Research article

Fluorescent quenching probes based *SAA 1* genotyping with a fully automated system

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ABSTRACT

Objective: The aim of the present study is to develop and validate a reliable and simple application for genotyping serum amyloid A1 (*SAA1*).
Methods: The specific nested PCR was performed to amplify a product of *SAA1* gene. Two quenching probes (QPs) were designed for detecting two single nucleotide polymorphism (SNP) sites, rs1136743(C/T) and rs1136747(C/T) respectively for *SAA1* genotypes. The specific nested PCR and QPs of *SAA1* genotying was introduced into a fully automated genotyping system (I-densy, ARKRAY, Inc.), which enables the genotyping of *SAA1* from whole blood.
Results: Six genotypes of *SAA1* ($\alpha^{+/+}$, $\beta^{+/+}$, $\gamma^{+/+}$, $\alpha\beta$, $\alpha\gamma$ and $\beta\gamma$) could be determined by monitoring the fluorescence intensity of two QPs with melting temperature (TM) analysis. Total 121 clinical samples were *SAA1* genotyped in the fluorescent quenching probes based method with a fully automated I-densy system and were further sequence confirmed with a PCR direct sequencing approach.
Conclusion: This fully automated system is a rapid and reliable strategy for the *SAA1* genotyping and for its future clinical application.

1. Introduction

The serum amyloid A (SAA) is a family of differentially expressed apolipoproteins. Some of them are acute-phase SAAs (A-SAAs) which secrete and increase by as much as 1000-fold during the acute phase of inflammation, like *SAA1* and *SAA2*; the other is constitutive SAAs (C-SAAs), which was induced minimally during the acute-phase response and have only been found in human and mouse, like SAA4 [1,2]. Although the liver synthesizes both A-SAA and C-SAA, accumulating epidemiological data suggest that A-SAAs associates strongly with developing amyloidosis and cardiovascular diseases (CVDs) [3, 4, 5]. Despite the high sequence identity (over 93%) among different A-SAA isoforms, *SAA1* shows to be a major isoform and more pathogenic properties [6].

SAA1 is a 12KDa protein of 104 amino acids which is produced mainly from liver after stimulation by inflammatory cytokines, such as

interleukin-1 (IL-1) and tumor necrosis factor (TNF) [7]. Two single nucleotide polymorphisms at exon 3 constitute three kinds of *SAA1* allelic variants, resulting six genotype of *SAA1* in human ($\alpha^{+/+}$, $\beta^{+/+}$, $\gamma^{+/+}$, $\alpha\beta$, $\alpha\gamma$ and $\beta\gamma$) [8, 9]. Polymorphisms in the gene coding for *SAA1* have been identified as a risk factor of developing amyloidosis and estimate candidates for atherosclerosis [10, 11, 12, 13, 14]. The homozygous $\alpha^{+/+}$ is reported to be the most common genotype among patient group with amyloidosis in Caucasoid population, while homozygous $\gamma^{+/+}$ in Japanese population. Furthermore, *SAA1* genotype is significantly associated with a higher NPC (nasopharyngeal carcinoma) risk, as the frequency of the $\beta^{+/+}$ genotype in NPC patients was 2-fold higher than in the healthy individuals [15].

As previously reported, the quenching probe (QP) method is extremely effective in detecting target genes using fluorescence quenching [9]. Through the addition specific primer sets, a gene amplification response is generated, after which QP was used to detect the specific gene
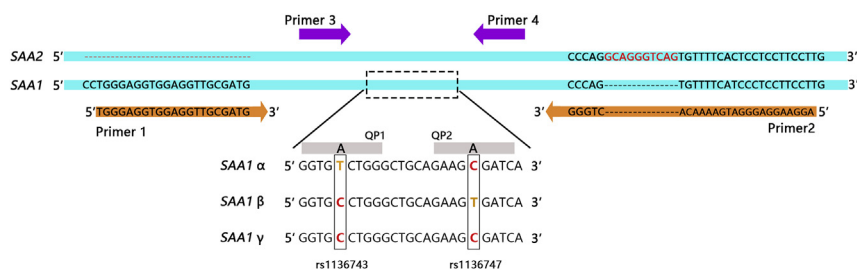

Figure 1. Schematic diagram showing the sequences and locations of primers used for QP methods and DS for *SAA1* genotyping. Primer 1 and 2 are used to specifically amplify *SAA1* and DS. Primer 3 and 4 are nested primers for QP method. Two single nucleotide polymorphisms (rs1136743 and rs1136747) associated to *SAA1* genotypes are framed in black boxes, which are identified by two QPs respectively. The QP1 was designed to be perfectly complementary to the thymine deoxynucleotide at rs1136743, and the QP2 was designed to be perfectly complementary to the thymine deoxynucleotide at rs1136747.

arrangement quickly and easily by fluorometry. The present study reports a simple and reliable QP method for *SAA1* genotyping, with which the six genotypes of the *SAA1* were identified with a specific nested PCR and QPs to a fully automated genotyping system (I-densy, ARKRAY, Inc.). It could be a powerful tool for research study and future clinical application.

2. Material and methods

2.1. Genomic DNA extraction and construction of plasmids containing SAA1 allelic genomic DNA fragments

Ethical approval for this study and the use of human subjects was obtained from the research ethics committee of the Third People's Hospital of Zhenjiang, consistent with the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from all patients.

Genomic DNA was extracted from peripheral blood in QIAGEN QIAcube (Qiagen, Germany) with QIAamp DNA Blood Mini kit (Qiagen, Germany) following the manufacturer's instructions. DNA concentration was examined with NanoDrop 2000 spectrometer (Thermo Scientific, USA).

The DNA fragments of *SAA1* allelic variants were amplified from human genomic DNA by Primer 1 (TGGGAGGTGGAGGTTGCGATG) and Primer 2 (AGGAAGGAGGGATGAAAACACTGGG) (Sangon Biotech Co., Ltd., China) and were inserted to pBlueScript II (+) DNA vector at an EcoRV site as positive controls.

2.2. Specific nested PCR and detection of SAA1 genotype by QP method

Primer 1 and Primer 2 were designed and synthesized to specifically amplify *SAA1* gene product theoretically, which was about 800 base pair long, shown in Figure 1. Additionally, another nested primer pair, Primer 3 (GGAACTATGATGCTGCCAAAA) and Primer 4 (GCTCGTCTCCCTCCTGACTG) (Sangon Biotech Co., Ltd., China), amplified an internal fragment covering the SNP region of *SAA1*, coupling with two QPs (QP1-FAM and QP2-TAMRA) (Sangon Biotech Co., Ltd., China) synthesized for detecting two SNP sites of *SAA1*, rs1136743 and rs1136747 respectively. After the PCR was complete, melting temperature (Tm) analyses were performed. The SNPs were identified by the difference in Tm, which could be used to identify the six genotypes of *SAA1* ($\alpha^{+/+}$, $\beta^{+/+}$, $\gamma^{+/+}$, $\alpha\beta$, $\alpha\gamma$ and $\beta\gamma$).

2.3. Detection of SAA1 genotype by PCR direct sequencing

The *SAA1* genomic DNA sequence was amplified using the forward Primer 1 and Primer 2, shown above. The PCR products were subjected to direct sequencing (Sangon Biotech Co., Ltd., China).

3. Results

3.1. Establish a QP method to genotype SAA1 gene

Two single nucleotide polymorphisms at exon 3 of *SAA1* gene, rs1136743 and rs1136747, constitute three different *SAA1* allelic variants: *SAA1*α, *SAA1*β and *SAA1*γ. The genomic sequence of *SAA1* and *SAA2* were aligned to find out region with significant difference since high identity between *SAA1* and *SAA2* genes. According to DNA sequence alignment between *SAA1* and *SAA2*, we designed and synthesized one pair of primers (Primer 1 and Primer 2) to specifically amplify *SAA1* gene resulting in an around 800 base pair long product. On top of that, a quenching probe (QP) method was applied with another nested primer pair (Primer 3 and Primer 4) to amplify an internal fragment covering SNP region of *SAA1* combined with two QPs labeled with two different fluorescent dyes (FAM and TAMRA) for detecting two SNP sites of *SAA1*, rs1136743(C/T) and rs1136747(C/T) respectively, as shown in Figure 1.

As shown in Table 1, theoretical melting patterns and peak temperatures of the six genotypes of *SAA1* ($\alpha^{+/+}$, $\beta^{+/+}$, $\gamma^{+/+}$, $\alpha\beta$, $\alpha\gamma$ and $\beta\gamma$) were listed. Theoretically, the mismatched cytosine deoxynucleotide would be detected as a single fluorescent peak at a relatively lower temperature compared to the perfectly matched thymine deoxynucleotide where the QP1-FAM was designed to be perfectly complementary to the thymine deoxynucleotide at rs1136743 site. Similarly, the cytosine or the thymine deoxynucleotide at rs1136747 would be detected as single peak at low or high temperature, respectively. Thus, combining the detection results of the two SNP sites, we would be able to identify the six genotypes of *SAA1* ($\alpha^{+/+}$, $\beta^{+/+}$, $\gamma^{+/+}$, $\alpha\beta$, $\alpha\gamma$ and $\beta\gamma$). Of particular notes, by QP method for SNP detection, *SAA1*$\alpha\beta$ genotype will have two peaks of low and high temperature at each SNP site, because *SAA1*$\alpha\beta$ is heterozygous at both SNP sites; *SAA1*$\alpha\gamma$ genotype will have two peaks at rs1136743, while *SAA1*$\beta\gamma$ genotype will have two peaks at rs1136747.

To validate the QP method for *SAA1* genotyping, three plasmids inserted *SAA1* allelic variants were used to prepare six genotypes of *SAA1*, which had already been proved by direct sequencing. As expected, two SNPs of *SAA1* could be identified by two QPs respectively in one reaction, which was able to identify the specific genotype of *SAA1*, shown in Figure 2. For homozygotes, *SAA1*$\alpha^{+/+}$ were detected as one peak at 61 °C for rs1136743 and one peak at 50 °C for rs1136747; *SAA1*$\beta^{+/+}$ were detected as one peak at 52 °C for rs1136743 and one peak at 59 °C for rs1136747; *SAA1*$\gamma^{+/+}$ were detected as one peak at 52 °C for rs1136743 and one peak at 50 °C for rs1136747. For heterozygotes, *SAA1*$\alpha\beta$ were detected as two peaks for rs1136743 and two peaks

Table 1. Theoretical melting temperature (TM) patterns of six *SAA1* genotype by quenching probe method.

Genotypes	TM (rs1136743)			TM (rs1136747)		
	Low	High	Allele	Low	High	Allele
$\alpha^{+/+}$	-	+	T	+		C
$\beta^{+/+}$	+	-	C	-	+	T
$\gamma^{+/+}$	+	-	C	+	-	C
$\alpha\beta$	+	+	C/T	+	+	C/T
$\alpha\gamma$	+	+	C/T	+	-	C
$\beta\gamma$	+	-	C	+	+	C/T

TM, melting temperature; +, indicating a fluorescence peak at the corresponding temperature; -, indicating no peak existing.
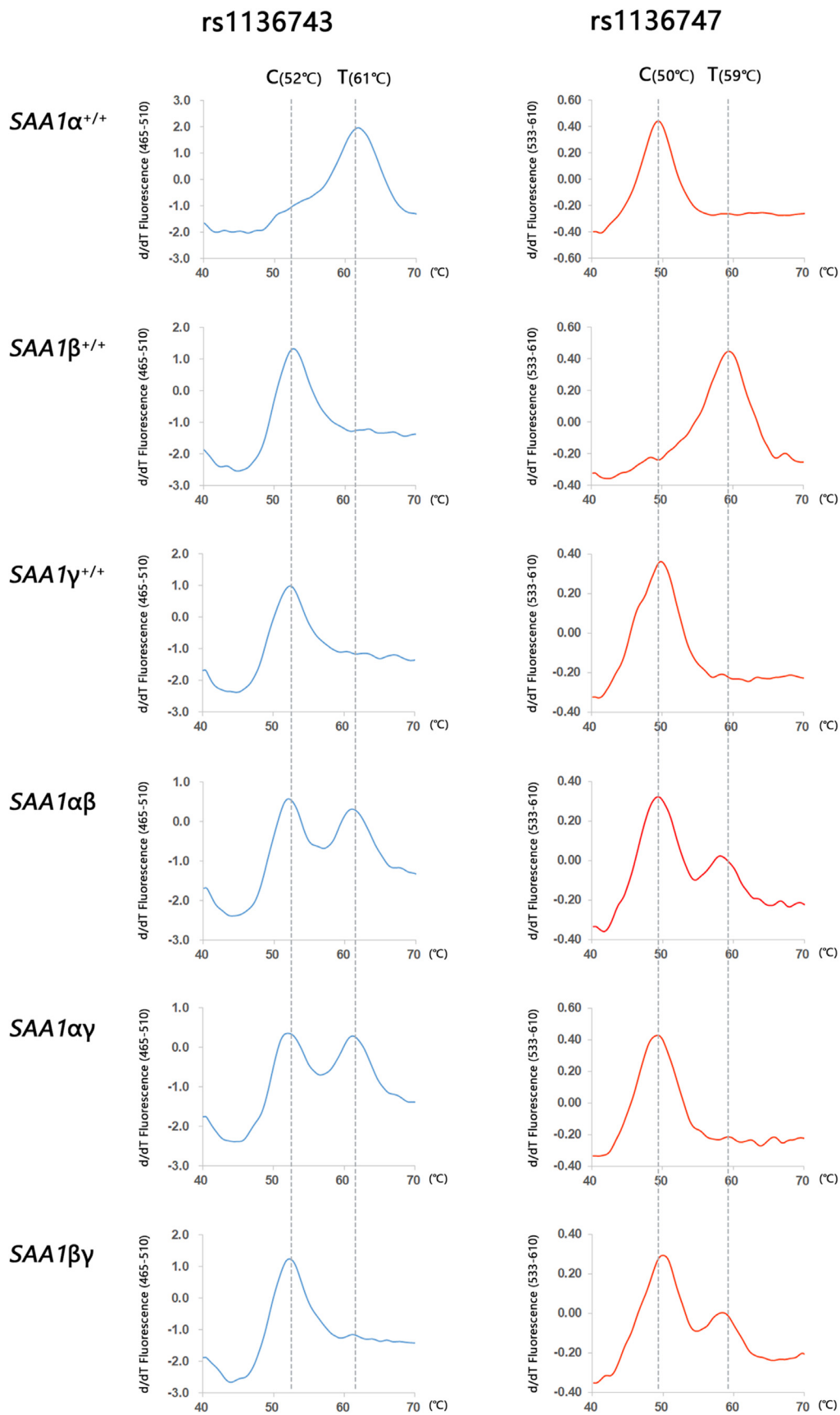
Figure 2. Validation of the QP method by plasmids inserted different *SAA1* allelic variants. For homozygotes, each genotype were detected as one peak at rs1136743 and one peak at rs1136747. For heterozygotes, *SAA1*αβ were detected as two peaks at rs1136743 and two peaks at rs1136747; *SAA1*αγ were detected as two peaks at rs1136743 and one peaks at rs1136747; *SAA1*βγ were detected as one peak at rs1136743 and two peaks at rs1136747.

for rs1136747; *SAA1*αγ were detected as two peaks for rs1136743 and one peaks at 50 °C for rs1136747; *SAA1*βγ were detected as one peak at 52 °C for rs1136743 and two peaks for rs1136747. Taken together, the fluorescent quenching probes based method with a fully automated I-densy system we reparted here could be used to identify the six genotypes of *SAA1* ($\alpha^{+/+}$, $\beta^{+/+}$, $\gamma^{+/+}$, αβ, αγ and βγ).

3.2. Whole blood sample testing in a fully automated system

In order to evaluate the clinical application of our fluorescent quenching probes based method with a fully automated I-densy system, the peripheral blood from anonymous donors were directly analyzed. At the meanwhile, the genomic DNA extracted from anonymous donors' peripheral blood was used for PCR direct sequencing, in which the forward Primer 1 and reverse-sequencing Primer 2 were used.

Total 121 clinical samples were tested by the fluorescent quenching probes based method with a fully automated I-densy system and PCR direct sequencing. The *SAA1* genotyping results from two methods were shown in Table 2. As expected, the genotyping results derived from our system and the PCR direct sequencing methods was identical. indicating that our QP method is reliable for *SAA1* genotyping. The samples were 12 (9.9%)$\alpha^{+/+}$, 2 (1.7%) $\beta^{+/+}$, 23 (19.0%) $\gamma^{+/+}$, 21 (17.4%) αβ, 31 (25.6%) αγ and 32 (26.4%) βγ, respectively. Taken together, the QP method on the fully automated I-densy system is a rapid and reliable strategy for the *SAA1* genotyping and future clinical application.

4. Discussion

Serum amyloid A 1 is produced mainly in liver after pro-inflammatory cytokines stimulation. Polymorphisms in the gene coding for *SAA1* have been identified as a risk factor of developing amyloidosis. Caucasian patients with $\alpha^{+/+}$ genotype have a higher risk of developing amyloidosis, probably because *SAA1* is more sensitive to degradation by MMP-1 [12].

To investigate *SAA1* genotype, current methods such as PCR direct sequencing (DS), restriction fragment length polymorphism (RFLP) and allele-specific PCR applied for the detection of associated SNP sites are insufficient in terms of convenience, easy operation. a reliable detection method that combines simplicity and ease of use is desirable.

Our data show that the fluorescent quenching probes based method with a fully automated I-densy system described above is practicable and reliable, making it suitable for genotyping *SAA1*. The invention is based on specific nested PCR amplification of the targeting SNPs of *SAA1* gene. The nested PCR could get rid of the interference of *SAA2*. The system we developed could determine the genotypes by monitoring the fluorescence intensity of two QPs. Furthermore, the fully automated system, I-densy, for whole blood samples testing, provides easy option for gene analysis.

In conclusion, we have determined that the nested PCR associated QP assay described here is a useful and reliable method for *SAA1* genotyping. It would be a powerful tool for research study and clinical application.

Table 2. Comparison of the quenching probe method and direct sequencing for genotypes of *SAA1*.

genotypes	QP*		DS**	
	n***	%	n***	%
$\alpha^{+/+}$	12	9.9	12	9.9
$\beta^{+/+}$	2	1.7	2	1.7
$\gamma^{+/+}$	23	19.0	23	19.0
αβ	21	17.4	21	17.4
αγ	31	25.6	31	25.6
βγ	32	26.4	32	26.4
Total	121	100	121	100

*, quenching probe method; **, direct sequencing; ***, number.

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