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High-Speed Detection of the Two Common Paraoxonase Polymorphisms Leu_{55}→Met and Gln_{192}→Arg by Real-Time Fluorescence PCR and Melting Curves

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Human paraoxonase (PON1) is a calcium-dependent esterase exclusively bound to apolipoprotein A-I and clusterin, containing high-density lipoprotein (HDL) particles that hydrolyzes organophosphates and aryl esters. Several studies have indicated that PON1 can prevent low-density lipoprotein (LDL) oxidation by hydrolyzing lipid peroxides in the lipoprotein, which is the crucial first step for atherogenesis. Therefore it may protect against the development of atherosclerosis. Serum PON1 activity has been shown to be decreased in familiar hypercholesterolemia and in diseases that are associated with accelerated atherogenesis. The *PON1* gene has two common coding region polymorphisms, Leu_{55}→Met and Gln_{192}→Arg. Both polymorphisms have been identified as independent risk factors for cardiovascular disease in diabetic and non-diabetic patients. We have established high-speed and easy-to-perform genotyping for the two most significant *PON1* gene polymorphisms, employing the LightCycler technology and melting curves. This technique eliminates PCR contamination related to sample handling and does not require digestion of PCR products with restriction enzymes and/or fragment separation on gels. Clin Chem Lab Med 2002; 40(4):337–340

Key words: Paraoxonase; Real-time fluorescence genotyping; Single nucleotide polymorphisms.

Abbreviations: apoA-I, apolipoprotein A-I; FRET, fluorescence resonance energy transfer; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PON1, human paraoxonase; RFLP, restriction fragment length polymorphism; T_m, melting point.

Introduction

Human paraoxonase (PON1) is a calcium-dependent esterase located in a subfraction of high-density lipoprotein (HDL) that contains apolipoprotein A-I (apoA-I) and clusterin. PON1 binds and hydrolyzes organophosphates and aryl esters (1, 2). Although its natural substrate is unknown, PON1 has been shown to prevent low-density lipoprotein (LDL) oxidation by hydrolyzing lipid peroxides in the lipoprotein. It therefore might prevent the development of atherosclerosis (3). The *PON1* gene is located at q21 to q22 on the long arm of chromosome 7 with other members of its supergene family and encodes for a 43–45 kDa protein that contains up to three carbohydrate chains. Serum PON1 activity has been shown to be decreased in myocardial infarction survivors (4) and in patients with non-insulin dependent diabetes mellitus (5), genetic HDL-deficiency (6), familiar hypercholesterolemia, and in patients on dialysis (7).

There are two common DNA polymorphisms within the *PON1* gene. One is Leu_{55}→Met (TTG→ATG), the other is Gln_{192}→Arg (CAA→CGA). The dominant effect on activity is exerted by the PON1–192 polymorphism. However, the PON1–55 polymorphism also has a smaller, but significant effect on activity (8). The reason for polymorphism-associated differences in PON1 activity is unclear. Both polymorphisms have been identified as independent risk factors for cardiovascular disease in diabetic and non-diabetic patients. PON1–192 allele frequencies in healthy Caucasian individuals reported by Akhmedova *et al.* were 51.7% Gln/Gln, 40.6% Gln/Arg and 7.7% Arg/Arg. For position 55, they were 49.3% Leu/Leu, 38.6% Leu/Met and 12.1% Met/Met (9).

To date genotyping for PON1 is performed by PCR, followed by digestion with restriction enzymes and restriction fragment length polymorphism (RFLP) analysis. These methods are widely used, though they are time-consuming and require optimization of the PCR reaction to eliminate nonspecific PCR products.

In contrast, real-time fluorescence genotyping by rapid cycle offers fast, accurate and simple point mutation analysis. Fluorescence monitoring of PCR amplification is based on the concept of fluorescence resonance energy transfer (FRET) between two adjacent dyes that are part of two oligonucleotide hybridization probes, in a high-speed thermal cycler which uses glass capillaries and hot air for heating. Generation of a PCR product results in the adjacent hybridization of the two hybridization probes, thus positioning both fluorophores close to each other. Excitation of the donor fluorophore results in an emission spectrum that is used to excite the acceptor fluorophore. The emission of the acceptor fluorophore is monitored on-line and reflects the accumulation of the PCR product. This technology enables the real-time detection of specific PCR products, followed by detection of mutations or sequence variations by identification of the melting be-

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havior of one of the two fluorescently labeled hybridization oligonucleotides. To this end, one hybridization probe matches the wild-type (common) sequence, or the mutant (variant) sequence, with the variable nucleotide in the middle of the sequence, and has fluorescein at its 3`end (detection probe); a second hybridization primer (anchor primer, phosphorylated at 3`end) is located distally by a distance of one to three nucleotides and is labeled with LC Red640 fluorophore at its 5`end. Cycling conditions are very fast because of the quick adaptation of the temperature in the glass capillary and fast temperature transition rates. Following PCR, the mixture is denatured and the temperature is lowered to 45 °C to enable binding to the hybridization probes, generating maximum fluorescence, then slowly increased to 75 °C to permit gradual melting of the detection probe which is monitored by the decline of the fluorescence. Melting curves are converted to melting peaks, allowing easy distinction of the common from the variant sequence (allele). Mismatch with the detection probe caused by a single point-mutation leads to a decrease in the melting point of the probe (10). Previously we successfully used LightCycler technology (Roche Molecular Biochemicals) for the identification of the apolipoprotein B3500 mutation, apolipoprotein E isoforms, prothrombin G20210A mutation, methylenetetrahydrofolate reductase C677T sequence variation, and α_1-antitrypsin Z and S allele mutations (11–13).

In the present study we describe a new sequence variation detection method in the *PON1* gene to identify these two polymorphisms, using real-time fluorescence PCR and melting curves.

Materials and Methods

We used genomic DNA isolated from EDTA blood from healthy individuals using QIAamp Blood Kit (QIAGEN, Hilden, Germany). The primer sequences for PCR and sequences of the hybridization probes are shown in Table 1. The antisense primer for PON1–55 and the sense primer for PON1–192 polymorphism were previously published by Akhmedova *et al.* (9) and Mackness *et al.* (5), respectively. The detection probe of PON1–55 is derived from the variant allele (Met). PCR and melting curve determination were performed in 20 µl volumes in glass capillaries (Roche Molecular Biochemicals, Mannheim, Germany). For PON1–55 and PON1–192 genotyping the following pipeting scheme was used: 9 µl of H_2O, 1 µl of 25 mmol/l $MgCl_2$, 2 µl each of 5 pmol/µl PON1–55F and PON1–55R or PON1–192F and PON1–192R, 1 µl each of 4 pmol/µl PON1–55DA and PON1–55A or PON1–192DA and PON1–192A, and 2 µl of DNA-Master Hybridization Probes (Roche Molecular Biochemicals, Mannheim, Germany), containing Taq polymerase, reaction buffer, and dNTP mixture, and 10 mmol/l $MgCl_2$ as a 10× concentrate. Genomic DNA (2 µl, 50–200 ng) was used for amplification. Fluorescently labeled hybridization probes were synthesized by TIB MOLBIOL (Berlin, Germany). The cycling program for PON1–55 genotyping consisted of initial denaturation at 94 °C for 600 s, followed by 50 cycles of denaturation at 94 °C for 0 s, annealing at 50 °C for 5 s, and extension at 72 °C for 2 s, with a ramping rate of 20 °C/s. The fluorescence was monitored at the end of each 5 s annealing phase. After amplification of the products we generated melting curves by denaturing the reaction at 94 °C for 150 s, keeping the sample at 45 °C for 30 s, and then slowly heating the sample to 75 °C with a ramp rate of 0.2 °C/s, simultaneously monitoring the decline in fluorescence with respect to temperature *(–dF/dT)* against temperature. Cycling conditions for PON1–192 genotyping were as follows: the initial denaturation was performed at 94 °C for 600 s, followed by 50 cycles of denaturation at 94 °C for 0 s, annealing at 57 °C for 5 s, and extension at 72 °C for 2 s, with a ramping rate of 20 °C/s. The fluorescence was monitored at the end of each 5 s annealing phase. After amplification of the products we generated melting curves by keeping the sample at 45 °C for 3 min, and then slowly heating the sample to 75 °C with a ramp rate of 0.2 °C/s, simultaneously monitoring the decline in fluorescence with respect to temperature *(–dF/dT)* against temperature. Amplification and detection occurred in the same closed tube in less than 40 min.

Results and Discussion

The fluorescence profiles generated from the DNA samples are illustrated in Figure 1. When the hybridization probes for the PON1–192 or PON1–55 isotype

Table 1 Sequences of PCR primers and hybridization probes.

Primers	Sequence[a]	Orientation	PCR product
PCR primers			
PON1–55F	ATTCTGAACCTATTAAAGAAGAGTGATG	Sense	240 bp
PON1–55R	CTTAAACTGCCAGTCCTAGAAAACG	Antisense	
PON1–192F	TATTGTTGCTGTGGGACCTGAG	Sense	199 bp
PON1–192R	GACATACTTGCCATCGGGTGAA	Antisense	
Detection probes			
PON1–55DA	GCTCTGAAGACATGGAGATA-F[b]		
PON1–192DA	TGACCCCTACTTACAATCCT-F		
Anchor probes			
PON1–55A	LCRed 640-GCCTAATGGACTGGCTTTCATTAGCTCTGT-ph[b]		
PON1–192A	LCRed 640-GAGATGTATTTGGGTTTAGCGTGGTCGTAT-ph		

[a]Sequences given 5` to 3` (accession number: AC004022). The detection probe of PON1–55 is derived from the variant allele (Met), the variant site is underlined. [b]F: fluorescein, ph: phosphoryl group.

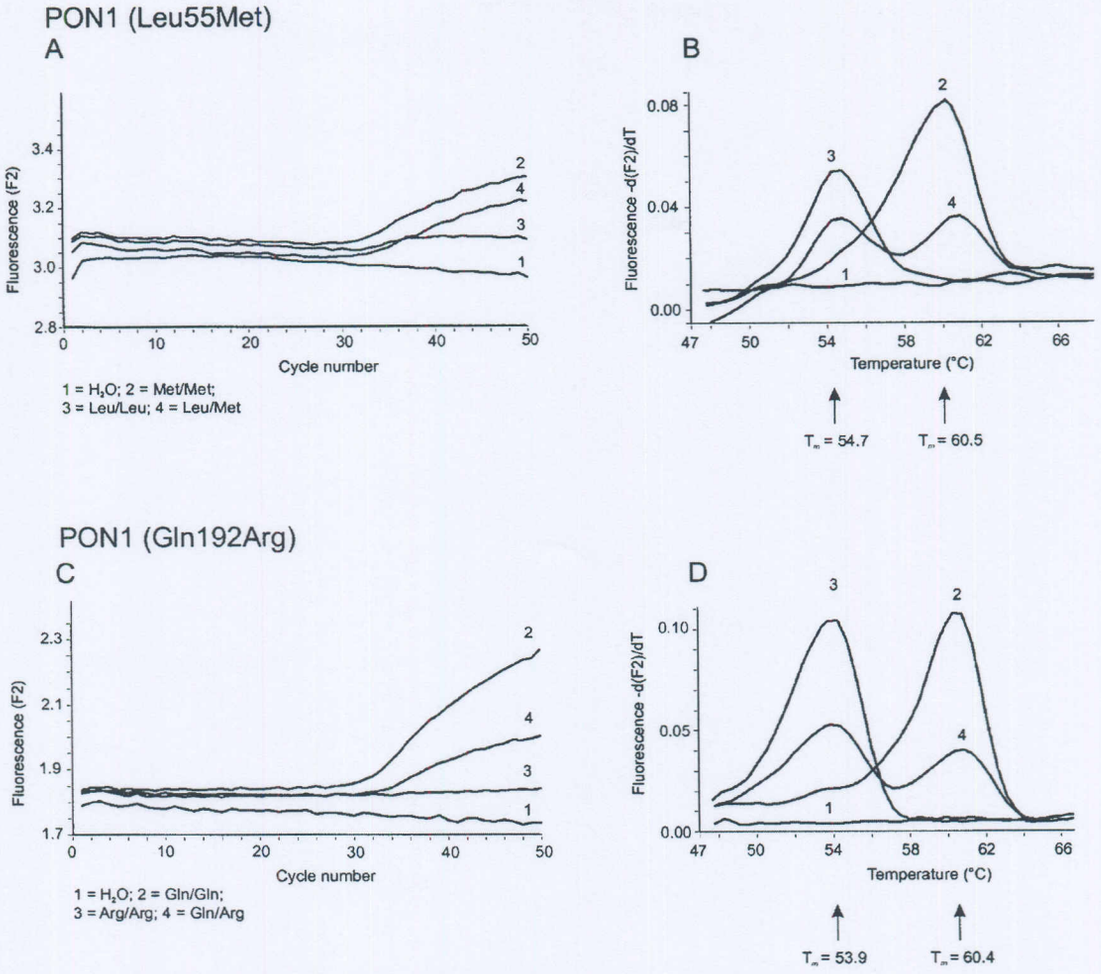

Figure 1 Fluorescence *vs.* cycle number (A, C) and melting peaks (B, D) for PON1–55 and PON1–192 genotyping. Melting curves converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature (*–dF/dT*) against temperature. The melting point (T_m) of the individual detection probes is shown by an arrow. The detection probe of PON1–55 is derived from the variant Met-allele.

were used, fluorescence increased constantly in the samples with DNA (samples 2, 3 and 4), whereas no fluorescence was detected in the H_2O control (sample 1) (Figure 1A and Figure 1C). Figure 1B and Figure 1D show the relationship between fluorescence and temperature in melting curve analyses of four samples after PCR. The melting point (T_m) of the PON1–55 homozygous variant type (Met) sample (curve 2) was at 60.5 °C, the heterozygous sample showed a two-phase melting behavior (curve 4), whereas the T_m of the normal allele sample (Leu) was at 54.7 °C (curve 3) (Figure 1B). The T_m of the PON1–192 normal allele (Gln) sample (curve 2) was at 60.4 °C, whereas the heterozygous sample (curve 4) produced two melting peaks at 53.9 °C and 60.4 °C. The melting point of the homozygous variant allele (Arg) sample (curve 3) was 53.9 °C (Figure 1D). Analysis of PCR products on agarose gels revealed the presence of the specific 240 and 199 bp PCR products. We have validated the technology by analyzing 50 healthy individuals for PON1–192 and PON1–55 polymorphisms. The genotypes determined with the LightCycler were identical to those obtained with conventional PCR and RFLP analysis.

In the last few years, several laboratories have reported the results of their studies on the relationship between PON1 polymorphisms and the presence of cardiovascular and other diseases using RFLP analysis. To our knowledge, genotyping of PON1–192 and PON1–55 polymorphisms using real-time fluorescence PCR and melting curves have not been previously reported. This technology is less time-consuming and more accurate and cost-effective than traditional PCR analysis and allows the simultaneous analysis of 32 samples within one run. It can also be adapted to analyze two mutations/polymorphisms on two different PCR products by dual color detection using LC Red640 and LC Red705 probes in the same capillary, thus reducing time, labor and reagent costs to a minimum.

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