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RESEARCH PAPER

Detection of Single Nucleotide Polymorphism Genotyping by Real-time Polymerase Chain Reaction Coupled with High Specific Invader Assay in Single Tube

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Abstract: A method was developed for genotyping of single nucleotide polymorphism (SNP) by real-time polymerase chain reaction (PCR) coupled with high specific invader assay. The concentrations of flap endonuclease 1 (FEN1 enzyme) and wild type detection probe were optimized to reduce the background signal. Under the optimal conditions including 1×buffer, 250 μM dNTPs, 0.5 μM each PCR primer, 0.05 μM invasive oligo probe, 0.125 μM wild detection probe, 0.5 μM mutant detection probe, 0.25 μM each fluorescence resonance energy transfer (FRET) probe, 1 U *Taq* DNA polymerase, 1.5 U FEN1, and approximate 40–200 ng of genomic DNA, the background signals of wild-type sample and mutant-type sample were dramatically decreased and the background interference to the esults was thus eliminated. The reaction program was initial denaturation at 95 °C for 3 min, followed by 10 cycles (95 °C for 20 s, 67 °C for 60 s, 70 °C for 30 s) and 35 cycles (95 °C for 20 s, 63 °C for 60 s, read the fluorescence signal, 70 °C for 30 s). In the experiment, a total of 21 cases of ALDH2*2, 19 cases of CYP2C19*2 and 19 cases of CYP2C19*3 were analyzed by the established method, and the results showed that the genotypes of ALDH2*2 were 10 cases of GG homozygote, 8 cases of GA heterozygote and 3 cases of AA homozygote; the genotypes of CYP2C19*2 were 9 cases of GG homozygote, 8 cases of GA heterozygote and 2 cases of AA homozygote; and the genotypes of CYP2C19*3 were 18 cases of GG homozygote and 1 case of GA heterozygote. These results were consistent with the detection results by pyrosequencing. The established method was specific, simple, short time-consuming and low cost, and could be used for SNP genotyping with no-pollution in single tube.

Key Words: Real-time polymerase chain reaction; Invader assay; Single nucleotide polymorphism; ALDH2 gene; CYP2C19 gene

1 Introduction

Single nucleotide polymorphisms (SNPs) are DNA sequence polymorphisms caused by single nucleotide variations in genome, including substitutions, transversions, insertions, and deletions. SNPs are the most common form of polymorphisms in the human genome, which are highly abundant and stable, and distributed throughout the genome and considered important factor for susceptibility to diseases and individual response to medicine[1]. Currently, the approaches for SNP genotyping are single strand conformation polymorphism (SSCP)[2], polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[3], matrix-assisted laser desorption/ionization

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time-of-flight mass spectrometry (MALDI-TOF MS)[4], pyrosequencing[5–11], Taqman technique[12] and so on. However, these techniques are time-consuming, complicated to operation, highly expensive and easy to be cross-contamination.

Invader assay is a method of signal amplification, by which a flap endonuclease 1 (FEN1) is used to cleave three-dimensional overlapping structure formed by hybridization of oligonucleotides to target DNA over the SNP site, thus the cleavage products, flap sequences, are generated, follows by a secondary cleavage of fluorescence resonance energy transfer (FRET) cassette to release a fluorescent signal[13]. Invader assay-based signal amplification technique possessed the merits of high specificity, no pollution and so on compared with common PCR-based target amplification[14]. The sensitivity of this method is low, therefore a PCR amplification is needed before reaction[15,16], but it easily lead to cross-contamination. In 2012, Zou *et al.*[15] established a novel technique of real-time PCR coupled with high specific invader assay for the detection of methylated genes in colorectal cancer by combining real-time-PCR with invader assay. The technique has the advantages including high sensitivity, good quantitative performance and non-polluting detection. Based on the previous researches, a method of real-time PCR coupled with high specific invader assay was developed for SNP genotyping. By optimization of the concentrations of FEN1 enzyme and detection probe, we successfully reduced the background signal and achieved accurate genotyping of the SNPs. The merits of this method were showed as follows: (1) Real-time monitoring of results. As PCR amplification and invader assay were carried out at the same time, it was possible to read the results in real time. (2) High specificity. FEN1 enzyme could recognize and cleave the three-dimensional overlapping structure formed by target sequence, invasive oligo probe and detection probe. Without overlapping structure, enzyme activity was weak. (3) Good versatility. FRET probes were universal for three SNPs. (4) No pollution. The step of opening the lip was omitted during operating so that the method could reduce the possibility of cross-contamination. (5) Easy operation and fast detection. Experimental operation was easy and fast because it just contained the preparation of template and reaction system.

Clinical studies show that nitroglycerin is widely used in the treatment of coronary heart disease. By the metabolism of a key enzyme aldehyde dehydrogenase 2 (ALDH2) in the body, nitroglycerin releases pharmacological active NO, which can consequently relax vascular smooth muscle[17]. But the ALDH2*2 polymorphism will affect the release of NO and reduce drug efficacy. Moreover, ALDH2 can make intermediate metabolite of alcohol, acetaldehyde, further metabolize into non-toxic products, while ALDH2*2 polymorphism will accumulate the acetaldehyde in the body[18], which finally damages the liver, brain and other tissues and prompts the risk of suffering gastrointestinal cancer, liver cancer, colorectal cancer, breast cancer, and so on[19]. In clinically, clopidogrel is widely used in diminishing platelet inhibition, and as a prodrug, it requires biotransformation of cytochrome P450 2C19 (CYP2C19) enzyme to generate its active metabolite to achieve the anticoagulant effect[20]. CYP2C19*2 and CYP2C19*3 polymorphisms will reduce enzyme activity, resulting in decrease in drug efficacy and increase in adverse cardiovascular events[21]. Thus, the detection of these specific genotypes helps to guide rational drug use. So we established the real-time PCR coupled with high specific invader assay for genotyping of ALDH2*2, CYP2C19*2 and CYP2C19*3.

2 Experimental

2.1 Instruments and reagents

Rotor-gene Q real-time PCR system was purchased from QIAGEN, Germany.

FEN1 enzyme was self-expression by laboratory[22]. *Taq* DNA polymerase and Wizard® Genomic DNA Purification Kit were obtained from Promega, USA. 3-(nmorpholino) propanesulfonic acid (MOPS) was purchased from Amresco, USA. $MgCl_2$ was from NEB, USA. Other reagents here were analytical grade. Experimental water was sterilized double distilled water. The primers and probes based on target sequence were designed by Universal Invader™ software. All oligo-nucleotides were synthesized by Shanghai Invitrogen Corporation, as shown in Table 1.

2.2 Method

The conditions of reaction included 1 × buffer (10 mM MOPS, 7.5 mM $MgCl_2$, pH 7.5), 250 μM dNTPs, 0.5 μM each PCR primer, 0.05 μM invasive oligo probe, 0.125 μM wild detection probe, 0.5 μM mutant detection probe, 0.25 μM each FRET probe, 1 U *Taq* DNA polymerase, 1.5 U FEN1, and approximate 40–200 ng genomic DNA.

The reaction program was initial denaturation at 95 °C for 3 min, followed by 10 cycles (95 °C for 20 s, 67 °C for 60 s, 70 °C for 30 s) and 35 cycles (95 °C for 20 s, 63 °C for 60 s, read the fluorescence signal, 70°C for 30 s).

3 Results and discussion

3.1 Principle of real-time PCR coupled with high specific invader assay for SNP genotyping

The schematic of real-time PCR coupled with high specific invader assay for SNP genotyping is illustrated in Fig.1. We first designed the primers of PCR amplification and the probes of invader assay, and ensured the melting temperature (T_m) of