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A novel method of detecting alpha-1 antitrypsin deficiency of Z mutant (GAG^{342}AAG) in a single PCR reaction using base-quenched probe

Li Qin [a], Guanghua Luo [a,*], Jun Zhang [a], Ning Xu [b,**]

[a] Comprehensive Laboratory, Changzhou Key Lab of Individualized Diagnosis and Treatment Associated with High Technology Research, The Third Affiliated Hospital of Soochow University, Changzhou 213003, China
[b] Division of Clinical Chemistry and Pharmacology, Department of Laboratory Medicine, Lunds University, S-221 85 Lund, Sweden

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ABSTRACT

Background: Alpha-1 antitrypsin (A1AT) is a protease inhibitor that protects the tissues from degradation by neutrophil elastase under certain pathological process. Alpha-1 antitrypsin deficiency (A1ATD) could associate with both lung and liver pathogenicities. Of all the deficiency alleles, Z mutant is the most common variant and causes severe complications. Here, we described a novel and quick method to detect Z mutant using the base-quenched probe technique in only one single PCR reaction.
Methods: Primers and probe were designed based on the base-quenched probe technique. Two vectors, representing the two genotypes, were constructed as amplification templates for validating the method. The Z mutant (GAG^{342}AAG) was analyzed according to the melting curve. Finally, the accuracy was confirmed by direct sequencing.
Results: Z mutant could be accurately distinguished from the wild type. The wild type resulted in high melting temperature (TM) (48.64 ± 1.33 °C), while when the Z mutation was present, the TM was shifted to an obvious low TM (41.38 ± 0.9017 °C). The sensitivity reached a low of 10^3 copies of template DNA with a clear melting valley and a complete concordance occurred between this method and the direct DNA sequencing.
Conclusion: The present described method is simple, quick and economic as well as suitable for large-scale genotyping studies and clinical testing of Z mutant in patients with emphysema and cirrhosis.

1. Introduction

Alpha-1 antitrypsin deficiency (A1ATD) is one of the most common potentially fatal hereditary diseases in recent years. The gene encoding alpha-1 antitrypsin (A1AT), called *SERPINA1*, is located on chromosome 14 at q31–32.3 [1]. More than 200 genetic alterations of *SERPINA1* have been reported, of which the Z and S mutants are the most common and clinically relevant in A1ATD [2,3]. The Z mutant is a single nucleotide alteration resulting in an amino acid substitution at position 342 (Glu^{342}, *GAG* → Lys, *AAG*) [4,5]. Many patients with clinical manifestations of A1ATD are diagnosed with the type PiZZ (Pi = Proteinase inhibitor) variant. Patients have many clinical implications primarily affecting the lungs and liver. Infants with prolonged jaundice after birth with conjugated hyperbilirubinemia and abnormal liver enzyme values are clinically suspected to carry the genotype [3]. Liver damage and cirrhosis will occur at childhood or even at ages above 50 [6]. Chronic obstructive pulmonary disease (COPD) is most commonly induced by smoking and genetic susceptibility. The most well studied COPD-inducing genetic condition is A1ATD [5]. The incidence of emphysema and liver disease in patients with A1ATD may relate to environmental and genetic factors.

The diagnosis of A1ATD is based on the analysis of *A1AT* genotype and phenotype. The determination of serum A1AT levels is useful as a screening test. As A1AT is also an acute-phase protein, serum levels of A1AT can be relatively increased in patients with acute or chronic inflammatory conditions such as infections, stress and some cancers [7,8]. This may in turn result in false normal plasma levels of A1AT in patients with A1ATD. Phenotype determination is required to confirm the diagnosis of A1ATD and is performed in patients whose A1AT levels are below the normal range or close to borderline levels [9]. Isoelectric focusing (IEF) is the classical method used to determine the so-called *A1AT* 'phenotype' and is considered a 'gold standard' for identifying A1AT variants [2,8]. However, the IEF method is laborious and time consuming. Molecular genotyping is effective, more cost efficient and can be utilized to identify individuals with S or Z mutants [10].

Conventional molecular genotyping methods to detect signal nucleotide polymorphisms (SNPs), including restriction fragment length polymorphism (RFLP) [11], single strand conformation polymorphism (SSCP) [12] and melt-curve analysis with fluorescence resonance energy transfer (FRET) probes using two fluorescent dyes specific to the

* Correspondence to: G. Luo, Comprehensive Laboratory, The Third Affiliated Hospital of Soochow University, Changzhou 213003, China. Tel.: +86 519 68870619.
** Corresponding author. Tel.: +46 46 173487; fax: +46 46 130064.
E-mail addresses: shineroar@163.com (G. Luo), ning.xu@med.lu.se (N. Xu).

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Table 1
Sequences of primers and probe of Z mutant (GAG^{342}AAG).

Primer/probe	Length	Sequence (5′ → 3′)
Sense primer	23 bp	5′ aaaggcgaccaatgaacaactgc 3′
Antisense primer	20 bp	5′ gccccagcagcttcagtccc 3′
Probe	14 bp	5′ ccatcgacgagaaa 3′-FAM

Underline indicates polymorphic nucleotide.

region of the gene where the mutation is located [13] are all limited by cumbersome protocols. Kaczor et al. [14] developed a genotyping method for the detection of alleles S and Z using dual-labeled target-specific fluorescent probes. However, this method also requires the design of two probes for the Z mutation site. In this paper, we describe a simple, fast, and economic method of identifying SNPs that requires only one fluorescent-labeled probe and does not invariably depend on the deoxyguanosine nucleotide [15]. We have previously used this method to detect various other SNPs (e.g. apoM, mitochondrial DNA) [16]. In this study, we applied it for detecting the Z mutant (GAG^{342}AAG) in A1ATD. Real-time fluorescence assay (as cited in the reference [14]) was used to validate our results.

2. Materials and methods

2.1. Materials

The Blood DNA isolation kit, Taq DNA polymerase, 4×deoxynucleoside triphosphates (4×dNTPs), 10×polymerase chain reaction (PCR) buffer and $MgCl_2$ were obtained from Shenergy Biocolor (Shanghai, China). Primers and probe (Table 1) were synthesized and fluorescence modified by Sangon (Shanghai, China).

2.2. Patient samples

145 patients were obtained from The Third Affiliated Hospital of Soochow University between July 2012 and April 2013 including 120 patients diagnosed clinically with COPD and 25 patients with liver cirrhosis. Additionally, 210 subjects were recruited from Kuitun Hospital in Kuitun, Xinjiang, including 130 subjects of Uygur and 80 subjects of Kazakh origin. All participants were informed by written consent.

2.3. Vector constructions

Two vectors, representing the two genotypes (G homozygote, A homozygote), were constructed as amplification templates for validating the method. Two 464-bp fragments of *A1AT* were synthesized by Sangon Biotech (Shanghai, China). These fragments were digested and connected into the vector PUC57, then amplified. The plasmid was then extracted and used as the amplification templates (Fig. 1).

2.4. Real-time PCR and melting curve analyses

Genomic DNA of blood samples was extracted by the 3S blood DNA isolation kit according to the manufacturer's instructions. Primers and probe of *A1AT* Z mutant were designed according to the sequence in the NCBI (NT-026437). A 464-bp fragment of the *A1AT* was amplified with specific primers from human genomic DNA. PCR reactions were performed as follows: total volume of 25 μl containing 2 μl of the genomic DNA template, 2.5 μl of 10×PCR buffer, 1.5 μl of 25 mM $MgCl_2$, 0.5 μl of 0.2 mM 4×dNTP, 1.25 U Taq DNA polymerase, 0.1 μl of 100 μM of each primer, and 0.2 μl of 10 μM probes. Thermal cycling for PiZ mutation was performed on a LightCycler (version 480II, Roche) under the following conditions: the cycling program consisted of 5 min of initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 1 s (temperature transition rate 4.4 °C/s), 65 °C for 45 s. The analytical melting program involved heating the amplicon/probe heteroduplex at 95 °C for 1 min, 35 °C for 2 min, and increased to 70 °C at a temperature transition rate of 0.06 °C/s, with continuous acquisition of fluorescence data. The emission of FAM is largely quenched by the adjacent base (A, T, C, or G), whereas FAM-labeled probe hybridizes to a part of the target sequence at a lower temperature (e.g., 35 °C) after the PCR. The probe subsequently melts off when the temperature is slowly increased. Concurrently, the fluorescence is largely increased. When mutated genotypes are present, it will occur at different temperatures compared with the wild-type genotype.

Mg^{2+} concentration is an important factor for controlling the specificity of PCR reactions and melting temperatures (TMs). Insufficient Mg^{2+} concentration resulted in a low yield of PCR products. However, excessive Mg^{2+} could increase non-specific products and

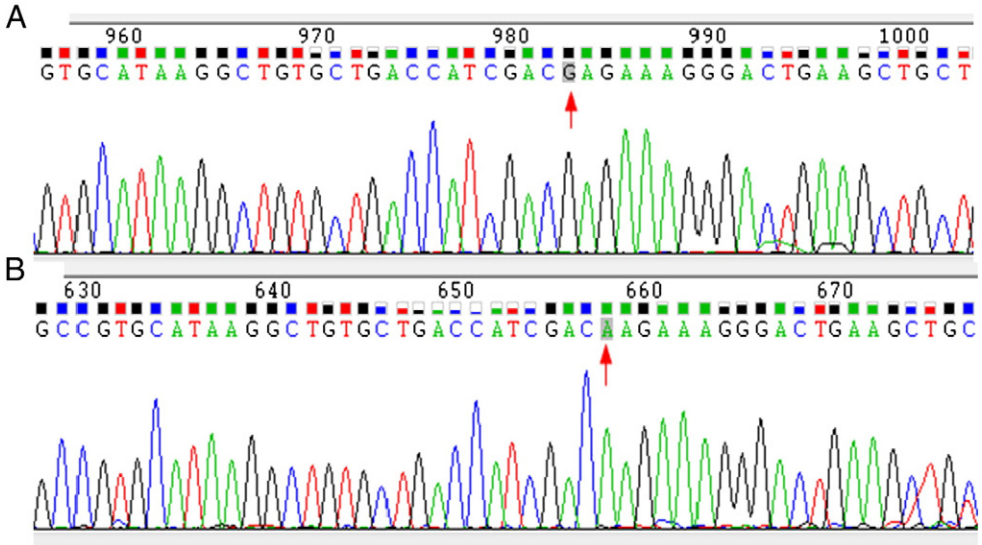

Fig. 1. Vector construction A: The sequences are identified as wild-type. B: The sequences are identified as PiZZ mutant. The red arrows show the mutant alleles.