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An Improved RSP Method to Detect *HpaI* Polymorphism in the Apolipoprotein C-I Gene Promoter

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Abstract

Background: An apolipoprotein C I gene promoter polymorphism (CGTT insertion at position -317) is associated with familial dysbetalipoproteinemia, cardiovascular diseases, and Alzheimer's disease. Restriction site polymorphism (RSP) assays were previously established to detect this polymorphism. In this study, we introduce an improved RSP assay to detect this polymorphism.

Methods: This method included newly designed primers and only one round of PCR amplification which yields one short and specific APOC I fragment followed by *HpaI* digestion. Briefly, It consists of three steps: 1) one round of PCR amplification of DNA sample, 2) *HpaI* enzyme digestion of PCR products, and 3) electrophoresis on an agarose gel to visualize the genotypes. This improved RSP method was applied to genotype 92 human samples collected from The Johns Hopkins Hospital.

Results: The observed allele frequencies for H1 and H2 from 92 genotyped human subjects were 0.707 and 0.293 respectively. The H2 allele frequency in the black subjects (0.350) was significantly ($p = 0.024$) higher than that in the white subjects (0.177). This method was more economical and convenient than the methods previously reported to detect this mutation in the APOC I gene.

Conclusions: This assay will be readily applied to screen large sample sizes for population studies in a simple and cost effective way.

Background

Coronary artery disease (CAD) is the leading cause of death in the western world. High concentrations of blood cholesterol or triglycerides and low high density lipoprotein (HDL) are among independent risk factors to CAD [1]. DNA sequence variations at several genes (APOE, APOB and APOC3) have been implicated as genetic determinants of plasma lipid levels [2]. APOC1 is a constituent of triglyceride-rich lipoproteins and HDL. The APOC1 gene lies in a gene cluster containing APOE and APOC2 on chromosome 19. The presence of the *HpaI* restriction site (CGTT insertion at position -317) in the APOC1 pro-

motor has a significant effect on APOC1 transcription [3,4]. In one population-based genetic association study, this polymorphism was associated with lipoprotein metabolism disorder [5]. Recently the association of APOC1 gene polymorphism with both late-onset Alzheimer's disease [6] and age-associated memory impairment [7] has been reported. Protection from obesity and insulin resistance also has been found in mice over expressing human APOC1 [8].

Nillesen et al [9] first designed one step PCR method followed by *HpaI* digestion genotyped this polymorphism.

However, 22 to 24 nucleotides out of 26 nucleotides in the 5'-upstream primer (5'-TTTGAGCTCGGCTCTTGAGACAGGAA-3') were identical to at least 9 different sequences from 9 different chromosomes, it yielded some non-specifically amplified fragments by PCR, thus made it difficult for the allele-calling. Later a two-step nested PCR followed by *HpaI* digestion was developed to detect this polymorphism [4]. However, these two rounds of PCR amplification consume more reagents and need more time and labor to perform, thus exacting higher cost compared to one round of PCR amplification as reported here. In this study, we designed a pair of new oligoes using the computer to amplify a shorter and a unique fragment (195 bp) of APOC1 gene promoter. Only one round of PCR is needed to efficiently generate the enough and specific PCR product for the genotyping. Our new protocol consists of only three steps: one PCR amplification, *HpaI* digestion and agarose gel electrophoresis. It's much simpler, less costly and more suitable for clinical diagnostic tests. This improved RSP method was applied to genotype 92 human samples collected from the Johns Hopkins hospital. Two alleles, H1 (APOC-I *HpaI*-negative) and H2 (APOC-I *HpaI*-positive), were typed. Our genotype results, that the H1-allele frequency is 0.707 and the H2-allele frequency is 0.293, are in good agreement with the findings of Xu *et al* (1999)[4] and Hubacek *et al* [2]. This new RSP assay should facilitate the screening of this mutation in large populations and contribute to the understanding of the molecular mechanism underlying the association of this mutation with a number of diseased conditions.

Methods

Subjects

Umbilical cord blood was obtained from 92 subjects consisting of 53 males and 39 females, who were born at The Johns Hopkins Hospital. Among them, 30 were white Caucasians, 60 were African Americans, and 2 were other races. The samples were collected for The Johns Hopkins Study on the Fetal Origin of Atherosclerosis directed by Dr. Peter O Kwiterovich. Informed consents were obtained from the parents of each subject.

DNA preparation

DNA was isolated from cord white blood cells by using Easy-DNA Kit (Cat. No. K1800-01, Invitrogene, Carlsband, CA) by following the manufacture's instruction. The amount of DNA in each sample was determined by measuring the optical density at 260-nm wavelength using a spectrophotometer (DU-640, Beckman Instrument, Inc., Fullerton, CA). DNA samples were stored at -80°C.

Genotyping

One third of the PCR product was digested with 5 U *HpaI*

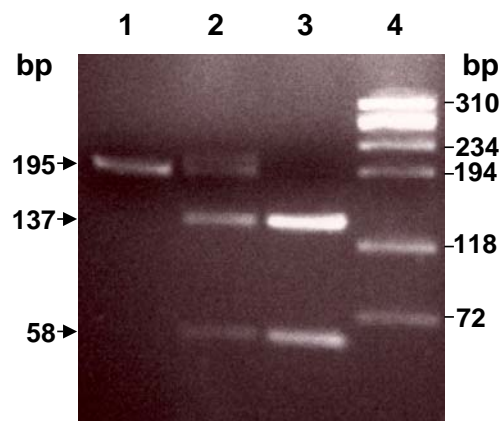


Figure 1
Gel patterns of *HpaI* polymorphism. Lane 1 shows the homozygous H1/H1 genotype, lane 2 is heterozygous H1/H2 and lane 3 is homozygous H2/H2. Lane 4 is a 72- to 1,353-bp DNA ladder.

Design and synthesis of PCR primers

The primers were designed using Primer 3 software (Primer 3, Ver.3, Whitehead Institute/MIT Center for Genome Research) based on the published human apoC1 genomic DNA sequence flanking the promoter region (GenBank accession: M20902). The primer sequences are as follows: 5' sense, 5'-ATC GAT CAC GAC CCT CTC-3', which corresponds to the nucleotide positions 38 to 55 (at -455 relative to the APOC1 gene start site); 3' antisense, 5'-TCC CCC ACT CAG AAT GTA GC-3', which corresponds to the nucleotide positions 233 to 214 (at -260 relative to the APOC1 gene start site). The 5' sense primer was the shortened version of the upstream internal primer published by Xu *et al*. [4]. The primers were synthesized on an Applied Biosystems model 381A synthesizer in the DNA Synthesis and Analysis Core Facility, The Johns Hopkins University School of Medicine.

PCR amplification

PCR was carried out in 25 µl total volume containing 50 ng genomic DNA, 5 pmol of each primer, 0.2 mM of each dATP, dTTP, dCTP and dGTP, 1.5 mM MgCl₂, and 1.25 U of Taq DNA polymerase (Cat. No. N808-0160, Perkin-Elmer, Foster City, CA). Thermal cycling was done in a Perkin-Elmer GeneAmp PCR System 9600 Thermal Cycler with an initial 2 min denaturation at 94°C followed by 35 cycles of denaturing at 94°C for 30 sec., annealing at 60°C for 30 sec., extending at 72°C for 1 min, and a final extension of 5 min at 72°C.

(Cat. No. 105S, New England Biolabs, Inc. Beverly, MA) for one and half hours in a 37°C water bath, followed by

Table 1: APOC1 Gene Allele Frequency in 92 Human Subjects

	N ^a	H1-allele	H2-allele	Total alleles ^b
Total	92	130 (0.707)	54 (0.293)	184 (1.000)
White	30	51 (0.823)	11 (0.177)	62 (1.000)
Black	60	78 (0.650)	42 (0.350)	120 (1.000)
P ^c			= 0.024	

^a In the study group, 53 males, 39 females; 30 white Caucasians, 60 African Americans, 2 are other races whose allele frequencies were not calculated separately. ^b There are 48 people with the H1/H1 genotype: 26 are the black, 21 are the white and 1 is other race; 34 with H1/H2: 26 are the black, 7 are the white and 1 is other race; and 10 with H2/H2: 8 are the black and 2 are the white. The observed heterozygosity was 0.370 (34/92). ^c Black vs White.

separation on a 2% agarose gel electrophoresis for 1 to 1.5 hours at constant voltage of 110 V. The gels were stained by 0.5 ug/ml of ethidium bromide. The image was captured on the thermal paper using the Eagle Eye II Still Video System (Stratagene, La Jolla, CA). The typical gel patterns of *HpaI* polymorphism are shown in Fig. 1. Lane 1 shows the homozygous H1/H1 genotype, lane 2 is the heterozygous H1/H2, and lane 3 is the homozygous H2/H2. Lane 4 is X174 DNA-*haeIII* Digest DNA ladder (Cat. No. 302-6S, Beverly, MA).

Statistical analysis

Statistical analysis with the Chi-square test was done using SigmaStat (ver2.03, SPSS, Inc., Chicago, IL, USA). Yates correction for continuity was used in calculating the test. P < 0.05 was set to be significantly different.

Results

***HpaI* polymorphism in APOC1 gene promoter**

An example of the gel pattern of each genotype is presented in Fig. 1. In the homozygous genotype H1/H1, where *HpaI* recognition sites are absent, no digestion of the PCR products occurs, and only one 195 bp band appears (lane 1). In homozygous genotype H2/H2, where *HpaI* recognition sites are present, the PCR products were completely digested into two small fragments: 137 and 58 bp, respectively (lane 3). In heterozygous genotype H1/H2, where the *HpaI* recognition site of one allele (H1) is absent and the *HpaI* recognition site of another allele (H2) is present, one allele was not digested and another allele was completely digested, so that all three possible fragments appear: 195, 137 and 58 bp, respectively (lane 2). This new protocol produces clear-cut results for genotypes.

Mutation frequency in the study group

92 USA subjects were genotyped (Table 1). The H1-allele frequency is 0.707 and the H2-allele frequency is 0.293. The observed heterozygosity was 0.370. The H1-allele frequency in the white subjects is 0.823 and the H2-allele frequency is 0.177. The H1-allele frequency in the black is

0.650 and the H2-allele frequency is 0.350. The H2 allele frequency in the black subjects (0.350) was significantly (p = 0.024) higher than that in the white subjects (0.177).

Discussion

An *HpaI* restriction fragment length polymorphism (RFLP) in the APOE-C1-C2 gene cluster on chromosome 19 was found to be strongly associated with familial dysbetalipoproteinemia (type III hyperlipoproteinemia). Smit *et al* first localized the polymorphic *HpaI* site of APOC1 gene promoter by using Southern blot analysis [10]. The exact localization was determined by building a restriction map of the APOE-C1-C2 gene cluster for the enzymes *EcoRI*, *BamHI*, *HindIII* and *SallI*, then subcloning and sequencing the *SacI* fragment, which contains the *HpaI* site. The *HpaI* site variably present at -317 relative to the APOC1 gene is produced by a 4-bp CGTT insertion [3]. This Southern blot method was valuable for the identification of the *HpaI* polymorphism, but is not convenient for a routine diagnostic purpose. One step PCR method designed by Nillesen *et al* [9] in 1990 for genotyping APOC1 *HpaI* polymorphism was not a convenient method because the 5'-upstream primer in its PCR reaction was highly homologous to several other gene sequences and generated some non-specifically amplified fragments, thus made it difficult for the allele-calling. Later a two-step nested PCR developed by Xu *et al* [4] exacted more reagents, more time and labor to perform. In this study, we designed a pair of oligoes to amplify a shorter and a unique fragment (195 bp) of APOC1 gene promoter. Only one round of PCR is needed to efficiently generate the enough and specific PCR product for the genotyping. Our new protocol consists of only three steps: one PCR amplification, *HpaI* digestion and agarose gel electrophoresis. It's much simpler, less costly and more suitable for clinical diagnostic tests. This type of RSP methods has been previously reported by our lab to detect other restriction site polymorphisms in genes related to cardiovascular and inflammation diseases [11-13].

We have applied this new RSP to detect the APOC1 *Hpa*I polymorphism in 92 human subjects (Table 1) derived from The Johns Hopkins Study on the Fetal Origin of Atherosclerosis. Our findings that the H1-allele frequency is 0.707 and the H2-allele frequency is 0.293 are consistent with the previous reports by Xu *et al*[4] who reported that in their population the allele frequencies for H1- and H2-allele were 0.72 and 0.27, respectively, and by Hubacek *et al*[2] who found that the allele frequencies of their population for H1- and H2-allele were 0.75 and 0.25, respectively. We found that the H2 allele frequency in the black subjects (0.350) was significantly ($p = 0.024$) higher than that in the white subjects (0.177). Xu *et al*[4] also reported that the H2 allele frequency in the black subjects (0.31) was higher than that in the white subjects (0.24). The larger and more population studies are needed to validate these observations.

Conclusions

Even until now, high throughput genotyping cores, which are in most cases dependent on the expensive equipments, are only available in major institutions and commercial companies. This new RSP method is readily useful in any individual lab. It will facilitate the screening of APOC1 gene promoter *Hpa*I polymorphism in large and more populations and contribute to the understanding of the molecular mechanism underlying the association of this mutation with a number of diseased conditions.

Competing interests

None declared.

Authors' contributions

LG carried out the PCR and *Hpa*I digestion, and drafted the manuscript. CT and TL helped agarose electrophoresis experiments and data collection. SQY designed the RSP method and directed whole project.

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