Is the ApoE Polymorphism Associated with Dilated Cardiomyopathy?

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Abstract. Apolipoprotein E (ApoE) is 34 kDa protein involved in the modulation of cholesterol transport and homeostasis. Polymorphism of the ApoE gene has been implicated in many chronic cardiovascular and neuronal diseases. ApoE $\varepsilon 4$ allele has been reported to be associated with increased risk of cardiovascular diseases such as myocardial infarction, hypertension, coronary heart disease, etc. Fifty patients with the end-stage dilated cardiomyopathy (DCM) and advanced congestive heart failure were examined in our study. For evaluation of ApoE polymorphism, novel approach of fast screening of ApoE gene polymorphism by combination of PCR and blotting (CVD StripAssay) was used. Individual genotypes were correlated with basic cardiologic clinical parameters. The reported frequency of this allele in Caucasian population is 14.7%. Our results showed that in patients with DCM frequency of the ApoE $\varepsilon 4$ allele is 40%. Frequency of the genotype $\varepsilon 2/4$ was 58% and $\varepsilon 3/4$ was 22%. Comparison with control Caucasian groups monitored by others clearly revealed that frequency of $\varepsilon 4$ alelle is increased in patients with advanced stages of DCM. This observation suggests association of ApoE polymorphism with severe form of DCM. Physiological consequences of this observation remain to be clarified.

Key words: Apolipoprotein E — Gene polymorphism — Dilated cardiomyopathy

Introduction

Apolipoprotein E (ApoE) is a major component of low-density and high-density lipoproteins. Three common alleles – $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ – encode for the three main isoforms – ApoE $\varepsilon 2$, ApoE $\varepsilon 3$, and ApoE $\varepsilon 4$ – circulating in the bloodstream. Accumulating evidence demonstrates that ApoE alleles are associated with both

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cardiovascular and Alzheimer's diseases (Bouthillier et al. 1983; Bretsky et al. 1999; Poirier 1999, 2000; Mahley and Rall 2000; Saunders et al. 2000), although the mechanism by which the ApoE locus is linked to these diseases remains unsolved. Among alleles, the $\varepsilon 4$ has been found to be associated with higher plasma cholesterol levels and is related to the risk of lipid disorder and coronary heart disease (Wilson et al. 1994; Contois et al. 1996).

ApoE is an important mediator of the transport and hepatic metabolic clearance of circulating cholesterol. When the ApoE polypeptide is dysfunctional or absent, circulating levels of cholesterol accumulate (Piedrahita et al. 1992; Plump et al. 1992). In addition to its role in lipid metabolism, ApoE has been assigned also to other functions, including anti-inflammatory role (Guo et al. 2004), a common pathological process for both cardiovascular and Alzheimer's diseases. The isoform $\varepsilon 4$ correlates with the increased risk for atherosclerosis (Corder et al. 1993) and amyloid plaque formation (Hofman et al. 1997). Moreover, elevated cholesterol uptake increases amyloid plaque formation or amyloid deposition (Sparks et al. 1994; Bales et al. 1997). Association of the ApoE alleles with essential hypertension (Liu et al. 2003), coronary heart disease (Viitanen et al. 2001) and myocardial infarction (Incalcaterra et al. 2004; Ranjith et al. 2004) has also been reported.

In our report we examined 50 patients with advanced congestive dilated cardiomyopathy (DCM) from Slovakia and correlated results with clinical variables of the disease. Here we present our data obtained by CVD StripAssay. This assay enables comfortable and highly time- and work-saving approach that could help to screen sufficient number of patients/probands and create database large enough to determine association of allele with risk of certain disease.

Materials and Methods

Patients

Fifty patients (43 males) of the age of 49.7±3.1 years sufferring from advanced DCM were included in the study. All patients were from Caucasian population of Slovakia. Patients were admitted for evaluation as potential heart transplant candidates. Diagnosis of DCM was based on echocardiography. Coronary angiogram was normal in all patients. An average left ventricular ejection fraction was $24.8 \pm 7.0\,\%$ and mean functional class according NYHA (New York Heart Association) classification was 2.9 ± 0.6.

Systolic and diastolic functions were evaluated using echocardiography. Systolic function was represented by left ventricular ejection fraction and was calculated from 2-D mode using Simpson rule. For evaluation of the diastolic function, relation of the peak early mitral valve flow velocity (E) to the peak velocity after left atrial systole (A) – (E/A) and deceleration time (DT) was measured.

Left ventricular end systolic and end diastolic diameters (LVESDi and LVEDDi) were measured and indexed on body surface area. To quantify functional capacity, maximal oxygen consumption (VO2max) was measured.

DNA extraction and purification

Blood samples were collected into EDTA and stored at −20 ℃ until DNA extraction. The Ethic Committee of the Slovak Institute of Cardiovascular Diseases, Bratislava, Slovakia, approved presented study and verbal consent was obtained from the participants prior to blood sample collection. DNA was extracted using proteinase K and sodium-dodecyl-sulphate digestion at 37 ℃ overnight and purified using phenol-chloroform extraction. The extracted DNA was precipitated using 1/10 volume of 3 mol·l^{-1} sodium acetate (pH 5.2) and 2 volumes of cold 96 % ethanol. The purity and concentration of DNA was measured spectrophotometrically (GeneQuant Pro, Amersham Biosciences). Purified DNA was solubilized in TE buffer (0.01 mol·l^{-1} Tris, pH 8.0; 0.001 mol·l^{-1} EDTA, pH 7.5) and stored at −20 ℃ until used for DNA amplification.

CVD StripAssay

For identification of gene polymorphisms associated with cardiovascular diseases (CVD) we used new CVD StripAssay (ViennaLab, Labordiagnostika, GmbH). The principle of the method is based on polymerase chain reaction (PCR) and subsequent reverse hybridization. The procedure consists of 2 steps: 1. PCR amplification with biotinylated primer pairs and 2. hybridization of amplified products with test strips carrying allele-specific oligonucleotide probes immobilized in parallel lines. Bound biotinyled sequences are detected by streptavidine-alkaline phosphatase and color substrate.

We prepared two PCR reactions – A (amplification mix A) and B (amplification mix B) that differed in primer pairs, and 100 ng of DNA for each. PCR reactions A and B were amplified at same conditions: denaturation at 94 ℃ for 2 min and 35 cycles of 94 ℃/15 s, 58 ℃/30 s, 72 ℃/30 s with final polymerization at 72 ℃ for 3 min (thermal cycler Biometra). PCR products from reaction A and B were mixed together with hybridization buffer, incubated for 5 min at room temperature and hybridized to the detection test strip. Hybridization was accomplished at 45 ℃. After series of stringent washes (according to the protocol of provider) the reaction was detected by color development directly on test strip. Results were evaluated from test strips using ApoE genotypes scale included in the kit.

Besides ApoE, strips can detect also following mutations: FV G1691A (Leiden), FV H1299R (R2), Prothrombin G20210A, Factor XIII V34L, β-Fibrinogen-455 G-A, PAI-1 4G/5G, GPIIIa L33P (HPA-1), MTHFR C677T, MTHFR A1298C, ACE I/D, ApoB R3500Q.

Results

Our data obtained from the selected group of 50 patients sufferring from the advanced congestive DCM and analyzed by CVD StrippAssay (Figure 1) showed that the most frequent ApoE genotypes were $\varepsilon 2/4$ (58 %) and $\varepsilon 3/4$ (22 %). There was no patient carrying both $\varepsilon 4$ alleles ($\varepsilon 4/4 - 0\,\%$). Distribution of the ApoE geno-

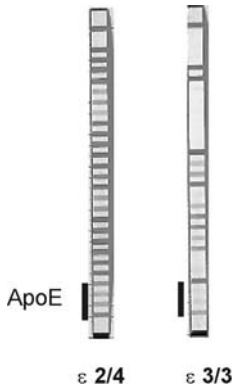

Figure 1. Representative figure of test strips (CVD StripAssay) with indicated ApoE evaluation.

types was correlated by Hardy Weinberg equilibrium ($p > 0.05$ by chi-square test). Frequency of the $\varepsilon 4$ alelle in patients with DCM reached 40 %, while $\varepsilon 2$ was 33 % and $\varepsilon 3$ was 27 %. Distribution of individual ApoE genotypes in DCM patients is shown in Figure 2. Our results on patients with the end-stage DCM were compared with two Caucasian control subject groups tested by Wong and collegues (2004) and Hubáček et al. (2003) (Table 1). Czech population screened by Hubáček et al. (2003) is identical to Slovak population. Both control groups were almost identical in data provided (two selected control groups represent majority of results published on healthy Caucasian population). Comparison of the ApoE polymorphism in patients with the end-stage DCM and healthy controls is shown in Table 1. Frequency of the $\varepsilon 2/4$ genotype in DCM was 27–34-times higher compared to controls and frequency of the $\varepsilon 4$ allelle increased almost 3-times in the group of patients compared to controls, which is strongly suggesting the involvement of $\varepsilon 4$ alelle in DCM.

As shown in Table 2, in DCM patients with individual ApoE genotypes, no significant differences were found in selected variables of the left ventricular morphology (LVESDi, LVEDDi), left ventricular systolic function (EF), left ventricular diastolic function (E/A, DT) or functional capacity (VO2max). However, an apparent trend of reduction of the time interval between the point of diagnosis of DCM to the advanced stage of disease in patients with $\varepsilon 2/4$ genotype was observed.

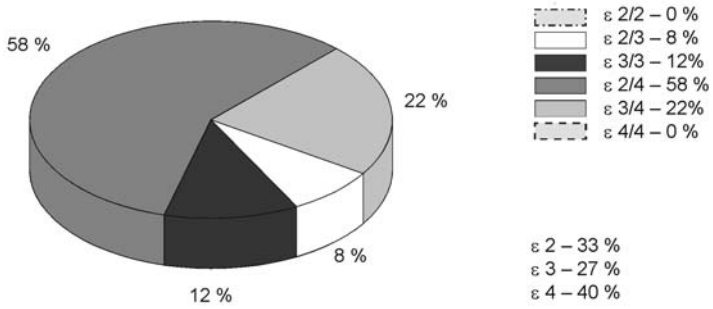

Figure 2. The percentual representation of individual ApoE genotypes and the frequencies of individual ApoE alleles in tested group of patients with DCM.

Table 1. Comparison of genotypes and allele frequencies in healthy subjects and subjects with DCM

ApoE	DCM (%) $n = 50$	Healthy (%) $n = 166$ (Hubáček et al. 2003)	Healthy (%) $n = 3003$ (Wong et al. 2004)
$\varepsilon 2/2$	0.0	0.6	0.9
$\varepsilon 2/3$	8.0	9.0	13.0
$\varepsilon 3/3$	12.0	66.9	59.3
$\varepsilon 2/4$	58.0	1.2	2.1
$\varepsilon 3/4$	22.0	18.1	22.5
$\varepsilon 4/4$	0.0	4.2	2.2
$\varepsilon 2$	33.0	7.1	8.5
$\varepsilon 3$	27.0	82.0	76.8
$\varepsilon 4$	40.0	10.9	14.7

DCM, dilated cardiomyopathy; ApoE, apolipoprotein E.

Table 2. The clinical characteristics of diferent ApoE genotypic groups (results are presented as mean ± S.E.M.).

	$\varepsilon 2/3$ ($n = 4$)	$\varepsilon 2/4$ ($n = 29$)	$\varepsilon 3/3$ ($n = 6$)	$\varepsilon 3/4$ ($n = 11$)
Age (years)	46.4 ± 3.7	48.0 ± 2.0	51.3 ± 3.6	53.2 ± 3.2
Sex (M/F)	4/0	24/5	5/1	10/1
LVESDi (mm/m^2)	34.9 ± 2.2	29.7 ± 1.4	29.0 ± 4.9	29.8 ± 1.9
LVEDDi (mm/m^2)	38.6 ± 1.9	33.7 ± 1.1	33.7 ± 4.6	36.8 ± 4.2
EF (%)	24.8 ± 2.5	24.1 ± 1.7	24.5 ± 2.2	24.6 ± 2.0
E/A	1.2 ± 0.3	1.9 ± 0.3	2.4 ± 0.5	1.9 ± 0.4
DT (ms)	180.0 ± 33.9	136.5 ± 12.2	136.0 ± 22.2	146.3 ± 17.4
VO2max	23.7 ± 2.7	20.1 ± 1.3	18.5 ± 2.6	18.7 ± 2.1
Time from D to A (months)	60.0 ± 20.2	52.0 ± 10.7	56.3 ± 17.4	80.4 ± 40.1

LVESDi, left ventricular end systolic diameter; LVEDDi, left ventricular end diastolic diameter; EF, left ventricular ejection fraction; E/A, relation of the peak early mitral valve flow velocity (E) to the peak velocity after left atrial systole (A); DT, deceleration time; VO2max, maximum amount of oxygen in milliliters used in 1 min *per* kg of body weight; Time from D to A, time interval from establishment of diagnosis of DCM to admittion to the hospital for decision making for heart transplantation.

Discussion

Molecular screening of the ApoE alleles and genotypes using DNA amplification was carried out by CVD StrippAssay. Test strips carrying allele-specific oligonucleotide probe, immobilized in parallel lines, allow hybridization of PCR amplification products and immediate recognition of ApoE alleles and genotypes that

could be detected after a colour development reaction directly on the test strip. For one patient, one strip is needed and besides ApoE polymorphism it recognizes 11 other gene polymorphisms. Thus, CVD StrippAssay system provides an alternative method for fast and effective screening of ApoE genotypes.

We observed high incidence of the $\varepsilon 4$ allele (40 %) in patients with advanced congestive DCM. Our results were compared with ApoE screening results of healthy Caucasian population published by others (Viitanen et al. 2001; Hubáček et al. 2003; Wong et al. 2004). In healthy Caucasian population, genotype $\varepsilon 2/4$ is quite rare – frequency 1–2 % (Hallman et al. 1991; Kao et al. 1995; Muros and Rodriguez-Ferrer 1996). We found much higher frequency of this genotype in patiens with the end-stage DCM (58 %, Figure 2). High frequency of the $\varepsilon 2/4$ genotype was followed by $\varepsilon 3/4$ genotype with frequency 22 %. $\varepsilon 4$ allele was found to be the most frequent (40 %), followed by $\varepsilon 2$ allele with frequency 33 % and $\varepsilon 3$ allele with frequency 27 % in examined group.

ApoE may be an important gene accounting for the genetic variation of plasma low density lipoprotein C concentrations in a population with CVD. Also, the ApoE $\varepsilon 4$ allele is a significant risk factor for coronary heart disease (Song et al. 2004). Our findings suggest that $\varepsilon 4$ allele on ApoE might be associated also with DCM. In order to clarify pathophysiological consequences of this findings in the context of advanced DCM, we correlated individual genotypes with the selected clinical parameters, such as LVESDi, LVEDDi, EF, E/A, DT, VO2max. No significant changes among individual genotypes were observed in any of these parameters. This observation is raising doubts about physiological/pathophysiological significance of the association of $\varepsilon 4$ allele with DCM. Since the "proinflammatory" (Lynch et al. 2003) and "proapoptotic" (DeKroon et al. 2003) effect of $\varepsilon 4$ has been reported, the possible involvement of this effect in progression of DCM might be speculated. Activation of inflammatory response and apoptosis is considered as motor of heart failure progression (Anker and von Haehling 2004; Saraste et al. 1999) and from these aspects, $\varepsilon 4$ allele encoding for ApoE $\varepsilon 4$ may play a role in this complex phenomenon. Nevertheless, more clinical parameters need to be correlated with occurrence of the $\varepsilon 4$ allele to determine the physiological importance.

Acknowledgements. This work was supported by Genomika SP 51/0280800/0280802 and APVT 51–027–404.

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Final version accepted: January 26, 2006