EVALUATION OF THE REAL-TIME PCR ANALYSIS OF APOLIPOPROTEIN E GENE CODON 112 AND CODON 158 POLYMORPHISMS

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APOLİPOPROTEİN E GENİ KODON 112 VE 158 POLİMORFİZMLERİNİN REAL TIME PCR ANALİZLERİNİN DEĞERLENDİRİLMESİ

Özet: *ApoE genotiplendirilmesi Alzheimer hastalığı ve koroner arter hastalığı riski altında olan apo E e4 allel taşıyıcılarının belirlenmesinde son derece yararlıdır. Genotiplendirmede en sık kullanılan yöntemler apoE geninde belirli bir fragmanın PCR ile çoğaltılması ve ardından restriksiyon enzimleri ile muamele edilerek jelde gösterilmesi esasına dayanır. Son çalışmalarda Rapid cycle kullanılarak lightCyclerda gerçekleştirilen Real Time Fluorescence PCR tekniğinin diğer konvansiyonel yöntemlerden üstün olduğu öne sürülmektedir.*

Bu çalışmada bu yeni teknik ile konvansiyonel bir yöntem olan restriksiyon enzim analizini100 olguda karşılaştırıldı.

Çalışmamızın sonuçlarına gore yeni geliştirilmiş olan Real Time PCR tekniğinin restriksiyon enzim analizine kıyasla daha hızlı, tekrarlanabilir, doğru ve güvenilir bir yöntem olduğu kanısına varıldı.

Anahtar Kelimeler: *ApoE, genotip, RFLP, PCR, yöntem karşılaştırma*

Summary: *Apo E genotyping is helpful in the identification of subjects carrying e4 allele who were at risk for Alzheimer's disease and coronary artery disease. The most common apoE genotyping methods involve the restriction enzyme digestion of PCR- amplified fragment of apoE gene. In recent studies it is claimed that the new developed method that uses rapid cycle PCR and Real Time fluorescence PCR with light cycler (Roche diagnostics) is superior than the conventional techniques.*

In this study we compared the new technique and the conventional method (restriction enzyme analysis) in 100 subjects.

According to the results of our study, this new Real Time PCR procedure has many advantages superior to restriction enzyme analysis. This new technology is fast, reproducible, reliable and accurate method than RFLP analysis.

Key Words: *ApoE, genotype, RFLP, PCR, method comparison*

INTRODUCTION

Apolipoprotein E (Apo E) is a 299 amino acid plasma protein that plays an important role in the metabolism of atherogenic lipoproteins (1,2). The Apo E gene locus, located on chromosome 19 is polymorphic. Three alleles called e2, e3 and e4 are inherited in a co-dominant fashion and lead to six genotypes (three homozygous e2/e2, e3/e3 and e4/e4 and three heterozygous e2/e3, e2/e4 and e3/e4) (3-5). The common alleles are the results of the variations within codons 112 and 158 of apo E gene. e2, e3 and e4 alleles contain TGC/TGC (Cys/Cys), TGC/CGC (Cys/Arg), and CGC/CGC (Arg/Arg), at these two sites respectively (6). The frequencies of e2, e3 and e4 alleles are relatively constant in white populations (85, 78% and 14%) and the most common allele in all populations studied to date is e3 (7). The polymorphism of apoE locus is a good indicator of plasma lipid status and closely related disorders (8,9). The e4 allele is asso-

ciated with higher plasma low-density lipoprotein (LDL) - cholesterol levels and has been found to be a risk factor for coronary artery disease and Alzheimer's disease (AD) (10,11). Whereas e2 allele seems to be protective against AD and is associated with lower LDL-cholesterol and longevity (12). Thus, Apo E genotyping draw the attention of researchers.

Conventional methods used for Apo E genotyping were mostly based on the restriction enzyme digestion of the PCR products and gel visualization (13,14). Also PCR methods such as allele specific oligonucletide hybridization, the amplification refractory mutation system, oligonucleotide ligation, heteroduplex analysis and single strand conformational polymorphism are used (15 -18). These kinds of methods are time-consuming and require additional procedures such as enzyme digestion, staining and electrophoresis after PCR amplification and leads to unequivocal genotyping. Recently, a new developed Apo E genotyping technique that uses rapid cycle PCR and Real-Time fluorescence PCR with LightCycler (Roche Diagnostics) has been introduced (19, 20). In recent reports, it is claimed that the new technique has many advantages superior to those conventional gel- based techniques. In this study, we investigated practicability and reliability of Real-Time fluorescence PCR method in a clinical routine setting.

MATERIALS AND METHODS

For the method comparison venous blood samples of 100 donors, who were admitted for cardiac catheterization were analyzed by PCR-RFLP and Real-Time PCR techniques. Donors gave their informed consent and the study was approved by the Ethics Committee of the Çukurova University, Adana. The blood samples of the subjects were obtained into EDTA. Genomic DNA from the leukocyte was isolated according to the method of Miller (21).

Standard genotyping protocol and LightCycler assisted Real-Time fluorescence PCR protocols of Apo E genotyping was performed on by using LightCycler Apo E Codon 112 and 158 mutation kit (Roche, Mannheim, Germany). The kit contained three reagent vials. In vial 1 mutation detection mixture with the sequence-specific amplification primers, the fluorophore-labeled hybridization probes [3'- Fluorescein labeled two anchor probes (for codon 112 and 158), 5'- Red 640 labeled (for codon 112) mutation probe and 5'-Red 705 labeled (for codon 158) mutation probe]. Vial 2 contains a ten fold concentrated mixture of taq polymerase, buffer, and dNTPs. Vial 3 consist of heterozygous control templates. The 18 microliter reaction mixture prepared according to manufacturers appointments and 2 microliter genomic DNA and control templates were loaded in to the precooled lightCycler capillaries. Quality control is performed by use of PCR grade H2O as negative control. Then sealed capillaries were centrifuged in microcentrifuge and stated in LightCycler rotor. The PCR was initiated with a denaturation by first heating for 60 seconds at 95°C. Forty-five cycles of denaturation for 0 seconds at 95°C, annealing for 10 seconds at 60°C and primer extension for 10 seconds at 72°C were applied for amplification. Fluorescence was measured at the end of the annealing period of each cycle. The final melting curve was recorded during the period of cooling to 42 °C for 4 minutes for maximum hybridization and then heating slowly at 0.1 C/s to 80 °C with the LightCycler Instruments. The fluorescence emitted by LC-Red 640 and LC-Red 705 was measured continuously in channels to entry during the slow temperature ramp to monitor the dissociation of the fluorophore-labeled detection probes from the complementary single- stranded DNA. These signals were then converted to melting peaks.

For standard PCR procedures the apo E gene polymorphism were investigated by RFLP (CfoI) according to the method of Wenham et al (22). The template DNA (0.4 mg per sample) was amplified using the primers11 5'-TCC AAG GAG CTG CAG GCG GCG CA-3' and 5'-ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A-3'. These primers (25 pmol of each) were added to a mixture containing 0.2

mmol/L each of the dNTPs 5ml of 10X Cetus buffer (pH 8,3), 10 ml of DMSO and 1.5 units of Taq DNA polymerase (Perkin Elmer Cetus). The PCR was initiated with a denaturation by first heating the samples for five minutes at 95°C. Forty cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 65°C and primer extension for 90 seconds at 70°C was applied for amplification. PCR products of Apo E locus were digested with CfoI restriction enzyme at 37°C for a night. The samples were examined by agarose gel electrophoresis (3% agarose) in 1XTBE buffer (0.1 M Tris, 0.1 M Boric acid, 2mM EDTA pH 8.25) at 150 V for 30 minutes and visualized under UV after ethidium bromide staining.

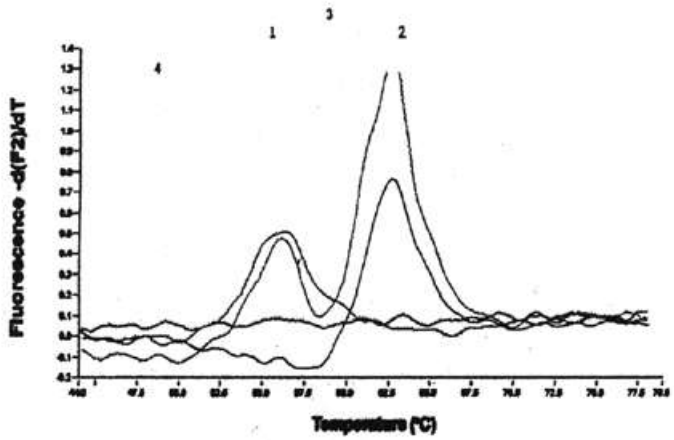

Figure I.

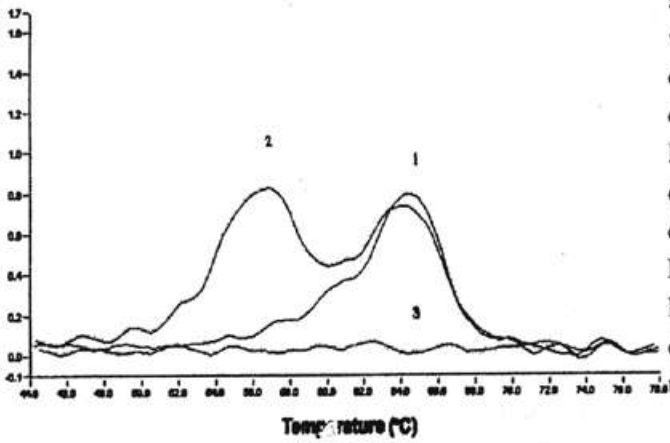

Figure II.

RESULTS

The distinction of apo E genotypes was obtained by an online melting curve analysis. The differential analysis of melting curves (-dF/ dT) for codons 112 and 158 are shown in figures 1 and 2. Figure 1 gives, the melting peaks of the samples homozygous CGC, homozygous TGC and heterozygous CGC/TGC for codon 112. In figure 2, the melting peaks of codon 158 homozygous CGC and heterozygous CGC/TGC samples are presented.

Therefore, the melting curve of e 3/3 genotype showed single peaks at 56.0°C and 64.5°C and e 4/4 genotype showed single peaks at 62.5°C and 64.5°C in codons 112 and 158 respectively.The heterozygous samples such as e 2/4 and e 3/4 contained both targets and thus generated both peaks at 56.0°C and 62.5°C in codon 112 and 66.0°C and 56.5°C in codon 158 respectively.

DISCUSSION

To evaluate the validity and reliability of this protocol we have compared the data obtained from fluorescence genotyping with results from genotyping using a previously published allele-specific restriction enzyme analysis (RFLP). In contrast to other studies which the methods were found to be in full concordance, out of 100 DNA sample we found a deviation of 9% between two genotyping methods (nine of the samples found as e2/e2 carriers with RFLP analyses were determined as e2/e3 by the real time PCR procedure) (19,20). The remaining 91% were all in full concordance between two methods. The deviated PCR- products were subjected to a single strand sequencing reaction. Sequencing was carried out according to the dye terminator method on an automated DNA sequencer (ABI PRISM 310 genetic analyzer, Perkin Elmer, Weiterstaat, Germany). The sequencing analyses results were in full concordance with the real time PCR.

The sequencing analyses revealed that the errors observed errors are probably due to the incomplete

digestion of the amplicons in RFLP protocol. Because it is well known that RFLP need PCR conditions of sufficient specificity to produce a clean amplification product that can be enzymatically digested and analyzed by electrophoresis and this procedure may be complicated by incomplete digestion of the amplicons. To date, Apo E genotyping by several PCR- based methods that has been time consuming due to several steps that needed to be sequentially performed, including PCR, restriction enzyme digestions, gel electrophoresis and documentation of results have been used. This present LightCycler assisted approach to amplify and simultaneously detect and document. Apo E polymorphisms online combines simple sample processing and rapid analysis. This approach neither requires post PCR restriction enzyme digestion nor gel electrophoresis, nor documentation of the electrophoretically separated DNA fragments. A major advantage is the rapid thermal cycling for PCR and online, real-time detection of the reaction kinetics. An additional advantage of this approach is that contaminating, non-specific PCR products do not affect the results. The genotyping of the 100 samples on the Light-Cycler was completed within 2 hours, whereas the RFLP protocol was performed within several hours including the enzyme digestion period and required several manual sample-processing steps.

In summary, our data lead us to conclude that the quality, reliability and accuracy and speed of LightCycler assisted analysis is superior and preferable to that of PCR restriction enzyme and gel based methods for Apo E genotyping.

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