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RESEARCH PAPER

Genotyping of Alcohol Dehydrogenase Gene by Pyrosequencing Coupled with Improved Linear-after-the-Exponential Polymerase Chain Reaction Using Human Whole Blood as Starting Material

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Abstract: Pyrosequencing is one of principal methods to detect genetic polymorphism currently, but the complicated sample pretreatment procedure limits its application in clinical tests. In order to simplify the process of pyrosequencing, on the basis of the linear-after-the-exponential-polymerase chain reaction (LATE-PCR), we improved the primer design method of LATE-PCR by increasing the length and the concentration of the excess primer and using whole blood as template for direct amplification, and established an improved LATE-PCR (imLATE-PCR) method on the basis of common rTaq polymerase and high-pH buffer (HpH Buffer). The amplification conditions and the amount of whole blood template were optimized and the influence of blood anticoagulant was investigated. By amplifying the whole blood in a single tube with one-step process to get the sequencing template, alcohol dehydrogenase (ADH) gene polymorphisms of 24 clinical blood samples were successfully detected, and the results could served as a guide for individualized medication clinically. The genotypes of ADH1B locus of 24 samples are 6 cases of AA homozygote, 14 cases of AG heterozygote, and 4 cases of GG homozygote. The genotypes of ADH1C are 20 cases of GG homozygote, 4 cases of AG heterozygote, with no cases of AA homozygote.

Key Words: Whole blood-polymerase chain reaction; Improved linear-after-the-exponential-polymerase chain reaction; Pyrosequencing; Gene polymorphism; Alcohol metabolism

1 Introduction

Pyrosequencing is a nucleic acid sequencing method based on the luminescence of fluorescein resulting from cascade enzymatic reactions triggered by inorganic pyrophosphate (PPi), which is released from the binding of complementary nucleotide to the single chain template and the subsequent elongation of the primer[1–3]. Up to now, pyrosequencing was applied in the detection of gene polymorphism[4], microbial typing[5], methylation analysis[6] etc. The large-scale DNA sequencing[7] was achieved in application. Nowadays, single-stranded templates for pyrosequencing are mainly prepared by solid-phase microsphere-based method[8], but with a complicated procedure and low efficiency. In addition, the use of biotinlyated primers and streptavidin-coated microspheres increases the detection cost, and cross-contamination of samples during the preparation of single-stranded primers often occurs. These shortcomings have greatly hindered the

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clinical application of pyrosequencing [9].

The asymmetric polymerase chain reaction (PCR) method can produce single-stranded PCR product, which may be used directly as sequencing template[10]. However, till now the conventional asymmetric PCR has not been widely used due to the low efficiency in producing single chain product. On the basis of asymmetric PCR, linear-after-the-exponential-PCR[11] (LATE-PCR) exhibited an amplification efficiency similar to symmetric PCR by optimizing primer concentration and Tm value. A high yield of single-stranded DNA (ssDNA) could be obtained to meet the requirements of single-stranded nucleic acid-based hybridization and real-time PCR[12]. However, LATE-PCR primer design requires strict criteria. With the progress of PCR amplification, the concentration of the amplicon strand increases, while excess primer concentration decreases, leading to the competition between excess primer and the amplified product, and the yield of ssDNA would be low if the amplicon sequence is long. Improved LATE-PCR (imLATE-PCR)[13] employs an excess primer with longer sequence and a restricted primer with shorter sequence to achieve a significant difference between the Tm values of the primers. In contrast to LATE-PCR, imLATE-PCR shows high flexibility in primer design and can produce longer ssDNA template, thus expanding the practical application scope of asymmetric PCR.

To increase the detection speed and reduce the possibility of sample contamination, in this study, whole blood was used as PCR template directly. Compared with conventional PCR, due to the elimination of DNA extraction step (and thus the corresponding chemical reagents and instruments used in DNA extraction), whole blood PCR has a high detection speed and a low detection cost. The simplified process avoided the risk of contamination which usually came from operations in DNA extraction process such as opening the lid repeatedly, adding the reagents, discarding the waste and transferring the templates during the DNA extraction. This method was more applicable in clinical test[14].

In this study, we coupled whole blood-based direct amplification with imLATE-PCR to establish a whole blood imLATE-PCR method using the common rTaq polymerase and high pH buffer (HpH Buffer). This method exhibited several advantages: (1) Rapidity and simplicity. By using whole blood as template, DNA extraction and single-stranded template preparation steps were eliminated, which could greatly increase the detection speed and simplify the operation process and save the detection time. (2) Low cost and environmentally friendly. Due to the elimination of DNA extraction and the ssDNA template preparation steps, expensive biotinylated primers, magnetic microspheres, and some organic reagents were not required, thus the cost was reduced. (3) Lower contamination. Cross-contamination, commonly occurring in the processes of reagents addition and solution transferring during DNA extraction and template preparation, was avoided. (4) High sensitivity. Satisfactory sequencing results could be obtained by using 0.1 μL whole blood, e.g. fingertip blood, thus facilitating the rapid clinical test.

Pyrosequencing-based detection of polymorphism of alcohol metabolism related genes ADH1B and ADH1C was carried out by using the proposed method for a quick evaluation of the alcohol metabolism in human, which is of great significance in prevention of a variety of diseases associated with drinking behaviour and supervision of common physical examination.

2 Experimental

2.1 Instruments and reagents

EDC-10 Gene amplification equipment was purchased from Dongseng biological technology Co. Ltd, China. Portable bioluminescence analyzer (Hitachi, Ltd., Central Research Laboratory, Japan) was used for pyrosequencing.

ATP sulfurylase and the *E. Coli* DNA polymerase Klenow fragment were expressed and purified in our lab. rTaq DNA polymerase and 500 bp DNA Ladder marker were purchased from TaKaRa (Dalian, China). Polyvinylpyrrolidone (PVP) and Quan-tiLum recombinant luciferase were purchased from Promega (Madison, USA). Apyrase-VII, adenosine 5'-phosphosulfate (APS) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). Streptavidin Sepharose™ beads were purchased from GE Healthcare (New Jersey, USA). dATPαS, dGTP, dTTP and dCTP were purchased from Amersham Pharmacia (San Diego, USA). Other chemicals were of analytical grade. All solutions were prepared with sterilized double-distilled water.

All primers were synthesized by Invitrogen (Shanghai, China), and the sequences are shown in Table 1.

2.2 Experimental

2.2.1 Primer design for ImLATE-PCR

Two polymorphism loci commonly occurring in Chinese people in alcohol metabolism related alcohol dehydrogenase (ADH) gene were investigated by imLATE-PCR in this study. The primers were designed using the software Primer 5.0 according to the following principles: $Tm^E - Tm^L \geq 5°C$; $Tm^A - Tm^E \leq 13°C$[13] (Tm^E means the *T*m value of excess primer, Tm^L means the *T*m value of limiting primer, Tm^A means the Tm value of amplicon). Software OligoAnalyzer 3.1 was used to calculate the Tm values of primers (Website URL: http://www.idtdna.com/analyzer/Applications/OligoAn-alyzer/). The sequences of PCR primers are listed in Table 1.

2.2.2 Whole blood imLATE-PCR amplification