Multiplex Mutagenically Separated Polymerase Chain Reaction Assay for Rapid Detection of Human Mitochondrial DNA Variations in Coding Area

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Recently, it has been recognized that information in the mitochondrial DNA (mtDNA) coding region can provide additional forensic discrimination with respect to the standard typing of the D-loop region, increasing the forensic power of mtDNA testing, which is sometimes rather limited. In the present study, we simultaneously typed ten single nucleotide polymorphisms (SNP) in the coding region by use of mutagenically separated polymerase chain reaction (MS-PCR) in the Chinese Chengdu population. This technique, in which different-size allele-specific primers were used, specifically amplified both alleles of mtDNA in the same reaction. Subsequent gel electrophoresis showed ten of the allelic products of different loci. Using multiplex MS-PCR, 30 primers were added simultaneously into one reaction tube to identify ten SNPs. The mtDNA variations of 160 individuals from the Chinese Chengdu population were examined and classified into 18 haplotypes. The multiplex MS-PCR method is suitable for large-scale screening studies of mtDNA variability because it is both rapid and economical.

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Single nucleotide polymorphisms (SNPs) in mitochondrial DNA (mtDNA) are widely used as markers in population genetic studies and forensic analyses. Mitochondrial DNA is present in at least hundreds of copies in each cell (1), which is an additional advantage when small or degraded samples are the only source of DNA available for analysis (2-4). For identification of individuals in forensics, nucleotide sequencing of the two hypervariable regions (HVR) I

and II at the origin of replication of mtDNA is the most widely used method for the analysis of mtDNA (5-7). However, the two routinely analyzed mtDNA hypervariable regions HVR I and II provide a limited power of discrimination in a forensic context and, in many cases, provide redundant information in evolutionary studies. Since the sequencing of the entire mitochondrial molecule is not practical, genotyping of additional SNPs in the coding regions of mtDNA has been suggested to increase the power of discrimination between individuals with identical HVR I/II types (7-11). Several methods are now available to detect the mtSNPs, such as direct sequencing, the SNaPshot assay (8), MALDI-TOF MS (11), and the Taqman assay (12). However, these techniques necessitate the use of expensive equipment such as an autosequencer. Therefore, the ability to utilize these assays may be limited in developing countries. Other available genotyping assays, such as the line probe assay (LiPA), selective polymerase chain reaction (PCR) or amplification refractory mutation detection system (ARMS), the oligonucleotide ligation assay (OLA), and the heteroduplex tracking assay (HTA) are available, but lower sensitivity, lower specificity, and complicated procedures limit their usage (13-15).

Therefore, developing a rapid and economical method for analyzing the mtSNPs is of great importance. In this study we describe the development of a simple, rapid, and economical method for analyzing the mtSNPs, the multiplex mutagenically separated PCR (MS-PCR) technique (16). The aim of this study was to apply the multiplex MS-PCR system to detect ten SNPs in one reaction tube simultaneously.

The principle of the technique involves using two allele-specific primers of different lengths that are separately complementary to a given DNA sequence except for a mismatch near the 3′end of the primers. These additional and deliberately designed differences can markedly reduce cross-reactions in subsequent PCR cycles. A typical MS-PCR test contains three primers in the PCR mixture. Both normal and mutant alleles are amplified in the same reaction tube. Subsequent gel electrophoresis shows one of the two allelic products at the same locus. The method is rapid, reliable, and non-isotopic and provides a within-assay quality control for the exclusion of false-negative results. Moreover, the results can be obtained in less than one working day.

Materials and methods

Preparation of DNA templates

The study included the total of 160 individuals from Chinese population from Chengdu, the capital of Sichuan province in southwest China. Genomic DNA was extracted from peripheral blood by the phenol-chloroform method (17). The blood samples were part of our routine casework and the individuals gave their informed consent.

Primer design

The multiplex system was designed to analyze the target sites 12705C/T, 8701A/G, 8584G/A, 10400C/T, 4883C/T, 10873T/C, 15301G/A, 14783T/C, 3010G/A, 5460G/A. These sites were selected from a mtSNP database (*http://www.mitomap.org/cgi-bin/tbl-7gen.pl*) (18) and previous reports (9,19), as sites of possible important mutations in the East Asian populations. The oligonucleotide primers for the MS-PCR analysis were designed from the nucleotide sequence of mtDNA shown in Table 1. The principle of the primer design was based on that used by Rust et al (16) and that of the ARMS method but with some modification, where primers were

Table 1. Primer sequences of 10 single nucleotide polymorphism loci

Haplogroup	Primer	Sequence (5'-3')*	Concentration of primers (μM/L)	Length of products (bp)
R	12705C	CTAAGATTAGTATGGTAATTAGGCAG	0.43	123
	12705T	GTAAGAAAGATTAGTATGGTAATTAGTAAA	0.53	127
	12705R	CTGTAGCATTGTTCGTTACATGGTC	0.43	
N	8701G	TTCGTCCTTTAGTGTTGTGTATTGC	0.05	106
	8701A	CAGGAACGTCCTTTAGTGTTGTGTAGGGT	0.05	110
	8701R	CCCCACCTCCAAATATCTCATCAAC	0.05	
M8	8584G	CCCGGAAATAGAATGATCAGTACTTC	0.16	97
	8584A	CCCCCCCTTAAATAGAATGATCAGTACCGT	0.27	101
	8584R	CCCTGAGAACCAAAATGAACGAAA	0.16	
M	10400C	CGTTTTGTTTAAACTATATACCAATGCG	0.16	88
	10400T	CATTGCTTTTGTTTAAACTATATACCAAGTCA	0.27	92
	10400R	AATCATCATCCTAGCCCTAAGTCTGG	0.16	
D	4883C	GAGATTTGGTATATGATTGAGCTG	0.16	82
	4883T	GGACTGATTTGGTATATGATTGAAATA	0.27	85
	4883R	TAAAAGGCACCCCTCTGACA	0.16	
N	10873C	GTTGATTTGGTTAAAAAATAGTCGG	0.16	77
	10873T	GTTCATGATTTGGTTAAAAAATAGGAGA	0.27	80
	10873R	CATAATTTGAATCAACACAACCACC	0.16	
N	15301A	GCTGATTCTTTACCTTTCACTTCATTTTA	0.16	73
	15301G	GATTCTTTACCTTTCACTTCATCCTG	0.16	70
	15301R	AAGAATAGGAGGTGGAGTGTTGCTA	0.16	
M	14783C	GGACCTCGATGAATGAGTGGTTAACTAG	0.53	67
	14783T	GGTCGATGAATGAGTGGTTAATCAA	0.27	64
	14783R	CCAATGACCCCAATACGCAAAA	0.27	
D4	3010G	CTTTAATAGCGGCTGCACCCTC	0.16	58
	3010A	AACAGTTAATAGCGGCTGCACAATT	0.16	61
	3010R	AGGGTTTACGACCTCGATGTTGG	0.16	
J1	5460A	AATGATAGGTAGGAGTAGCGTGGTAAGTGT	0.05	55
	5460G	GATAGGTAGGAGTAGCGTGGTAATGGC	0.05	52
	5460R	CACCCCATTCCTCCCCACACTCA	0.05	

*The mismatched bases are underlined.

designed in order to adjust the annealing temperatures and amplicon lengths to allow analysis in multiplex reactions.

Single MS-PCR conditions

Three primers at different concentrations (Table 1) were added into one reaction tube. PCR amplifications were carried out in a total volume of 37.5 μL of reaction solution containing 0.2 ng of genomic DNA, three primers for detection of one SNP locus, 10×PCR reaction buffer, 200 mmol/L dNTPs, 2.5 mmol/L $MgCl_2$, and 1.0 U Taq polymerase (MBI). The amplification reaction was conducted in a DNA Thermal Cycler 9600 (Applied Biosystems, Foster City, CA, USA). After an initial 5-minute denaturation at 94°C, 30 amplification cycles were carried out, each consisting of 20 seconds at 94°C, 20 seconds at 52°C, and 20 seconds at 72°C, followed by a final extension step of 5 minutes at 72°C.

Multiplex MS-PCR conditions

Thirty primers of ten SNPs loci were added into one reaction tube. The PCR cycling conditions were the same as for the single MS-PCR, except for the concentrations of the primers. The concentrations of the primers in the multiplex reactions were adjusted according to the productivity of the different primer pairs on the basis of those in a single reaction. Optimal concentrations of each primer are shown in Table 1. When any mtSNP failed to be analyzed due to the existence of a base substitution in the primer position or a problem with the quality of the DNA, it was analyzed by a single MS-PCR with the corresponding primer set.

Separation conditions

The PCR products were analyzed by vertical non-denaturing polyacrylamide gel electro-

phoresis with a discontinuous buffer system and visualized by silver staining. Two milliliter aliquot of the PCR product was separated by electrophoresis in a 6.5 cm native polyacrylamide gel (10% T, 5% C) containing 375 mM Tris-HCl buffer (pH 8.9) with running buffer (12.5 mM Tris, 96 mM glycine; pH 8.3). A reduced median network (Figure 1) was computed with the program NETWORK 4.2 (*http://www.fluxus-engineering.com/sharenet_rn.htm*) after star contraction.

Direct sequencing of PCR products

In addition to testing the robustness and accuracy of the MS-PCR method, a random set of the samples was typed by using direct DNA sequencing on an the ABI Prism 3100 sequencer with a BigDye Terminator Cycle Sequencing FS Kit (Applied Biosystems, Foster City, CA, USA).

Results

To develop a convenient method for the determination of haplotypes of a large number of DNA samples, we prepared ten primer sets for the amplification of products ranging from 52 to 127 bp in size and mixed them in a single tube. The MS-PCR assay was validated by testing 40 human mtDNA samples that had been previously characterized by sequencing. There was a complete agreement between multiplex MS-PCR and direct DNA sequencing. The specificities of multiplex MS-PCR reactions were confirmed by using the samples that had been previously characterized by direct sequencing. Each SNP obtained by single MS-PRC was correctly typed by vertical nondenaturing polyacrylamide gel electophoresis (Figure 2). The multiplex MS-PCR band patterns of human mtDNA haplotypes after separation on a polyacrylamide gel showed that each allele was correctly typed by comparing the PCR products with the known point

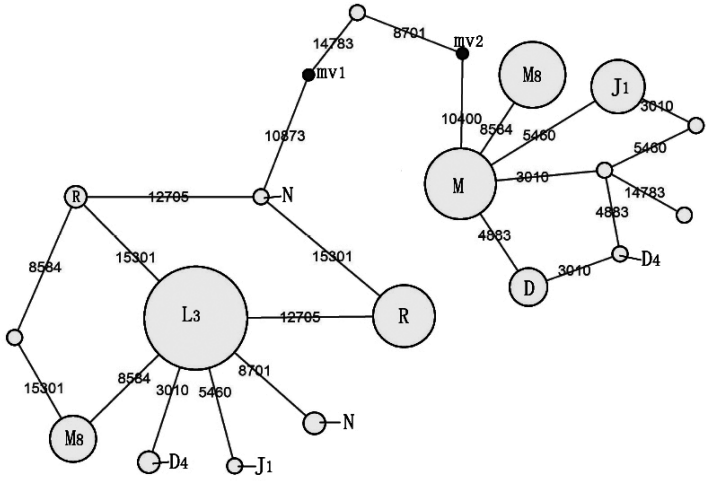

Figure 1. Reduced median network of a Chinese population typed for 10 single nucleotide polymorphisms in the coding region of mitochondrial DNA. The positions of single nucleotide polymorphisms according to the Cambridge Reference Sequence are indicated on the branches. The mv1 and mv2 are hypothesized sequences required to connect the existing sequences within the network with maximum parsimony.

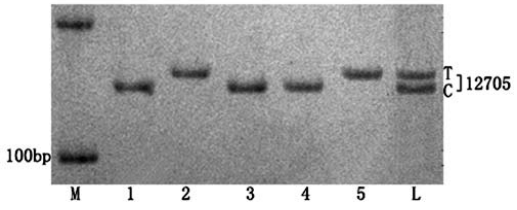

Figure 2. The results of a single mutagenically separated polymerase chain reaction for the detection of the 120705 single nucleotide polymorphism. Lanes 1, 3, 4 – 12705C; Lanes 2, 5 – 12705T; M – DNA marker; L – DNA ladder marker.

mutation (Figure 3). By multiplex MS-PCR analysis, the mtDNA variations of 160 individuals from Chinese population were examined and classified into 18 haplotypes. A phylogenetic network for the evolutionary relationship of mtDNA haplotypes in a reduced median network of 18 different haplotypes revealed that haplogroups M and N is the central haplogroup from which 4 clusters radiate-M8,J1,D,R (Figure 1).

Discussion

Mitochondrial DNA polymorphisms are now widely used as a maternally inherited mark-

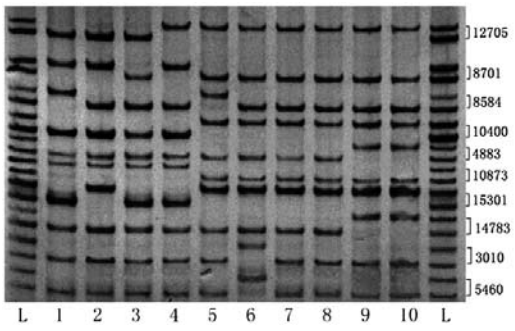

Figure 3. Multiplex mutagenically separated polymerase chain reaction band patterns of mitochondrial DNA haplotypes. L – DNA ladder marker.

er to elucidate the course of human evolution and migrations and for human identification. Several methods are now available to detect the mtSNPs, such as ARMS, restriction fragment length polymorphism (RFLP), HTA, LiPA, and OLA. ARMS is a PCR-based point mutation assay in which wild-type and mutant detection tests are performed in individual reaction tubes. Since there is no competition between the two detection primers as in MS-PCR, ARMS consequently suffers from lower specificity. The RFLP analysis is one of the most widely used techniques for detecting known point mutations in the coding region of mtDNA (20). In this method, some ambiguities remain with respect to the actual mutation site. HTA, LiPA, and OLA require multiple post-PCR manipulations to obtain results, making these assays laborious and time consuming. Furthermore, the polymorphisms in the probe-target region are critical in these assays, and unexpected mutations in the targeted region may cause false results. Commonly, PCR-based assays have the same limitation for the detection of mtSNPs.

To preserve the simplicity and increase sensitivity and specificity, two improvements were introduced in primers of MS-PCR. Designing the detection primer length difference of about 3-4 bp and introducing intentional mismatches to increase the specificity of the assay are the main features of multiplex MS-PCR. It is a relatively new PCR-based method for the identification of previously known allelic mutations in the nucleic acid sequence. The method is based on the introduction of artificial mutations into the PCR-primer binding regions of the amplified DNA in an allele-specific manner, using allele-specific primers with mutagenic positions at different distances from the 3′ end, based on previous studies (21-23). This mismatch maximized the difference between the rate of substrate formation for the loci of the target alleles, and that of the non-target alleles. To avoid the filling-up effect of the shorter primer, Rust et al (16) mutagenized a base of the longer primer that correlated with the 5′ end of the shorter primer. This mutagenic base inhibited the filling effect caused by using shorter PCR products as primers. We used some primers that had identical sequences to normal primers in that region and found that these primers worked very well, with no filling effect, thus obviating the need for a mutagenic change in this region for MS-PCR.

Concentrations of the primers were extremely critical. Initially, we used the identical concentration of primers to do PCR, and after checking the results of the PCR, we adjusted the concentrations of the different primers as required. The primer concentration was increased if too few products were produced, and it was decreased if too many products were produced. By adjusting the concentrations of various primers, the competition for these primers varied and resulted in differences in PCR yield and specificity. The products from MS-PCR are specific for their respective alleles and can be directly identified by polyacrylamide gel electrophoresis without further manipulation. This method was originally used to detect one mutation in one reaction tube. In this study, we extended the method to detect ten SNP loci in mtDNA simultaneously in one reaction tube.

In the multiplex MS-PCR, misinterpretations due to individual differences in setting up the test can be avoided because of the use of a master mixture of positive and negative controls for the reaction. A reaction in which at least one of the allele-specific bands is not obtained is regarded as incorrect. The multiplex MS-PCR has another advantage in that the competition and mutagenically allele-specific separation in one reaction tube can improve specificity, compared with the reactions in several tubes.

Rust et al (16) found that the longer of the allele-specific primers was used at a lower concentration rather than the shorter one. They thought this may be related to annealing advantages of longer primers at high temperatures. However, we found the opposite results. From Table 1 it is apparent that in all cases the longer of the allele-specific primers was used at a higher concentration. These may be two allele-specific primers with only 3 ~ 4 bases difference in size.

In conclusion, multiplex MS-PCR is very simple and rapid, because it only requires PCR amplification in a single tube and polyacrylamide gel electrophoresis in combination with silver staining. Not only is this multiplex MS-PCR method reliable and non-isotopic, but it allows for haplotypes of a large number of DNA samples to be obtained in less than one working day.

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