Optimization of Rapid Screening Assay by Mitochondrial DNA Single Nucleotide Polymorphisms in Hypervariable Region for Thais

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ABSTRACT

This study aims to optimize the rapid screening assay by SNPs minisequencing in hypervariable region of Thai individuals. Fifteen SNPs based on mtDNA genetic variation in hypervariable region I, II and III were selected. The DNA samples were obtained from buccal swab samples of Thai healthy individuals. The samples were extracted using NucleoSpin® Tissue kit and then were amplified using single duplex polymerase chain reaction (PCR) technique. The duplex PCR products were analyzed by agarose gel electrophoresis. It was found that duplex PCR products band size of HVRI and HVRII-III were revealed at 433 and 624 bp, respectively. The SNPs minisequencing assay was optimized using ABI PRISM® SNaPshot™ Multiplex Kit. The results showed that the typing of all 15 SNPs in samples was achieved without artefacts peak. It was revealed that the profiles of multiplex minisequencing reaction showed overlap of electropherogram peaks in the SNP 152 and 195. It was interesting to note that the observed and the expected product sizes were slightly different. In conclusion, the result showed that the duplex PCR products could be used for SNPs minisequencing. HVRI and HVRII-III amplification products can be also used for mtDNA sequencing in order to confirmed if needed. Moreover, the optimized of SNPs minisequencing provide appropriate method for the analysis of 15 SNPs in Thai population.

Keywords: Forensic sciences, Hypervariable regions, Minisequencing, Mitochondrial DNA, Single nucleotide polymorphisms

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Introduction

Using mitochondrial DNA (mtDNA) analysis for personal identification is an important and a powerful method in forensic sciences in several cases, including the identification of missing people, victims of mass disasters and forensic casework. It is used for the analysis of highly degraded samples or samples with insufficient nuclear DNA for conventional short tandem repeat (STR) analysis such as hair shafts, highly decomposed human remains and old or charred bones (Ginther *et al.* 1992; Holland *et al.* 1993). Due to mitochondrial DNA characteristics have several advantages, including its presence in a high copy number per cell, maternal inheritance and hypervariable region (HVR), which are different for each individuals (Hopgood *et al.* 1992).

The standard method of mtDNA analysis is the nucleotide sequencing of polymerase chain reaction (PCR) products from the hypervariable region I (HVRI) and II (HVRII) in control region followed by comparison to the revised Cambridge Reference Sequence (rCRS) (Andrews *et al.* 1999). However, personal identifications using mtDNA sequencing are rather expensive, time consuming and technically complex.

Several high-throughput technologies are now available for SNP typing methodologies, such as SNaPshot minisequencing, LightCycler, TaqMan assay, Molecular beacons, DNA microarrays and Invasive cleveage (Sobrino *et al.* 2005). However, the minisequencing technologies are the most popular methods in forensic research, especially the SNaPshot, because the detection performed on an automatic capillary electrophoresis instrument, that it is also used in majority of forensic laboratories. Currently, single nucleotide polymorphisms (SNPs) in both of coding and control regions by minisequencing has been studied in several populations (Chaitanya *et al.* 2014; Chemale *et al.* 2013; Köhnemann and Pfeiffer, 2011; Paneto *et al.* 2011; Ren *et al.* 2014; Sobrino *et al.* 2005). However, SNPs minisequencing study in Thais has not been reported and fully investigated.

Objective of the study

This study aims to optimize the rapid screening assay by SNPs minisequencing in hypervariable region of Thai individuals, in order to assist in the exclusion of mismatch samples and as a presumptive test prior to confirmatory mtDNA sequencing.

Research Methodology

Samples and DNA extraction

Buccal swab samples are collected from unrelated Thai individuals who lived in Thailand. Informed consent has been signed by all the participants. This study has been approved by the Research Ethics Committee of Chiang Mai University, Faculty of Medicine (code No. NON-2557-02272 (2015)). Genomic DNA extractions from buccal swab samples were carried out using the NucleoSpin® Tissue kit (MN, Germany) following the manufacturers recommendation. One microliter of the genomic DNA sample was analyzed for DNA integrity using agarose gel electrophoresis stained with ethidium bromide.

Selection of SNPs

Fifteen SNPs were selected based on mtDNA genetic variation in hypervariable region I, II and III from the mtDB Human Mitochondrial Genome Database

available at http://www.mtdb.igp.uu.se/ ((Ingman and Gyllensten, 2006) and previous reports.

Duplex PCR amplification

All the samples were used as templates to amplify the HVR products in a single duplex PCR using primer L15978 (5'CACCATTAGCACCCAAAGCT3'), H16410 (5'GAGGATGGTGGTCAAGGGAC3'), L15 (5'CACCCTATTAACCACTCACG3') and H619 (5'GG-TGATGTGAGCCCGTCTAA3'). PCR was carried out in a total volume of 40 µL with 20 µL of Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, USA), 10 µM of each primer and 2 µL of the genomic DNA extracted from samples. Thermal cycling conditions were as follows, pre-incubation for 10 s at 98°C, followed by 30 cycles of 5 s at 98°C, 5 s at 59°C and 20 s at 72°C and finally 1 min at 72°C. Amplifications were performed in a Thermal Cycler Mastercycler® Personal (Eppendorf, USA). The duplex PCR products were analyzed by agarose gel electrophoresis stained with ethidium bromide. The PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (MN, Germany) following the manufacturers recommendation. Finally, the purified PCR products were analyzed by agarose gel electrophoresis stained with ethidium bromide in order to detect quantity and purification of the PCR products.

SNP minisequencing reaction

SNPs minisequencing primers for the selected SNPs were designed using Primer3web software version 0.4.0 available at http://www.bioinfo.ut.ee/primer3-0.4.0/ (Rozen and Skaletsky, 2000) and tested for primer-primer interactions and hairpin structures using Autodimer Software (Vallone and Butler 2004). All primers were purchased from Sigma-Aldrich Co. LLC. (Malaysia). Primers were designed by adding a d(GATC)n tag at their 5' end in order to avoid overlap between the final SNPs minisequencing products. The SNPs minisequencing primers were shown in Table 1. The SNPs minisequencing reaction was performed using the ABI PRISM® SNaPshot™ Multiplex Kit (Applied Biosystems, USA). The reaction was performed in a total volume of 10 µL contaning 2.5 µL of SNaPshot Multiplex Ready Reaction Mix, each SNPs minisequencing primers (final concentration; 0.2 µM for singleplex reaction and between 0.2-1.6 µM for multiplex reaction) and 1 µL of purified PCR products. Thermal cycling conditions were 25 cycles of 10 s at 96 ºC, 15 s at 50 ºC and 30 s at 60 ºC. The SNPs minisequencing products were then purified using 1 µL of shrimp alkaline phosphatase or SAP (GE Healthcare, USA) and incubation at 37 ºC for 60 min followed by enzyme inactivation at 80 ºC for 15 min. Finally, fragments were sequenced by First BASE Laboratories Sdn Bhd (Selangor, Malaysia), perform fragment analysis on ABI Sequencer and analyze the results using GeneMapper® ID v4.0 Software.

Results and Discussion

In this study, in the SNPs minisequencing reaction, a primer that anneals to its target DNA immediately adjacent to the SNP is extended by a DNA polymerase with a single nucleotide that is complementary to the polymorphic site. This method is based on the high accuracy of nucleotide incorporation by DNA polymerases (Sobrino *et al.* 2005). One of the most common commercial technologies based on mini-

Table 1 MtDNA SNPs minisequencing primer information.

SNP Primer name	Primer specific sequence (5'-3')	Primer tag 5'-d(GATC)n	Final Conc. (µM)	Primer length (nt)	Alleles (dye)
L73*	GTATTTTCGTCTGGGGGGT	$(dGATC)_5$	0.2	39	A (green), G (blue)
L146*	AGTATCTGTCTTTGATTCCTGCC	$(dGATC)_5$	0.2	43	T (red), C (black)
H152* reverse	ATTGAACGTAGGTGCGATAAATAAT	$(dGATC)_6$	0.2	49	T (green), C (blue)
L195*	TTCAATATTACAGGCGAACATAC	$(dGATC)_7$	1.6	51	T (red), C (black)
L199	CAATATTACAGGCGAACATACTTAC	$(dGATC)_7$	0.4	53	T (red), C (black)
L489	CTCCCATACTACTAATCTCATCAA	$(dGATC)_9$-GA	0.6	62	T (red), C (black)
L16126*	GCCAGCCACCATGAATATTG	$(dGATC)_{11}$	0.8	64	T (red), C (black)
L16129*	CCAGCCACCATGAATATTGTAC	$(dGATC)_5$-GA	0.2	44	G (blue), A (green)
L16172*	ACCTGTAGTACATAAAAACCCAA	$(dGATC)_{11}$	1.6	67	T (red), C (black)
L16183	TACATAAAAACCCAATCCACATCAAA	$(dGATC)_{10}$-GAT	1.0	69	A (green), C (black)
L16189*	CAATCCACATCAAAACCCCC	$(dGATC)_6$	0.2	44	T (red), C (black)
L16223*	CAAGCAAGTACAGCAATCAACC	$(dGATC)_6$-GA	0.2	48	C (black), T (red)
L16304	ACAAACCTACCCACCCTTAACAG	$(dGATC)_{12}$	1.2	71	T (red), C (black)
L16311*	CCCACCCTTAACAGTACATAG	$(dGATC)_8$-GA	1.0	55	T (red), C (black)
L16362*	ATTACAGTCAAATCCCTTCTCG	$(dGATC)_9$	0.6	58	T (red), C (black)

*SNPs minisequencing primers follow by Chemale *et al.* (2013)

sequencing reaction followed by electrophoresis and fluorescence detection is the SNaPshot™ kit from Applied Biosystems (USA). The minisequencing multiplex single base extension (SBE) reaction uses fluorescent ddNTPs. An unlabeled primer is positioned with the 3' end at the base immediately upstream to the SNP site and is extended with a single ddNTP labelled with a fluorescent dye. Each ddNTP is assigned one fluorescent dye. Multiplex reactions can be accomplished by separation of the minisequencing products using tails at the 5' end of the minisequencing primers with differing lengths of non-human sequence. It is possible to perform a 10-plex according to the manufacturer's protocol, but larger multiplexes have been developed and it is needed to optimize the design and concentration of the minisequencing primers for PCR and SNPs minisequencing reaction of each set of SNPs (Álvarez-Iglesias *et al.* 2007; Chaitanya *et al.* 2014; Chemale *et al.* 2013; Huang *et al.* 2010; Köhnemann *et al.* 2008; Paneto *et al.* 2011; Ren *et al.* 2014; Sobrino *et al.* 2005).

For selection of SNPs, Salas and Amigo (2010) proposed a simulation-based method exploring combinations of different sets of SNPs of mtDNA in order to yield the maximum levels of discrimination power. This simulated approach indicates that no more than 12 SNPs is sufficient to account for 95% of the maximum level of high diversity for almost all population groups. However, admixed populations, such as African-Americans, need to double this amount

(22 SNPs) to reach similar values of diversity. In this study, 15 SNPs were selected from the previous studies and the mtDB Human Mitochondrial Genome Database to optimize and increase the discrimination power for Thais population.

Before the analysis the SNPs minisequencing in Thai individuals, the conditions of PCR amplification of HVRI, HVRII, and HVRIII were optimized and performed in a single reaction in order to reduce the number of steps and samples consumption. The PCR amplification was performed in the same PCR reaction (single duplex PCR) using primers for all three regions. From our result, it was found that all of the duplex PCR products band size of HVRI and HVRII-III from Thai individuals were revealed at 433 and 624 bp without nonspecific products, respectively with highly yielding and quantity (data not shown). Before using the duplex PCR products in SNPs minisequencing reactions, all duplex PCR products were purified to removed primers and unincorporated dNTPs that might affect the SNPs minisequencing reaction (Salas *et al.* 2005). Purified PCR products were analyzed by agarose gel electrophoresis to determine the yielding and purity. From our result, it was revealed that the duplex PCR amplification can also saving time and reagents and the purified duplex PCR products suitable to use as template for SNPs minisequencing reaction.

Then, SNPs minisequencing primers were first tested individually in the singleplex reaction using the purified duplex PCR products (Figure 1). A SNPs minisequencing reaction was then multiplexed with the primers for the 15 selected positions. The primer combination for typing of all 15 SNPs in samples was achieved, no artefacts were observed (Figure 2). From our result, it was suggests that the profiles of our multiplex reaction with 15 SNPs showed overlap of peaks in the SNPs 152 and 195. However, the SNPs minisequencing primer of 152 was designed to bind to heavy chain and 195 to light chain. Moreover, SNPs minisequencing primers were designed for 152T and 152C could display green and blue peak, respectively. In contrast, SNPs minisequencing primers were designed for 195T or 195C could display red and black peak, respectively. Therefore, we could detect the alleles by its estimated size and its dye color.

It has to be noted that the SNPs observed size which determined by the automated sequencer and the expected size of certain products were slightly different. This was due to differences in electrophoretic mobility mainly determined by the fragment size and nucleotide composition. Figure 1 was shown electropherogram of control reaction. The mobility of fragments labeled with dR110 (blue) dye was obviously faster than that of the same size fragments labeled with dR6G (green) dye. Identically, the mobility of fragments labeled with dTAMRA™ (black) dye was slightly faster than that of the same size fragments labeled with dROX™ (red) dye.

For the effect of the nucleotide composition on mobility was higher in short oligonucleotides than in longer oligonucleotides. Moreover, some fragments display stronger fluorescent signal than others in the electropherogram due to minisequencing chemical component. Therefore, the same SNP is detected by the automated sequencer at different peak heights depending on the inserted ddNTPs. However, this effect

does not have impact on interpretation of the electropherogram.

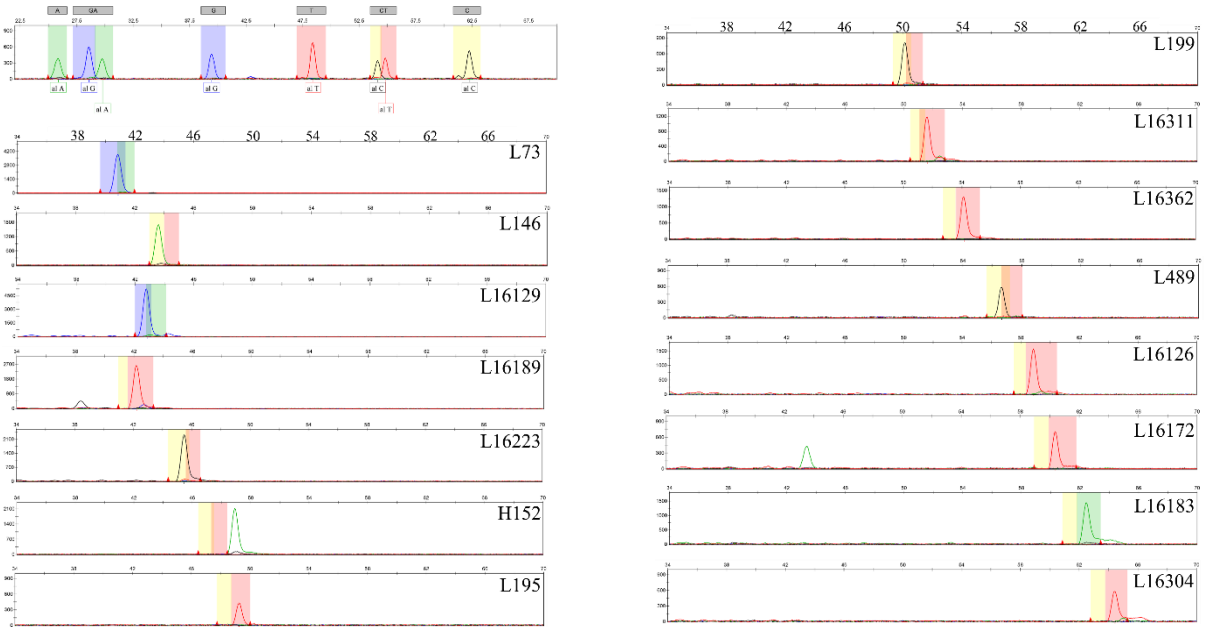

Figure 1 Electropherogram of the multiplex control reaction products and the singleplex SNP minisequencing reaction.

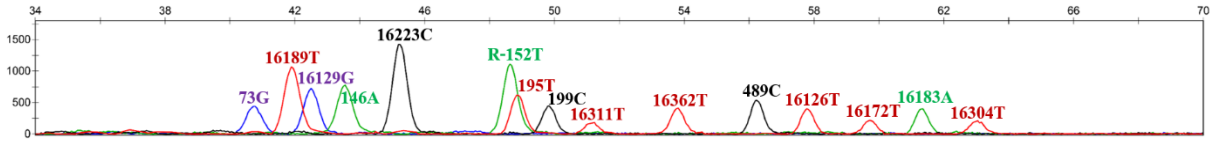

Figure 2 MtDNA SNPs typing electropherogram. Sample typed with 15 mtDNA SNPs assay. MtDNA SNP position and nucleotide genotyped are present. Reverse primers are labeled with an R before the mtDNA SNP position and nucleotide genotypes are converted to its complementary strand.

Conclusion

In conclusion, the result showed that the purified duplex PCR products could be used for SNPs minisequencing. Moreover, HVRI and HVRII-III amplification products can be also used for mtDNA sequencing in order to confirm if needed. Moreover, the optimization of SNPs minisequencing provide appropriate method for the analysis of 15 SNPs in Thai population. For further study, the SNPs minisequencing in 100 Thai individuals will be performed in both regions with SNaPshot assay with 15 SNPs. Finally, the results will be compared to revised Cambridge Reference Sequence (rCRS) and calculated with forensic statistical significance in order to determine haplogroup in Thai population.

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