Determination of the Apo E Genotype Using LightCycler™ Apo E Mutation Detection Kit

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Apolipoprotein E (Apo E) is found to be genetically polymorphic. There are 3 common alleles designated as E2, E3 and E4. Polymorphism of the Apo E DNA is associated with the risk increase of many diseases such as dyslipidemia, cardiovascular diseases, Alzheimer's diseases, etc. Hence, the interest in genotyping of the Apo E is now rising for the purpose of pre-diagnosis. The aim of this study was to characterize the Apo E DNA polymorphism using LightCycler Apo E mutation detection kit (Roche) in the Thai normal healthy subjects. The genomic DNA was extracted from the blood of 133 normal healthy subjects using DNA extraction kit (Roche). Exon 4 of the Apo E gene was amplified by the extracted genomic DNA using the real-time PCR. The simultaneous analysis of the two polymorphic codons (Codons 112 and 158) in a single reaction was conducted by using the two reporter dyes with the different excitation and emission spectrum LightCycler-Red 640 (LC-Red 640) and LC-Red 705 followed by the color compensation software to correct the temperature-dependent crossover among the emission spectra of the dyes. The different genotypes were then determined by performing the melting curve analysis in the two different channels. The results showed that the allele frequencies of the Apo E2, E3 and E4 were 0.26, 0.63 and 0.11 respectively and the genotype frequencies of the E2/3, E2/4, E3/3,E3/4 and E4/4 were 47.37, 5.26, 32.33, 14.28 and 0.75% respectively. This study found that E2/3 was the most common genotype of the Apo E. In conclusion, the LightCycler (LC) allelic discrimination method to genotype the Apo E is rapid, simple, reliable and strongly recommended for being successful diagnostic tests in the future.

Keywords: *Apolipoproteins E, DNA, Mutation, Polymorphism, Genetic, Reagent kits, Diagnostic*

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Apo E, an apolipoprotein involved in lipid metabolism, acceptably is genetically polymorphic[1]. There are 3 common alleles designated as E2, E3 and E4[2,3]. The molecular bases of the Apo E polymorphism are cysteine (TGC) and arginine (CGC) interchanges at the 112th and 158th residues of the gene that code the mature Apo E polypeptide of 299 amino acids[2,3]. The polymorphisms of the Apo E DNA are related to the risk increase of many diseases[4,9]. The Apo E4 allele (arg 112/158) indicates a higher risk of the coronary heart diseases and also constitutes a major risk of Alzheimer's disease[6,9] while the Apo E 2 allele (cys112/158) seems to have a protective effect against the Alzheimer's disease[6].

The three common apo E alleles lead to six common phenotypes originally disclosed by isoelectric focusing and immunoblotting[10]. However, with this methodology, the different degrees of protein posttranslational modification may give a rise to the misleading results, particularly in the pathological states[11]. These ambiguities can be circumvented by the use of genotyping methods, for example, allele-specific oligonucleotide hybridization[12], restriction enzyme analysis[13,14], the amplification refractory mutation system[15], oligonucleotide ligation assay[16], heteroduplex analysis[17], or single-strand conformation polymorphism (SSCP)[18]. These systems are generally

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time-consuming and difficult to automate because they require post amplification procedures such as restriction enzyme digestion and/or electrophoresis. The allele-specific restriction analysis (ASRA) procedure, one of these methods, has been most widely adopted although it has some drawbacks for the large-scale DNA diagnosis, particularly in relation to the complex electrophoretic pattern that results from the partial enzymatic digestion[19].

This study described the determination of the apo E genotyping using a commercially available fluorescence resonance energy transfer (FRET) with the LightCycler[20]. The simultaneous analysis of the two polymorphic Codons: 112 and 158 in a single reaction was conducted by using the two reporter dyes with the different excitation and emission spectra, LightCycler-Red 640 (LC-Red 640) and LC-Red 705 followed by the color compensation software to correct the temperature-dependent crossover, *i.e.* crosstalk among the emission spectra of the dyes.

In this assay, a 265-bp fragment harboring Exon 4 of the apo E gene was amplified by the specific primers from human genomic DNA (gDNA). The amplicon was detected by fluorescence using the specific pairs of hybridisation probes. The detection probes covering Codons 112 and 158 were 5' labeled with LC-Red 640 and LC-Red 705, respectively. The corresponding anchor probes were fluorescein-labeled at their 3' ends. When a pair of hybridization probes hybridized the same strand internal to the unlabeled PCR primers, the probes came in closely to the proximity producing FRET. During FRET, the acceptor fluorophores LC-Red 640 and LC-Red 705 emit fluorescence which was measured in the exact temporal coincidence in Channels 2 and 3 respectively of the optical system of the LightCycler, using a linear arrangement of dichroic bandpass filters[20].

According to the intensity of the FRET signal depending on the amount of specific PCR product generated, this detection strategy allowed the monitoring of the amplification process on a per-cycle basis. Moreover, the homogeneous genotyping was achieved by the analysis of the melting behavior when detecting probes that covered the polymorphic codons. When the fluorescence was monitored as the temperature increased to the melting point (T_m) of the probe/single-stranded PCR product duplex, the characteristic melting profile for each genotype was obtained under the condition of the presence and type of base pair mismatch in the heteroduplex. Consequently, the presence of a particular mismatch was reflected in the specifically lower T_m for the hybrid in the melting curve graph generated by the LightCycler software.

However, when the two fluorophores were measured simultaneously, the fluorescence overlaps between channels must be corrected to obtain the signals that were directly correlated with the amount of hybridized probes. This "crosstalk compensation" was achieved by using the color compensation module integrated into the LightCycler software package.

Material and Method

Genomic DNA extraction and amplification using real-time PCR

One-hundred and thirty-three normal healthy Thai subjects were recruited from both sexes: 122 females and 11 males. Five milliliters of the blood were taken according to the informed consent for gDNA purification using the DNA extraction kit (Roche Diagnostic, Mannheim, Germany). Exon 4 of the Apo E gene was amplified by the extracted gDNA using the commercially available LightCycler™ Apo E mutation detection kit (Roche Diagnostic, Mannheim, Germany).

In this analysis, a 265-bp fragment containing Exon 4 of the apo E gene was amplified from the extracted human gDNA. The reaction mixture (20 µl) containing 50 ng of the gDNA was prepared in a glass capillary following the manufacture's procedure. The cycling program was carried out after the preheating step at 95°C for 1 min through 45 cycles (denaturation at 95°C for 1 sec, annealing at 60°C for 10 sec, extension at 72°C for 10 sec) with the maximum ramp rate of 20°C/sec. Fluorescence was measured when ending the annealing step of each cycle in order to monitor the amplification. The fluorescence emitted by LC-Red 640 and LC-Red 705 was measured continuously in Channels 2 and 3 during the slow temperature ramp to monitor the dissociation of the fluorophore-labeled detection probes from the complementary single-stranded DNA. The fluorescence signals recorded in the respective channels were then converted to the melting peaks by plotting the negative derivatives of the fluorescence with respect to temperature vs. temperature ($-dF/dT$ vs. T). The resulting melting peaks in the different fluorescence channels were observed to discriminate between the homozygous and the heterozygous genotypes.

Results

During the amplification cycles, the fluorescence signals in both channels increased as the products were

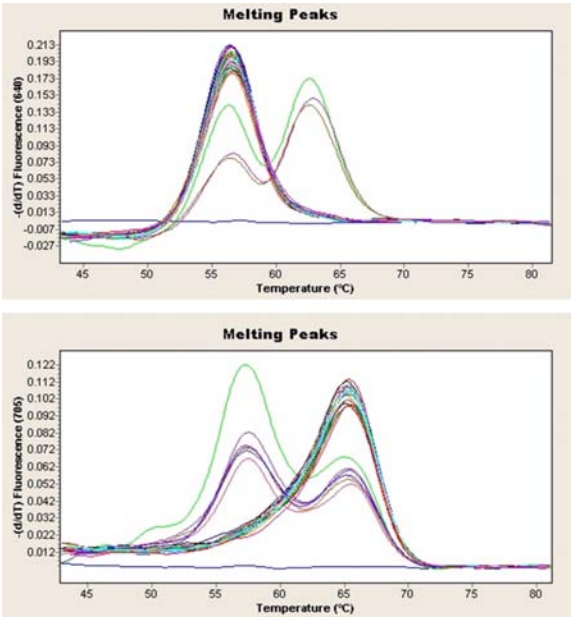

Fig. 1 Apolipoprotein E genotyping using the derivative melting curve plots for Codons 112 (A) and 158 (B). Data A and B were recorded simultaneously during the melting transition of the detection probes in Channels 2 and 3, respectively. The temperature transition was programmed at 0.1°C/s with the continuous fluorescence acquisition at the maximum speed for each sample from 42°C to 80°C. The melting curve plots of the fluorescence signal (*F*) vs. temperature (*T*) were transformed into the derivative melting curve plot of $-dF/dT$ vs. temperature. The derivative melting curves were shown as a sample homozygous for the E2 allele (- - - -), a sample homozygous for the E4 allele(- - -), and a sample with the genotype E2/E4 (——). The melting analysis of the no-template control (- - - - -) was also performed

accumulated. The process of hybridization and melting of the detection probes to the target were monitored by the melting curve analysis (Fig. 1, 2). The detection probes for Codons 112 and 158 matched the alleles coding for arginine (sequence CGC). Accordingly, when Codon 112 with a DNA homozygous for the sequence CGC was examined, the T_m was 61.5°C whereas the DNA coding for cysteine (sequence TGC) produced the markedly lower T_m of 55.5°C. Heterozygous samples contained both types of targets and, thus, generated both peaks (Fig. 1A). The fluorescence signal acquired in Channel 3 was used to genotype the Codon 158. However, according to the interference of the fluorescence produced by LC-Red 640, which was also recorded in Channel 3, the Channel 3 signals needed to be corrected for the contribution of LC-Red 640 by the crosstalk compensation module of the LightCycler system. Through this crosstalk compensation, the alleles coding for arginine and cysteine at Codon 158 were distinguishable, with T_ms at 66 and 58°C, respectively (Fig. 1B).

Fig. 2 showed the derivative melting curve obtained through the rapid-cycle PCR and FRET with the LightCycler. In Panel A, the melting peaks of F2 and F3 of Genotype E3/E3 were shown. In Panel B, the melting peaks of F2 and F3 of Genotype E3/E4 were reported. In Panel C, the melting peaks of F2 and F3 of Genotype E2/E3 were shown. Panels D and E showed the melting peaks of F2 and F3 of Genotypes E2/E4 and E4/E4, respectively.

The genotype frequencies of the E2/3, E2/4, E3/3, E3/4 and E4/4 from 133 normal healthy Thai subjects were 48.36, 5.26, 32.33, 14.28 and 0.75%, respectively (Table 1). However, the observed phenotypes of the 133 samples deviated from Hardy-Weinberg

Table 1. Genotype frequencies of the Apo E in 133 normal healthy Thai subjects determined by the melting curve analysis

Genotype	Codon 112 probes		Codon 158 probes		Case (n)	Frequency (%)
	TGC T_m 55.5°C	CGC T_m 61.5°C	TGC T_m 58.0°C	CGC T_m 66.0°C		
E2/2	*	-	*	-	-	-
E2/3	*	-	*	*	15	11.28
E2/4	*	*	*	*	6	4.51
E3/3	*	-	-	*	92	69.17
E3/4	*	*	-	*	19	14.29
E4/4	-	*	-	*	1	0.75

*, melting peak

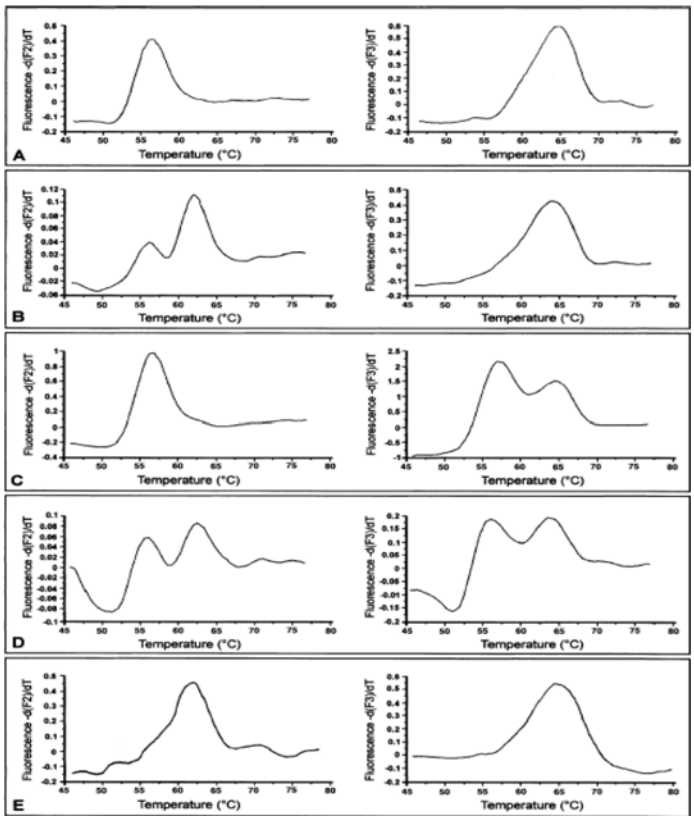

Fig. 2 The derivative melting curve obtained through rapid-cycle PCR and FRET with the LightCycler. In Panel A, the melting peaks of F2 and F3 of genotype E3/E3 were shown. In Panel B, the melting peaks of F2 and F3 of genotype E3/E4 were reported. In Panel C, the melting peaks of F2 and F3 of genotype E2/E3 were shown. Panels D and E showed the melting peaks of F2 and F3 of genotypes E2/E4 and E4/E4, respectively.

Table 2. Allele frequencies of the Apo E gene from several populations

Population	No. of subjects	E2	E3	E4	Hardy-Weinberg distribution		Different from the Thai population		Reference number
					χ^2; df = 5	p-value	χ^2; df = 2	p-value	
Thai	133	0.079	0.820	0.103	19.76	<0.005	-	-	This study
Thai	171	0.080	0.800	0.120	27.27	<0.005	36.62	36.62	21
Australia	424	0.068	0.759	0.172	33.30	<0.005	75.28	<0.001	22
Austria	469	0.090	0.789	0.117	4.13	NS	53.86	<0.001	23
Canada	203	0.078	0.770	0.152	1.61	NS	41.98	<0.001	24
Finland	615	0.041	0.733	0.227	7.09	NS	149.00	<0.001	25
Finland	203	0.062	0.695	0.244	2.26	NS	61.02	<0.001	26
Germany	1,000	0.078	0.783	0.139	7.15	NS	86.90	<0.001	26
Greenland	178	0.014	0.781	0.205	4.00	NS	129.17	<0.001	27
Singapore (Malay)	118	0.114	0.767	0.119	5.27	NS	17.39	<0.001	23
Singapore (Indian)	142	0.046	0.827	0.127	2.01	NS	49.69	<0.001	23
Trinidad	268	0.147	0.694	0.159	12.30	<0.05	49.69	<0.001	28

NS = not significant

equilibrium (χ^2 = 19.76, df = 5, $p < 0.005$). The allele frequencies of the apo E from the Thai subjects in this study were compared with the ones from the other foreign populations[21-27] (Table 2). It was found that the allele frequencies of the apo E from the Thai subjects were significantly different from the others ($p < 0.005$).

Discussion

Polymorphisms of the Apo E gene were associated with the increasing risks of many diseases[4-9]. Therefore, the identification of the Apo E DNA polymorphism was significantly important. In the past, one of the most widely used methods to determine the Apo E DNA polymorphism was the digestion of the amplified Apo E DNA with a restriction endonuclease[13]. Unfortunately, that method was time-consuming and unautomated as it required polyacrylamide gel electrophoresis following the application of PCR-RFLP. Recently, Roche Diagnostic has developed the Apo E Mutation Detection Kit using FRET with the LightCycler™ which easily allows the real time detection of the fluorescence of the amplified fragment before discriminating the alleles of the Apo E gene based on exploiting the different binding ability that the detection probes have with the wild type and mutant Apo E genes[29]. When the PCR is completed, the melting curve is performed to distinguish the different allelic variants by means of different melting temperatures. Accordingly, the genotyping of the Apo E in 133 normal healthy Thai subjects was investigated by using the Apo E Mutation Detection Kit with the LightCycler™ in this study.

The advantages of using the kit were as follows. Firstly, the very minute an amount of 32 DNA sample can be performed in the capillaries for the PCR reactions at a time, the whole process takes only 40 minutes; secondly, this method is used to avoid handling the neurotoxic polyacrylamide gel, and finally, this method showed 100% in accord with PCR-RFLP[30].

E3 was considered the most prominent genotype of the Apo E followed by E2 and E4. The genotype frequencies of the Apo E gene were significantly different from the other populations ($p < 0.005$; Table 2). This implied that these polymorphisms of the Apo E gene might be influenced by ethnicity. However, the allele frequencies of the Apo E DNA polymorphisms reported in this paper were significantly different from another study of the Thai population[21]. Poldee[21] reported that the calculated allele frequencies from the pooled data, *i.e.* normolipidemic and primary hyperlipidemic subjects might be enriched by those with hyperlipidemia and may not be the entire representatives of the normal Thai population.

This study additionally showed that the genotype frequencies of the Apo E gene significantly deviated from Hardy-Weinberg expectations (χ^2 = 19.76; df = 5, $p < 0.005$; Table 2). This phenomenon could be a chance for further investigation.

In conclusion, the Apo E Mutation Detection Kit using the LightCycler™ is rapid, simple, reliable and useful for the routine determination of the Apo E genotype for the diagnostic purposes.

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ความหลากหลายของยีน apolipoprotein E, โดยใช้ชุดทดสอบสำเร็จรูป Light Cycler apolipoprotein E mutation detection kit

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ความหลากหลายของยีน apolipoprotein E ที่มีอยู่ 3 แบบ คือ E2, E3 และ E4 จะเกี่ยวข้องกับการเพิ่มความเสี่ยงในการทำให้เกิดโรคต่าง ๆ เช่น ไขมันในเลือดสูง โรคหลอดเลือดหัวใจ โรคอัลไซเมอร์ เป็นต้น การศึกษานี้ จะทำการตรวจหาความหลากหลายของยีน apolipoprotein E ในคนไทยปกติจำนวน 133 คน โดยใช้ชุดทดสอบสำเร็จรูปที่เรียกว่า Light Cycler apolipoprotein E mutation detection kit ซึ่งใช้วิธี Realtime PCR หลังจากที่ทำการแยกดีเอ็นเอจากตัวอย่างเลือด โดยใช้ชุดแยกดีเอ็นเอสำเร็จรูปแล้ว จะทำการเพิ่มจำนวนยีนเพื่อตรวจสอบความหลากหลายของยีน apolipoprotein E ที่โดยใช้ Codon ตำแหน่งที่ 112 และ 158 ของเอ็กซอนที่ 4 ไปพร้อมกัน โดยใช้สีย้อม 2 ชนิดคือ ไลท์ไซเคลอร์เรด 640 และ 705 ซึ่งมีการเรืองแสงที่ความยาวคลื่นแตกต่างกัน จากนั้นจึงทำการวิเคราะห์ข้อมูลโดยใช้ซอฟท์แวร์ พบว่าความถี่ของอะลีล อี 2, อี 3 และอี 4 ที่พบในคนไทยปกติจะมีค่าเท่ากับ 0.26, 0.63 และ 0.11 ตามลำดับ ส่วนความถี่ของจีโนไทป์ อี 2/3, อี 2/4, อี 3/3, E3/4 และ อี 4/4 จะมีค่าเท่ากับ 47.37, 5.26, 32.33, 14.28 และ 0.75% ตามลำดับ จะเห็นได้ว่า อี 2/3 เป็นจีโนไทป์ที่พบมากที่สุดในตัวอย่างที่ใช้ในการศึกษานี้ ดังนั้นการตรวจหาความหลากหลายของยีนอะโปไลโปโปรตีน อี โดยใช้ชุดทดสอบสำเร็จรูปไลท์ไซเคลอร์อะโปไลโป โปรตีน อี มิวเตชั่นดีเทคชั่นคิท จะมีข้อได้เปรียบคือ ง่าย รวดเร็ว ให้ผลแม่นยำ และน่าจะใช้กันอย่างแพร่หลายต่อไป ในอนาคต