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Research paper

Discrimination of mitochondrial DNA 10400 locus by SNP-operated on/off Switch ☆

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Abstract

Objective: To apply reformed AS-PCR, which combined phosphorothioate-modified primers with exo$^+$ polymerase, in single nucleotide polymorphism discrimination of mitochondrial DNA 10400 locus. **Methods:** We used the mtDNA 10400 locus to design unmodified and 3′ phosphorothioate-modified allele-specific primers for PCR, which was performed using polymerases with and without 3′ exonuclease activities. The effects of these primers on primer-extension were evaluated by agarose gel electrophoresis. **Results:** The unmodified primers were extended by both exo$^-$ and exo$^+$ polymerase irrespective of whether the primers were matched or mismatched with the templates. However, the 3′ phosphorothioate-modified primers with a terminal mismatch triggered an "off-switch" of exo$^+$ polymerase when compared to exo$^-$ polymerase. **Conclusion:** The "on/off" switch constituted by the combination of 3′ phosphorothioate-modified primers with exo$^+$ polymerase is a cost-effective, high-throughput and reliable method for SNP typing, which will be of enormous application in association studies by single nucleotide polymorphism screening.

Key words: SNP; exo$^+$ polymerase; phosphorothioate-modification; allele-specific PCR; mitochondrial DNA

INTRODUCTION

Single nucleotide polymorphisms(SNPs) are single base-pair alterations in the DNA sequence that represent a major source of genetic heterogeneity. They are widely distributed in nuclear DNA and mitochondrial DNA(mtDNA), such as the mtDNA T10400C locus in the coding region[1]. They are exceedingly common polymorphisms, accounting for approximately 90% of all known sequence variations, and are estimated to occur every 100-300 base pairs[2]. Their abundance, despite their simplicity and rather limited polymorphic content, is the main reason for their current enormous employment in the biomedical field: SNPs are used as markers to identify the genes that underlie complex diseases and to realize the full potential of pharmacogenomics in analyzing variable responses to drugs[3]. In the forensic field, the interest in SNPs is also continuously increasing, due to the potential advantages in paternity testing because of the low mutation rates and especially in the analysis of degraded samples by use of short amplicons[4]. Therefore, efficient screening of known SNPs is of paramount importance in maximizing the value of sequence data to fundamental research and individualized medicine.

Although more than 20 different algorithms have been developed for SNP analysis, better SNP assays with more efficiency and accuracy are urgently needed for clinical application and for genomewide SNP screening. Among these assays, strategies using allele-specific polymerase chain reaction(AS-PCR) with Taq DNA polymerase and 3′ allele-specific primers play an important part in the development of SNP screening methods[5-8]. AS-PCR relies on the difference in extension efficiency of DNA polymerase between primers with matched and mismatched 3′ ends. DNA polymerase extends a primer only when the 3′ end is perfectly complementary to the DNA templates, and generates no products from mismatched primers(***Fig. 1***). The

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principle of this technique involves using two primers, one for each allele of a SNP. By detecting which primer forms the products, the genotype of a sample can be determined. However, AS-PCRs are actually compromised by their low reliability. Many researchers discovered that Taq DNA polymerase could yield identical products even when the 3′ end of primers is non-complementary to the templates[9]. These false positive results given by the mismatched primer extensions disturbed the genotype judgment, which remains one of the major obstacles to the effective application of AS-PCR.

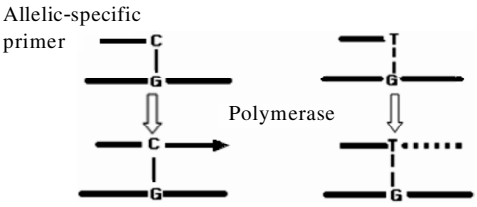


Fig. 1 Illustration of allele-specific PCR: The 3′ end of the primers is complementary to each allele of the SNP. When there is a perfect match the primer is extended

It has been well known for decades that high fidelity DNA polymerase with 3′ exonuclease activities exhibit higher nucleotide identification ability when compared to exo⁻polymerase(Taq) for a genetic analysis. The high fidelity of exo⁺ polymerase in DNA replication is a consequence of mismatched nucleotide removal before extension according to the hybridized templates[10,11]. Furthermore, in initial reports, primers at the 3′ end with a single phosphorothioate(PS)-modification could effectively protect the oligodeoxynucleotide degradation from exo⁺polymerase[12,13]. Here, we applied 3′ PS-modified primers and high fidelity polymerase(exo⁺ polymerase) in routine AS-PCR and tested its nucleotide discrimination ability by detecting the mtDNA 10400 SNP locus.

MATERIALS AND METHODS

Materials

Chelex-100 was purchased from Sigma Chemical Co. (St Louis, MO, USA). Agarose was purchased from Promega Corp.(Madison,Wi,USA). All phosphorothioate-modified and unmodified primers were synthesized commercially by Shanghai Sangon Biological Engineering Technology&Services Co. Ltd. (Shanghai, China). Taq and Pfu DNA polymerase were obtained from Tiangen Biotech Co. Ltd. (Beijing, China). Pfu is a high fidelity polymerase with internal 3′ to 5′ exonuclease activities while Taq is a low fidelity polymerase lacking this function.

Primer design

According to revised Cambridge Reference Sequence (rCRS), the primers for mtDNA 10400T were designed as follows:the forward primer(5′ taattatacaaaaaggattagactgagcT3′) and reverse primer (5′ gaagtgagatggtaaatgctag3′). The expected size of products was 149 bp. An 88 bp fragment was amplified for mtDNA 10400C with the forward primer (5′ aatcatcatcctagccctaagtctgg3′) and reverse primer(5′ cgttttgtttaaactatataccaattcG3′). The allele-specific primers, mtDNA 10400T forward primer and mtDNA 10400C reverse primer, were both 3′ phosphorothioate-modified and unmodified. So there were four pairs of primers for subsequent PCR and the primers from mtDNA 10400T were considered to be mismatched primers of mtDNA 10400C, and vice versa.

Preparation of DNA templates

MtDNA extracted from peripheral blood by a chelex100 method[14] was typed with direct DNA sequencing(Shanghai Sangon Biological Engineering Technology&Services Co., Ltd., Shanghai, China). The samples 1 and 2, of which mtDNA 10400 locus is T and C respectively, were chosen as the templates(***Fig. 2, 3***). The blood samples were from our routine casework and all the individuals gave their informed consent.

PCR

Four pairs of primers were used to amplify S1 and S2, respectively. The volume of each PCR system was 20 μl, comprised of 10 × PCR reaction buffer, 2 μl; 2.5 mmol/L dNTPs,0.96 μl; 25 mmol/L $MgCl_2$,1.2 μl;

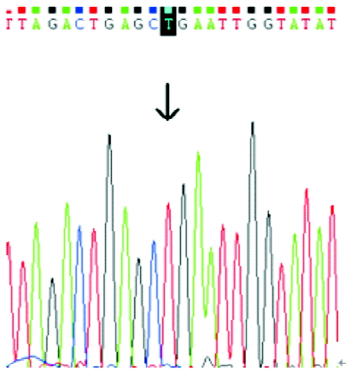


Fig. 2 The representative DNA sequence of S1 at 10 400 locus

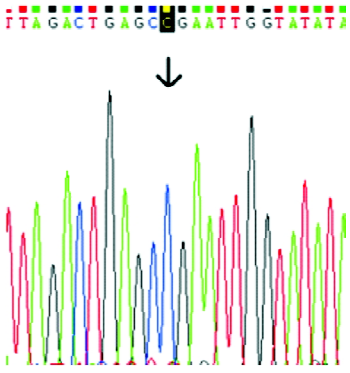


Fig. 3 The representative DNA sequence of S2 at 10 400 locus

10 μmol/L primer,0.32 μL;template DNA,about 2 ng, and 1.0 U Taq or Pfu DNA polymerase. The amplification reaction was conducted in a DNA Thermal Cycler PTC-200(MJ Research Inc., Waltham, MA, USA). After an initial 5-minute denaturation at 95℃, 36 amplification cycles were carried out, each consisting of 20 seconds at 95℃, 20 s at 52℃, and 20s at 72℃, followed by a final extension step of 3 min at 72℃.

Separation conditions

For each template, PCR products of unmodified and PS-modified primers were added to different lanes and then detected by 2% agarose gel electrophoresis with 1 × TAE running buffer. The results were analyzed by the ultraviolet gel imaging system(Lab Works, Perkin Elmer Life and Analytical Sciences, Inc., Waltham, MA, USA) after ethylene bromide staining.

RESULTS

Analysis of S1 PCR products

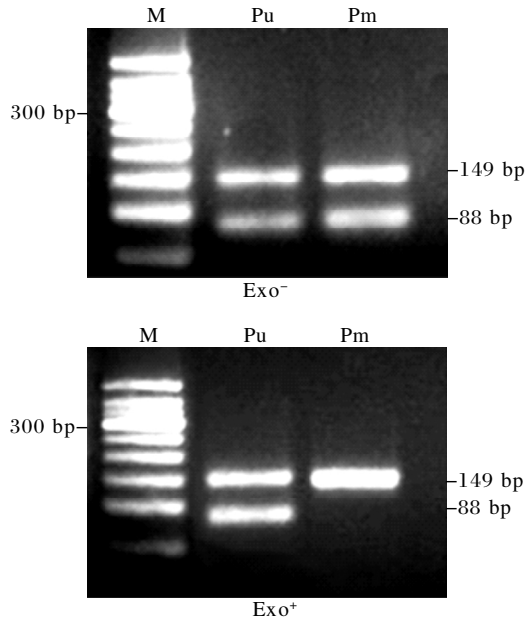
When using Taq DNA polymerase lacking 3′ exonuclease activities, the matched (149 bp) and mismatched (88 bp) primers were both extended, irrespective of whether the primers were PS-modified or unmodified. The 3′ terminal phosphorothioate-modification had no effects in improving the specificity of exo⁻ polymerase-catalytic reaction. The false positive products resulted from the 3′ mismatched primer extensions and disturbed the judgment of S1 genotype. When applying Pfu DNA polymerase with 3′ exonuclease activities, the non-specific amplification of unmodified primers could also not be avoided. However, an on/off switch effect was observed from the phosphorothioate-modified allele-specific primers:DNA polymerization only occurred from matched PS-primers, whereas mismatched PS-primers were not extended just as an off-switch was triggered on. Subsequent gel electrophoresis showed allelic products at the 149 bp position, which suggested the correct genotype of S1(***Fig. 4***).

Analysis of S2 PCR products

The same phenomenon was observed with S2 as with S1. As shown in ***Fig. 5***, Pfu DNA polymerase only allowed perfectly matched PS-primers(88 bp) to be extended, but not the mismatched PS-primers.

DISCUSSION

The main problems of PCR are its fidelity and specificity[15]. In regular AS-PCR, polymerases used in allele-specific primer extension are exclusively enzymes lacking 3′ exonuclease functions, which could also yield identical products when there was a non-complementary base to the templates. The conventional allele-specific primers may couple with various templates even under strictly optimized conditions. Actually, the



M represents 50 bp Marker; Pu for unmodified primers, and Pm for modified primers; Left panel:AS-PCR products from exo⁻ polymerase; Right panel:AS-PCR products from exo⁺ polymerase

Fig. 4 Genotype pattern of S1 tested by AS-PCR

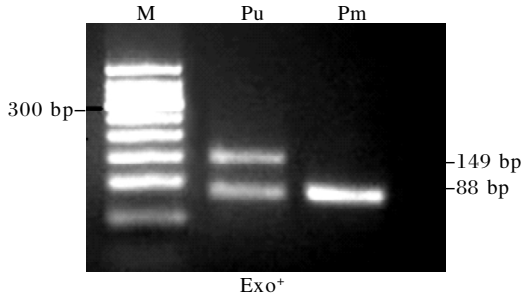


Fig. 5 Genotype pattern of S2 tested by AS-PCR

mispriming in AS-PCR is well known to be a major problem that can not be avoided, especially in multiplex template extension. That is the reason why in our study we observed unmodified primers with a 3′ terminus mismatch extended by exo⁻ polymerase. A strategy used in initial reports to improve the specificity is the introduction of another artificial mismatched base at the 3′ terminus of allele-specific primers[16], which is also not ideal in practice. Moreover, the false positive products were not efficiently eliminated when we using high fidelity polymerase(Pfu) to replace low fidelity polymerase(Taq) with conventional allele-specific primers. The reason possibly lies in that high fidelity polymerase with internal 3′ to 5′ exonuclease activities may first recognize the 3′ terminus mismatch, then repair, and finally allow the extension reaction to continue after a successful excision of mismatched bases. So we obtained template-dependent products from exo⁺ polymerase, whereas exo⁻ polymerase generated primer-dependent products with the mismatched nucleotides

still preserved[17,18]. Both template-dependent and primer-dependent products are responsible for the high false positives of AS-PCR.

Phosphorothioate-modification renders the oligonucleotide nuclease-resistant, a strategy widely used in antisense technology [19,20]. With the use of polymerase having 3′ to 5′ exonuclease activities and 3′ phosphorothioate-modified allele-specific primers, we improved the specificity of AS-PCR greatly and reduced false positives remarkably in our study. Exo$^+$ polymerase worked efficiently in conjunction with 3′ phosphorothioate-modified primers as a SNP-operated on/off switch in DNA polymerization. For 3′ allele-specific primers with phosphorothioate modification, matched primers turned on DNA polymerization and mismatched primers turned it off. In detail, high fidelity polymerase with 3′ exonuclease activities could discriminate the non-complementary bases and kept the state of trying to excise them before extension. When primers were non-complementary to the templates, the exonuclease-resistant property of phosphorothioate-modification blocked the mismatch excision during proofreading and mediated a premature termination of DNA polymerization in the end. As a result, it switched off the extension reaction of mispriming, whereas only perfectly matched primers could be extended from an on-switch effect. This novel on/off switch mechanism of extension ensured that products all came from perfectly matched primers and were amplified with an extremely low error rate. Moreover, the most significant advantages are ease of optimization and increased specificity for detecting rare alleles relative to routine allele-specific amplification. With this switch controlling DNA polymerization, we read the genotype of samples that are absolutely consistent with the sequencing results. The crucial structural components of the on/off switch are:(Ⅰ)allele-specific primers with 3′ terminal exonuclease-resistant modification and (Ⅱ)DNA polymerases having 3′ exonuclease activities. That is the reason why in our study low fidelity polymerase(Taq) lacking 3′ exonuclease activities failed to participate in this mechanism in our study. Now we are trying to extensively apply this strategy in detecting more mtDNA polymorphism loci, as well as autosomal SNPs.

MtDNA is a useful tool in paternity testing and identity evidence[21]. Owing to its small genome and short fragment, SNPs are always used for the definition of mtDNA haplogroups instead of STRs[22,23]. The interest of forensic researchers in autosomal SNPs is also continuously increasing, especially for the reason that they can be analyzed in short amplicons. However, SNPs have some limitations. First, the number of SNPs required is around four times the number of STRs(on average), so around 60 well balanced SNPs are necessary to have a similar discrimination power to the new multiplexes in use in the forensic field[24,25]. Second, the cost advantages are still not clear when compared to STRs employed in the commercial kits that are currently available. Therefore, both national and international organizations are in urgent need of a cost-effective, high-throughput, highly accurate method for efficient screening of known SNP sites.

The strategy of using phosphorothioate-modified allele-specific primers and exo$^+$ polymerase in our study was shown to be a suitable and economic method, either for application in the development of SNP screening assays or for positive controls in practical SNP assays. The perfect-matched primer on/mismatched primer off switch mechanism embodies powerful nucleotide identification ability, and, in addition to their high accuracy in genotyping, another advantage offered is their adaptability to different types of platforms, which provide opportunities for a wide range of visualization technologies, including routine PCR, real time PCR, multi-well plate PCR, as well as single base extension microarrays and electrochemical microarrays for SNP detection without the need for gel electrophoresis or enzymatic digestion. It is thus reasonable to expect that high-throughput and cost-efficient SNP assay formats will become more easily designed in the future with this technology. The versatility of SNP-operated on/off switch might also greatly enhance mutation analysis in the post-genomic era, as well as accelerate other fundamental and clinical applications, such as allele frequency estimation in pooled DNA and rare mutation detection for cancer diagnosis and therapeutic prediction[26]. We believe that great and immediate benefits will be achieved for the biomedical research and healthcare communities by using a wider application of SNP assays that are mediated by the on/off switch and by further studies to introduce additional genetic assays using this mechanism.

In conclusion, we describe a single-base mismatch-operated on/off switch in DNA polymerization through proofreading phosphorothioate-modified primer-3′-termini. With the combination of a polymerase having 3′ exonuclease activities and allele-specific primers with 3′ phosphorothioate-modifications, DNA polymerization occurred only from perfectly matched primers. Single-base pair mismatch turned off polymerization. This convenient and efficient method is devised from the high fidelity, in primer extension, of a polymerase with proofreading function. Because this on/off action is actually operated by base-pairing status at the single-base level between the 3′ terminal nucleotides of the

primers and their related templates, it has great potential in SNP assays and will greatly facilitate genetic and biomedical studies in the postgenomic era.

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