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Development of a universal chemiluminometric genotyping method for high-throughput detection of 7 *LDLR* gene mutations in greek population

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Abstract

Objectives: Familial hypercholesterolemia (FH) is caused by mutations in the LDL receptor (*LDLR*) gene. We report the application of a universal method with high allele discrimination properties to the simultaneous genotyping of 7 *LDLR* mutations in Greeks, in dry-reagent format.

Design and methods: We genotyped mutations C858A, C939A, G1285A, T1352C, G1646A, G1775A, C/T81G. Unpurified amplicons from a multiplex PCR that produced fragments encompassing all 7 mutations were subjected to probe extension reactions in the presence of fluorescein-modified dCTP, and a microtiter well-based assay of extension products with a peroxidase–antifluorescein conjugate and a chemiluminogenic substrate. We used lyophilized dry reagents and assigned genotypes by the signal ratio of normal-to-mutant-specific probe.

Results: We standardized the method and optimised all steps for specificity. The method was validated by genotyping blindly 119 (833 genotypings). Results were fully concordant with other methods used as standards.

Conclusions: This method is accurate, simple, rapid and robust. The microtiter well format allows genotyping of a large number of samples in parallel for several mutations.

Keywords: Chemiluminometric genotyping; Mutation detection; Familial hypercholesterolemia; *LDLR*

Introduction

Familial hypercholesterolemia (FH) is a monogenic autosomal dominant disorder characterized by elevated low density lipoprotein (LDL) cholesterol, tendon xanthomas, and premature coronary heart disease [1]. FH is caused by mutations in the LDL receptor (*LDLR*) gene, with more than 800 mutations known worldwide [2]. The frequency of heterozygous FH is 1:500 and 1:1,000,000 for homozygous FH. In Greeks and Greek-Cypriots, a total of 27 mutations have been reported [3–10]. According to one representative study, six *LDLR* mutations account for 60% of cases while the three most common mutations account for 49% of cases [4].

Traditionally, FH clinical diagnosis is based on family history, elevated LDL cholesterol, and tendon xanthomas. A serious problem in clinical diagnosis is that absolute total or LDL cholesterol cut-off points cannot be applied. In addition, total and LDL cholesterol do not allow unequivocal diagnosis of FH in patients with borderline lipid profiles, and partial or early clinical features of FH, especially children [11–13]. Mutation detection provides the only definite diagnosis and is also important in prevention and early treatment during the asymptomatic stage of the disease since atherosclerosis begins in childhood. Although the ability to perform DNA genotyping of the *LDLR* gene has become an important requirement, not many diagnostic assays exist, mainly because of the size of the *LDLR* gene which spans 45 kb and contains 18 exons [14].

Molecular diagnosis is currently accomplished by two approaches: screening of the *LDLR* gene for any possible mutations or screening of the gene for specific reported mutations. The former is useful in heterogeneous populations and may be accomplished using single-strand conformation polymorphism (SSCP) analysis [15–18], denaturing gradient gel electrophoresis (DGGE) [19,20], and denaturing HPLC [21,22]. More recently developed methods include sequencing of the *LDLR*

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cDNA region [23], a SSCP/heteroduplex method followed by capillary electrophoresis [24], and melt-microplate array diagonal gel electrophoresis [25]. Some of the above methods have been applied to genetic screening in heterogeneous populations [26] as has sequencing [27–30].

The second approach in molecular diagnosis of FH involves detection of specific reported mutations known to occur in a particular population. For example, in Spain, a DNA array for identification of 117 known *LDLR* mutations was recently developed [31]. Diagnosis is even simpler in populations in which a limited number of mutations (1 to 3) is responsible for a large percentage of cases [32,33]. Accordingly, since a small number of mutations are found in Greece, a strategy tailored to specific mutations is more efficient than screening the entire gene. Recently, a method to detect the three most common mutations using the NanoChip® microelectronic array technology system was developed [34].

In this study we developed a rapid and simple method for the simultaneous detection of 7 *LDLR* mutations in the Greek population that uses microtiter wells which are already adopted by clinical laboratories and requires only 70 min for completion. The method comprises (*a*) a multiplex PCR to simultaneously amplify 5 fragments spanning all 7 mutations of the *LDLR* gene (*b*) a probe extension (PE) reaction of the amplified products in the presence of a fluorescein-modified nucleotide, without prior purification of the PCR product and (*c*) detection of PE products on streptavidin-coated microtiter wells by use of a horseradish peroxidase (HRP)-labeled antifluorescein antibody in combination with a chemiluminogenic substrate, which provides high sensititvity of the whole assay thus allowing only 4 cycles of the PE reaction at previous step. The method was validated by analyzing 119 samples, corresponding to 833 genotypings.

Materials and methods

Instrumentation

Lyophilization of PCR and PE reaction mixtures was carried out using the Lyophlex 0.8 lyophiliser from BOC Edwards. PCR amplification reactions were performed in either a 96-well GeneAmp® PCR system 9700 from Applied Biosystems or in the Mastercycler gradient from Eppendorf. PE reaction was also performed in the Mastercycler gradient. Microtiter well-based chemiluminometric genotyping assays were performed using the Stat Fax 2200 shaker and automated washer Stat Fax 2600 from Awareness Technology. Chemiluminescence measurements were carried out using the PhL microplate luminometer from Aureon Biosystems GmbH. A digital camera, Kodak DC 120 and the Gel Analyser Software were from Kodak.

Materials

All oligonucleotides, used as primers for amplification of the 5 regions of the *LDLR* gene, as well as biotinylated genotyping probes for PE reactions, were from ThermoElectron. The sequences of all oligonucleotides and their respective functions are given in Table 1. Deoxynucleotide triphosphates (dNTPs) were from Promega. White, flat bottomed Maxisorp microtiter wells were from Nunc and streptavidin was from Roche. Tth DNA polymerase was purchased from Biotools, SmarTaq was from HyTest and Vent exo$^-$ DNA polymerase was from New England Biolabs. Fluorescein-OBEA-dCTP (F-dCTP) was from Millipore. Antifluorescein antibody conjugated to horseradish peroxidase (antiF-HRP) was from Biodesign and the chemiluminogenic substrate for horseradish peroxidase (CHMI) was obtained from BioFX.

Table 1
Primers and biotinylated oligonucleotides used in PCR and probe extension reactions, respectively

Name	Sequence (5′→3′)	Mutation detected
2_up	GGGAATCAGACTGTTCCTGATCGGATG	–
2_dn	ACGTCTCCTGGGACTCATCAGAGCCA	–
6_up	CAAGCAAACTGAGGCTCAGACACA	–
6_dn	CGCCCAGCATCGCTTCATTTTTT	–
9_up	TGCAGGATGACACAAGGGGATGG	–
9_dn	CTCTGTCAAGCTGGGTGCTGAGGCA	–
11_up	CCACCAGCTTCATGTACTGGACTGA	–
11_dn	ATTAGTCTGCCGTGGTGGCACGTGT	–
12_up	CAGCACGTGACCTCTCCTTATCCA	–
12_dn	TGCATCTCGTACGTAAGCCACACCTC	–
858_N	CCCAACAAGTTCAAGTGTCACAGC	C858A
858_M	CCCAACAAGTTCAAGTGTCACAGA	
939_N	GGTCAGATGAACCCATCAAAGAGTGC	C939A
939_M	GGTCAGATGAACCCATCAAAGAGTGA	
1285_N	ACCTCCGTGTCCAGAGCGACCAC	G1285A
1285_M	ACCTCCGTGTCCAGAGCGACCAT	
1352_N	GGTCTGACCTGTCCCAGAGAATGAT	T1352C
1352_M	GGTCTGACCTGTCCCAGAGAATGAC	
1646_N	TCAAGAAAGGGGGCCTGAATGG	G1646A
1646_M	TCAAGAAAGGGGGCCTGAATGA	
1775_N	TCCAAGATGGTCTTCCGGTTGCCCC	G1775A
1775_M	TCCAAGATGGTCTTCCGGTTGCCCT	
C81_N	GGCACTGGAACTCGTTTCTTTCG	C/T81G
T81_N	GGCACTGGAACTCGTTTCTTTCA	
81_M	GGCACTGGAACTCGTTTCTTTCC	

Samples

161 genomic DNA samples from individuals were included in this study. Blood collections were performed with informed oral consent of the participants. Genomic DNA was isolated by use of the QIAamp DNA Blood Mini Kit (Qiagen).

Chemiluminometric genotyping by probe extension

Polymerase chain reaction

PCR mixture containing 3 mM $MgCl_2$, 300 μM deoxynucleotide triphosphates, 5 pmol each of primers 2_up and 2_dn, 30 pmol of primers 6_up and 6_dn, 7.5 pmol of primers 9_up and 9_dn, 12 pmol of primers 11_up and 11_dn, 13.5 pmol of 12_up and 12_dn and stabilizers in a total volume of 20 μL, was lyophilized overnight. Lyophilized mixtures were reconstituted just before use by the addition of 30 μL of a solution containing DNA polymerase buffer, DNA polymerase and DNA template. After reconstitution the mixture contained 75 mM Tris–HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$,