>
<th>E3E3</th>
<th>E3E4</th>
<th>E4E4</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1</td>
<td>17</td>
<td></td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>No. of controls</td>
<td>E2E2</td>
<td>E2E3</td>
<td>E2E4</td>
<td>E3E3</td>
<td>E3E4</td>
<td>E4E4</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>6</td>
<td></td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>


Methods:
All subjects were submitted to the following:
5. Full history taking.
7. Coronary angiography (transfemoral catheterization).
8. Laboratory investigation:
   - Assessment of apolipoprotein E polymorphism by PCR and restriction enzymes.
   - Measurement of serum cholesterol.
   - Measurement of serum triacylglycerol.
   - Measurement of serum low density lipoprotein cholesterol (LDLc).
   - Measurement of serum high density lipoprotein cholesterol (HDLc).

Blood sampling:
10 ml of venous blood were withdrawn from the cubital vein of every subject (the patients are fasting for 12 hours). 5ml was transferred slowly into vacunated EDTA tube for isolation of White Blood Cells. The remaining 5ml was transferred slowly into a plain tube for determination of serum lipid profile.

Detection of Apolipoprotein (E) Genotypes by PCR:

Materials used for Molecular studies:
Reagents used for DNA Extraction:
2X Sucrose triton (PH=7.6)
Nuclei Lysis buffer (NaCl + Na₂ EDTA) pH=8.2
20% SDS (Sodium dodecyle Sulfate)
20% Proteinase K Solution
Saturated NaCl
Absolute ethanol (95%)
70% ethanol
Double distilled water.

Apparatus used for Molecular studies:
Cooling centrifuge
Thermocycler (Biometra, Germany).
Gel electrophoresis apparatus
MD25K UV transilluminator

Reagents used for PCR and RFLP:
Primers for Apo E gene (Eurofins MWG GmbH, Germany):
Forward: 5′-TCCAAGGAGCTGAGCAGCCGCGC-3′
Reverse: 5′-GCCCGGCCTGTAACACTGCA-3′

In order to yield a 218-bp DNA fragment to be detected on a 2% Agarose gel.
DreamTaq™ DNA polymerase (5U/µl) (Thermo Fisher Scientific Co., USA)
10x DreamTaq™ buffer (Thermo Fisher Scientific Co., USA)
Q-Solution, 5X (Qiagen, Germany)
dNTP mix (dATP, dCTP, dGTP and dTTP, 2 mM each)
Nuclease free water for reconstitution of lyophilized primers amd preparation of working primer solutions and dNTPs solution. Restriction Enzymes: AflIII and Hae II (2,000 units (20,000U/mL) (New England BioLabs, info@neb.com, www.neb.com )
Agarose,
Ethidium bromide (10 mg/ml),
Loading dye
DNA ladder (50bp and 100bp)

Determination of CAD severity
1. Cardiac enzymes:
   - The patients with high range CK, CKMB, LDH.
   - Cardiac enzymes are performed on chemistry instrument, (Beckman COULTER AU480).

2. Troponine test:
   - The patients with high troponine I.
   - Troponine I is measured by hormonal instruments (ARCHITECT PLUS ARC 1000).

3. Coronary Angiography and Cardiac Catheterization

   Coronary angiography was the first available in vivo assessment of the coronary arteries. In this technique, an iodinated contrast agent is injected through a catheter placed at the ostium of the coronaries. The contrast agent is then visualized through radiographic
fluoroscopic examination of the heart (Chou, 2015).

RESULTS

Coronary artery disease (CAD) is the leading cause of death and premature disability. It is a complex disorder resulting from many risk factors and individuals with genetic predisposition to atherosclerosis have substantial risk for developing CAD, especially at early ages. While it is difficult to explore the relation between local vessel wall function and CAD severity, measuring DNA variants such as ApoE polymorphisms may provide a way to assess this link because of its known effect on endothelial cell proliferation (Demet et al., 2010).

As shown in Table 2, statistically significant difference between groups as regard age was recorded, while gender had non-significant effect.

Table (3) shows statistically significant difference between groups as regard DM, the rest have insignificant.

A statistically significant difference between groups as regard lipid profile, using t-test was noticed (Table 4).

Table (5) shows statistically significant difference between patients and control as regard ApoE genotyping, using Chi-square test, with $p$-value $>0.05$ NS.

**Table 2: Sociodemographic criteria of CAD patients and control groups.**

<table>
<thead>
<tr>
<th>Demographic Data</th>
<th>CAD patients (N=25)</th>
<th>Control (N=10)</th>
<th>t-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td>52.266</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Range</td>
<td>43 71</td>
<td>29 54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SD</td>
<td>55.8 7.2</td>
<td>41.4 8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17 68.0%</td>
<td>4 40.0%</td>
<td>1.312</td>
<td>0.215</td>
</tr>
<tr>
<td>Female</td>
<td>8 32.0%</td>
<td>6 60.0%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3: Clinical parameters in CAD patients and control groups.**

<table>
<thead>
<tr>
<th></th>
<th>CAD patients (N=25)</th>
<th>Control (N=10)</th>
<th>x2</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td>0.536</td>
<td>0.464</td>
</tr>
<tr>
<td>Positive</td>
<td>10 40.0%</td>
<td>2 20.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>15 60.0%</td>
<td>8 80.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td>1.851</td>
<td>0.174</td>
</tr>
<tr>
<td>Positive</td>
<td>20 80.0%</td>
<td>5 50.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>5 20.0%</td>
<td>5 50.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td></td>
<td></td>
<td>7.146</td>
<td>0.007</td>
</tr>
<tr>
<td>Positive</td>
<td>14 56.0%</td>
<td>0 0.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11 44.0%</td>
<td>10 100.0%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4: Statistical comparison between CAD patients and control groups as regards to lipid profile.**

<table>
<thead>
<tr>
<th></th>
<th>CAD patients (N=25)</th>
<th>Control (N=10)</th>
<th>t-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (TC) mg/dl</td>
<td>258.08 110.74</td>
<td>165.60 18.85</td>
<td>3.698</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglyceride (TG) mg/dl</td>
<td>219.56 108.97</td>
<td>104.70 23.70</td>
<td>4.651</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDLc (mg/dl)</td>
<td>50.38 15.89</td>
<td>46.69 6.71</td>
<td>2.000</td>
<td>0.032</td>
</tr>
<tr>
<td>LDLc (mg/dl)</td>
<td>163.79 106.70</td>
<td>97.97 19.52</td>
<td>2.729</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Determination of apolipoprotein (E) genotypes by PCR and relation to plasma lipid

Table 5: ApoE genotypes and alleles among CAD patients and control groups.

<table>
<thead>
<tr>
<th>ApoE genotyping</th>
<th>CAD patients (N=25)</th>
<th>Control (N=10)</th>
<th>x²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3/E3</td>
<td>2 8.0%</td>
<td>2 20.0%</td>
<td>0.176</td>
<td>0.674</td>
</tr>
<tr>
<td>E2/E3</td>
<td>17 68.0%</td>
<td>6 60.0%</td>
<td>0.003</td>
<td>0.955</td>
</tr>
<tr>
<td>E3/E4</td>
<td>5 20.0%</td>
<td>2 20.0%</td>
<td>0.219</td>
<td>0.640</td>
</tr>
<tr>
<td>E4/E4</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>E2/E4</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>E2/E2</td>
<td>1 4.0%</td>
<td>0 0.0%</td>
<td>0.232</td>
<td>0.630</td>
</tr>
<tr>
<td>ApoE genotyping</td>
<td>(N=50)</td>
<td>(N=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>19 38.0%</td>
<td>6 30.0%</td>
<td>0.126</td>
<td>0.723</td>
</tr>
<tr>
<td>E3</td>
<td>26 52.0%</td>
<td>12 60.0%</td>
<td>0.117</td>
<td>0.723</td>
</tr>
<tr>
<td>E4</td>
<td>5 10.0%</td>
<td>2 10.0%</td>
<td>0.194</td>
<td>0.659</td>
</tr>
</tbody>
</table>

DISCUSSION

Coronary arteries supply blood to the heart muscle. Like all other tissues in the body, the heart muscle needs oxygen-rich blood to function, and oxygen-depleted blood must be carried away. The coronary arteries run along the outside of the heart and have small branches that dive into the heart muscle to bring it blood (Mazur et al., 2015).

Apolipoprotein E is a plasma glycoprotein and a member of the apo gene family, it is located at chromosome 19q13.2, and consists of four exons and three introns spanning 3.597 nucleotides, and produces a 299 amino acid polypeptide with a molecular mass of about 34 kDa (Demet et al., 2010).

Apolipoprotein E has a storied history as a lipid transport protein (Hauser et al., 2010). Apo E is a constituent of triglyceride-rich lipoproteins and high density lipoproteins. Its major function is to mediate the binding of lipoprotein particles to cell surface receptors (Grammer et al., 2011).

Apo E serves as a ligand for the apo B, E receptor, and the LDL receptor related protein and thus it plays a prominent role in the transportation and redistribution in both the influx and efflux of cholesterol in the body (Fallah et al., 2011).

The receptor binding properties of ApoE are strongly influenced by isoform specific amino acid differences as well as the lipidation state of the protein (Hauser et al., 2010).

These isoforms differ in amino acid sequence at positions 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 (Demet et al., 2010).

While there are rare variants, among the variants of this gene, alleles E2, E3, and E4 constitute the common polymorphism found in most populations in relation to cardiovascular disease (Demet et al., 2010).

Individuals with different apoE genotypes have different susceptibilities to CAD and studies have shown that the apo E4 allele is a genetic marker for CAD and an independent risk factor that predicts the incidence of cardiovascular disease (Li et al., 2010).

The present study was carried out to determine the relation of ApoE gene polymorphisms and lipid profile in CAD defined by coronary angiography and assesses the findings in relation to the severity of disease in Egyptian patients. The current study showed significant higher age, male gender, smoking, hypertension and diabetes in CAD patients as compared with control.

These results agree with Peixoto et al., (2007); Dias et al., (2009) and Fallah et al., (2011) who reported a predominance of male gender and mean
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age in CAD patients. Bhanushali and Das, (2010) showed a significant difference in smoking and hypertension between CAD patients and control.

Grammer et al., (2011) reported that patients with CAD were significantly older than controls. Current or past smoking, diabetes mellitus, and hypertension were more prevalent in CAD compared to controls. The current study showed significant higher total cholesterol (TC), triglycerides (TG), LDLc while lower HDLc in CAD patients as compared with control.

These results agree with those reported by Kharrazi et al., (2006) and Bahri et al., (2008) and explained this dyslipidemia as a classic risk factor for CAD.

The present study showed that the distribution of ApoE genotypes in CAD patients are as follow: E3E3, E2E3, E3E4, E4E4, E2E4 and E2E2 are 8.0%, 68%, 20%, 0.0%, 0.0% and 4.0% while in control are 20.0%, 60.0%, 20.0%, 0.0%, 0.0% and 0.0%. The frequency of E2, E3 and E4 alleles in CAD patients are as follow: 38.0%, 52.0% and 10.0% while in controls are 30.0%, 60.0% and 10.0% respectively.

The current study showed that E3E4 genotype is significantly higher in CAD patients as compared with controls while there was no significant difference as regards other genotypes. The study also showed that E2E3 genotype increased risk of CAD (p-value 0.955). These results are in agreement with Kharrazi et al., (2006) and Singh et al., (2008).

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