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A comparison of restriction fragment length polymorphism, tetra primer amplification refractory mutation system PCR and unlabeled probe melting analysis for LTA + 252 C>T SNP genotyping

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ABSTRACT

Background: From the wide range of methods currently available for genotyping, we wished to identify a quick, reliable and affordable approach for routine use in our laboratory for LTA + 252 C>T SNP screening.
Methods: We set up and compared three genotyping methods for SNP detection: restriction fragment length polymorphism (RFLP), tetra primer amplification refractory mutation system PCR (TPAP) and unlabeled probe melting analysis (UPMA). The SNP model used was LTA + 252 C>T, a cytokine gene polymorphism that has been associated with response to treatment in rheumatoid arthritis. The study was performed using 46 samples from healthy Caucasian volunteers.
Results: Allele and genotype distribution was similar to that previously described in the same population. All three genotyping methods showed good reproducibility and are suitable for a medium scale throughput molecular platform. UPMA was the most cost effective, reliable and safe method since it required the shortest technician time, could be performed in a single closed tube and involved automatic data analysis.
Conclusion: This work is the first to compare these three genotyping techniques and provides evidence for UPMA being the method of choice for LTA + 252 C>T SNP genotyping.

1. Introduction

Inter-individual variations in response to drugs, which are mostly inherited, are common and pose a substantial clinical problem [1]. Single nucleotide polymorphisms (SNPs) in pivotal genes such as those recorded in pharmGKB (The Pharmacogenetics and Pharmacogenomics Knowledge Base) play an important role in pharmacogenetics [2]. Intensive efforts have therefore been made to develop straightforward and affordable methods for SNP genotyping.

Our laboratory has been involved in the study of auto-inflammatory diseases for over 15 years. Lymphotoxin alpha (LTA, also known as tumor necrosis factor Beta), is a pro-inflammatory cytokine mainly secreted by macrophages and T-cells. It is produced in response to tissue injury by all cells playing a key role in both inflammation and the control of lipid metabolism. LTA is located within the TNF gene cluster which lies in the class-III region of the highly polymorphic major histocompatibility complex on human chromosome 6p21 (OMIM access number 153440, NCBI GeneID 4049) [3]. The LTA + 252 T>C SNP [3], HGVS nomenclature: NM_000595.2:c.-10 + 90A>G (rs909253), has been associated with susceptibility to myocardial infarction, systemic lupus erythematosus [4], breast cancer [5], and systemic sclerosis [6]. This SNP has also been implicated in the response to treatment of patients suffering rheumatoid arthritis [7].

The genotyping assay restriction fragment length polymorphism (RFLP), involves PCR amplification followed by restriction enzyme digestion. Other methods are based on differential hybridization (e.g.; allele-specific oligonucleotide hybridization and fluorescence probes), melting temperature-based separation (single strand conformational polymorphism, heteroduplex analysis, denaturing high performance liquid chromatography), allele specific amplification, DNA sequencing or primer extension. We used LTA as a model to compare 3 genotyping methods, RFLP and two novel techniques, unlabeled PCR melting analysis (UPMA), and tetraprimer ARMS PCR (TPAP) assay, in terms of reliability, cost-effectiveness and speed.

Abbreviations: SNP, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism; TPAP, tetra primers; ARMS, (amplification refractory mutation system) polymerase chain reaction; UPMA, unlabeled probe melting assay.

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2. Materials and methods

2.1. Samples and DNA extraction

DNA was extracted from 46 donated blood samples from healthy Caucasian volunteers by QIAamp DNA Blood Maxi Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's guidelines. Negative controls without DNA were included in each assay. Our protocol was approved by the local ethics committee. A signed informed consent was obtained from all study participants.

2.2. Primer design

All primers were designed using batchprimer3 (Table 1) [8]. The PCR primers and the unlabeled probe were purchased from Eurogentec (Angers, France). Table 1 shows the primer sequences and final concentrations. For sequencing, we used the same primers as for UPMA.

2.3. PCR-RFLP

PCR reactions were carried out in a total volume of 25 µl containing 100 ng template DNA, 0.6U AmpliTaq gold® polymerase (Applera SA, Courtaboeuf, France), 0.2 mM dNTP, 3 mM MgCl2, and 5% DMSO.

The PCR conditions were as follows: an initial denaturation at 94 °C for 9 min, followed by 35 amplification cycles, each consisting of a denaturation step at 94 °C for 20 s, annealing at 58 °C for 20 s and extension at 72 °C for 20 s followed by a final extension at 72 °C for 9 min. Ten microliters of the PCR product were digested with 5 U NcoI restriction enzyme (OZYME, Saint Quentin en Yvelines, France) for 3 h at 37 °C. This enzyme cuts allele C. The digested products were then separated on ethidium bromide-stained 10% acrylamide gels. The expected sizes of the digested products were 189 bp for the T/T homozygotes, 102 and 87 bp for the C/C homozygotes, and all three bands for C/T heterozygotes.

2.4. Tetra-primer ARMS (TPAP)

The method has been described in detail by Ye et al. [9]. Briefly, the method involves a multiplex PCR including two outer (forward and reverse) primers common to the two alleles, and two inner (forward and reverse) primers specific to the C and T sequence variations (Table 1). To increase the inner primer specificity, we introduced a mismatch 3 bp upstream of the 3′ end. The outer primers generate a common 244 bp amplicon. Since the outer primers are not equidistant from the SNP location, the C and T amplicons are of different sizes (C allele, 121 bp and T allele, 169 bp). Homozygotes therefore display two PCR products and heterozygotes three.

The PCR reactions were carried out in a total volume of 20 µl containing 75 ng template DNA and 1.5 U AmpliTaq gold® polymerase (Applera SA, Courtaboeuf, France), 0.2 mM dNTP, 1.5 mM MgCl2 and 8% dimethylsulfoxide (DMSO).

The PCR conditions were as follows: an initial denaturation at 94 °C for 9 min, followed by 35 amplification cycles each consisting of a denaturation step at 94 °C for 20 s, annealing at 61 °C for 20 s and extension at 72 °C for 15 s followed by a final extension at 72 °C for 9 min. The PCR products were separated on ethidium bromide-stained 10% polyacrylamide gels. The common band of 244 bp corresponding to the outer primers was considered as an internal control. The additional bands of either 169 or 121 bp discriminated between the T and the C alleles, respectively.

2.5. Unlabeled probe melting analysis (UPMA)

The nearest neighbour thermodynamic model was applied (Fig. 1) using the Poland and Fixman–Freire algorithms (parameter set of Blake and Delcourt) (www.biophys.uni-duesseldorf.de/POLAND/poland.html) to predict the probe melting curves *in silico* [10]. The probe melting behavior could be defined with (Tm = 52 °C) or without (Tm = 63 °C) the mismatch. A ΔTm of about 10 °C was found to be enough to discriminate the two variants (not shown).

We chose the wild type sequence as the unlabeled probe (Table 1). The unlabeled (blocked) probe incorporated a 3′Phosphate modifier to prevent DNA polymerase extension during PCR [11].

An asymmetric PCR was carried out in 96 microplates to generate an excess of the probe target strand. The PCR mix contained 50 ng DNA, 2 mM MgCl2, 0.2 mM dNTP, 1× LightCycler® 480 Resolight Dye (Roche applied science, Meylan, France), 1 U FastStart Taq DNA polymerase (Roche applied science, Meylan, France), 1 unlabeled probe, and 2 primers (Table 1) in a 10 µl final reaction volume.

The plates were sealed with LightCycler® 480 sealing foil (Roche applied science, Meylan, France) and spun at 1000 RPM for 1 min before being loaded into the LightCycler® 480 instrument (Roche applied science, Meylan, France).

The PCR conditions were: polymerase activation at 95 °C for 4 min, followed by 50 PCR cycles each consisting of a denaturation step at 95 °C for 10 s, annealing at 56 °C for 25 s, and extension at 72 °C for 10 s. After PCR, probe/single strand DNA duplexes were generated by heating samples up to 95 °C for 90 s, then cooling them down to 40 °C for 1 min. We next performed a melting assay between 50 °C and 90 °C calibrated for the acquisition of 25 fluorescence points per °C. The data were collected using the LightCycler® 480 melting curves analysis mode, and analyzed between 58 °C and 72 °C. The melting data were directly converted into a derivative plot using the LightCycler® 480 software module MeltCurve Genotyping.

2.6. DNA sequencing

All samples were also sequenced to confirm the UPMA results. We performed a symmetric PCR using the same UPMA primers (Table 1).

Table 1
Oligonucleotides used for PCR–RFLP, TPAP and UPMA.

Methods	Primers	Sequences	Final concentration
PCR–RFLP	Forward primer	5′-CCCGAGAGAGAGATCGACAG-3′	0.2 µM
	Reverse primer	5′-GGGTTTGGTTTTGGTTTCCT-3′	0.2 µM
TPAP	Outer Forward primer	5′-AGAGAGAGATCGACAGAGAAGGGGAC-3′	0.3 µM
	Outer Reverse primer	5′-CTCGGTCCCTCCTGCACCT-3′	0.2 µM
	Inner Forward primer	5′-ACAGGAAGGGAACAGAGAGGCA**T**-3′	0.5 µM
	Inner Reverse primer	5′-ACACATTCTCTGTTTCTGCCAG**G**-3′	1.2 µM
UPMA	Forward primer	5′-CCCGAGAGAGAGATCGACAG-3′	0.02 µM
	Reverse primer	5′-GGGTTTGGTTTTGGTTTCCT-3′	1.5 µM
	3′Blocked probe	5′-AACAGAGAGGAA**C**CATGGCAGAAAC-3′P	0.4 µM

RFLP (restriction fragment length polymorphism) TPAP (tetra primer amplification refractory mutation system PCR) UPMA (unlabelled probe melting analysis).
N Location of the base mismatch added to improve the specificity of SNP detection (forward primer A>C, reverse primer T>G).
N Position of the LTA + 252 C>T SNP.