RESEARCH ARTICLE

Tetra Primer ARMS PCR Optimization to Detect Single Nucleotide Polymorphisms of the *CYP2E1* Gene

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Abstract

Single nucleotide polymorphism (*SNP*) detection has been used extensively for genetic association studies of diseases including cancer. For mass, yet accurate and more economic *SNP* detection we have optimized tetra primer amplification refractory mutation system polymerase chain reaction (*ARMS PCR*) to detect three SNPs in the cytochrome P450 2E1 (*CYP2E1*) gene locus; i.e. rs3813865, rs2070672 and rs3813867. The optimization system strategies used were (1) designing inner and outer primers; (2) determining of their optimum primer concentration ratios; and (3) determining of the optimum *PCR* annealing temperature. The tetra primer ARMS *PCR* result could be directly observed using agarose gel electrophoresis. The method succesfully determined three SNPs in *CYP2E1* locus, the results being consistent with validation using DNA sequencing and restriction fragment length polymorphisms (RFLP).

Keywords: SNP detection - tetra primer ARMS PCR - CYP2E1 gene

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Introduction

Single nucleotide polymorphisms (*SNP*) is the simplest form of polymorphism found in human genome; on which on one nucleotide substituted by another one, insertion or deletion. *SNP* basically found in more than 1% of the entire population. The *SNP* is estimated to occur in every 500-1000 base pairs in the human genome, and may occur both in coding and noncoding region (Gray et al., 2002; Shastry, 2002). Although many *SNPs* showed no effect to cell abnormalities, but many researches by statistical calculation found that *SNPs* can be used as a biological marker for analyzing the disease risks (Fareed and Afzal, 2012; Iversen et al., 2014).

Polymorphisms in the gene-encoding carcinogen-metabolizing enzymes have been analyzed in many studies to determine the risk of various cancers (Danko and Chaschin, 2005). The procarcinogens are activated into reactive carcinogens by phase I enzymes (mostly by Cytochrome P450 enzyme superfamily), and these reactive carcinogens are detoxified by phase II enzyme. *DNA* damage produced by this reactive carcinogens would be a determinant for cancer development (Danko and Chaschin, 2005; Rendic and Guengerich, 2012). Therefore, genetic polymorphisms study in the gene responsible for carcinogens activation is considered important in genetic association studies of cancer (Danko and Chaschin, 2005; Xue et al., 2014).

Functional studies reported that *SNP* rs2031920/ rs3813867 (determined by RsaI/PstI restriction enzyme) of cytochrome P450 2E1 (*CYP2E1*) gene changed the transcriptional activity (Hayashi et al., 1991), as another *SNP* of the gene located at 5' flanking region and promoter (Huang et al., 2012). Several studies have been associated RsaI/PstI and risk of lung, oral, larynx, head and neck, gastric, and colorectal cancer, as well as nasopharyngeal carcinoma (Guo et al., 2010; Zhan et al., 2010; Anantharaman et al., 2011; Feng et al., 2012; Khlifi et al., 2013). Other study reported other *SNPs* located at the 5' flanking region or promoter of this gene which may associated with certain of cancer (Jia et al., 2009; Heathfield et al., 2014).

The *SNP* detection methods was developed since Restriction Fragment Length Polymorphism (*RFLP*) involves many steps, *DNA* sequencing needs expensive instruments, and robust *SNP* genotyping assay depends on consideration of funding (Kwok and Chen, 2003). Tetra primer Amplification Refractory Mutation System Polymerase Chain Reaction (*ARMS PCR*) was optimized to find rapid, efficient, economic and low tech laboratories method for detecting *SNPs* or mutation (Ye et al., 2001). Tetra primer *ARMS PCR* uses two set of primers, the outer and inner primers. The outer primers have the same function as primer in conventional *PCR*, i.e. to isolate and amplify *DNA* sequence of interest; while the inner primer used to detect allelic variation. The reaction of tetra primer *ARMS PCR* is run in a single tube and in one *PCR* step, and genotype variation from *SNPs* of interest could be visualized directly using common agarose gel electrophoresis (Akhlawat et al., 2014; Medrano and

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de Oliveira, 2014). Although often hampered by time required for optimization, previous reference offered strategic method to optimize tetra primers *ARMS PCR* (Ye et al., 2001; Akhlawat et al., 2014; Medrano and de Oliveira, 2014), as shown within this report on the optimization of three *SNPs* at the *CYP2E1* gene.

Materials and Methods

DNA extraction

DNA was extracted from buffycoat using genomic *DNA* purification mini kit from Geneaid (Geneaid Biotech. Ltd., Taipei, Taiwan). The quantity and quality (*DNA* fragment intactness) of extracted *DNA* was checked by spectrophotometer (Pharmacia Biotech GeneQuant II), and run in 1% gel agarose (added with ethidium bromide) electrophoresis.

SNPs target informations

Three *SNPs* of *CYP2E1* gene optimized in this study were rs3813865, rs2070672, and rs3813867. *DNA* sequence containing the *SNPs* were identified using accesible National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/*SNP*). The position, region and biological function of these *SNPs* were provided in Table 1.

Primer design

Two special set primers were designed by using accesible primer1 program (http://primer1.soton.ac.uk/public_html/primer1.html) developed by Ye et al. (2001). The specificity of primers and their melting temperatures were checked by using BLAST (http://www.ncbi.nlm.nih.gov/blast). The primers used for tetra primer Amplification Refractory Mutation System Polymerase Chain Reaction (*ARMS PCR*) for rs3813865, rs2070672 and rs3813867 detection provided in Table 2. The schematic illustration of tetra primer *ARMS PCR* to detect allele variation of rs3813865 was shown in Figure 1.

Table 1. SNPs ID, position, region and biological function

SNP ID	Position*	Region	Biological Function
rs3813865 (G>C)	-16533	5' flanking	upstream variant
rs2070672 (A>G)	-352	promoter	upstream variant
rs3813867 (G>C)	-1293	5' flanking	patogenic allele

*SNPs position was according to NM_000773.3:c in NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NM_000773.3)

Optimization steps of tetra primer ARMS PCR

Key elements in optimizing tetra primer *ARMS PCR* were included determining ratio of outer and inner primers, as well as the annealing temperature, while minimizing unspecific band. These factors were solved by using gradient *PCR* system (different annealing temperatures in one go) along with the use of different outer and inner primer ratios in the optimizing process.

The total volume of *PCR* reaction was 20 ul, and it was contained of 10 µl KAPATaq 2x ready mix (KAPA Biosystem Inc., Wilmington, MA), optimized ratio of outer primer and inner primer (each primer has concentration 10 nm/µl), and 1-3 µl genomic *DNA* (20-50 ng/µl). *DNA* amplification was applied by using *PCR* machine BIORAD C1000. Optimum outer and inner primers ratio for tetra primer *ARMS PCR* for rs3813865, rs2070672, and rs3813867 were 1:2, 1:2 and 1:3, respectively. The optimum *PCR* condition were as follows: 95°C for 2 minutes for cycle preparation, followed 35 cycle of denaturation at 95°C for 30 seconds, annealing for 30 seconds with different temperatures for rs3813865 (54°C), rs2070672 (58°C), and rs3813867 (56°C), polymerization at 72°C for 1 minute, and final extension at 72°C for 2 minutes. For result visualization, a mixture of 5 µl *PCR* product and 2 µl loading dye was run in 3% gel agarose (1st Base, Singapore) stained with ethidium bromide (Molecular Biology Grade, Cat. No. ETBC1001), against *DNA* marker 100-1500 bp (1st Base, Singapore).

Validation assay from tetra primer ARMS PCR result

Validation assay was done in two ways; (1) *DNA* sequencing for rs3813865 and rs2070672; (2) Restriction fragment length polymorphism (*RFLP*) for rs3813867. For *DNA* sequencing, conventional *PCR* was generated to amplify region contains rs3813865 and rs2070672, using the same outer primers of tetra *ARMS PCR* (Table 2). Fifty microliter (50 ul) of *PCR* product were sent to 1st Base Sequencing Laboratories (Selangor, Malaysia) for the process.

Table 2. PCR primers used in the tetra primer ARMS PCR for CYP2E1 polymorphisms detection

SNP ID	Primer set and sequences	Tm (°C)	Ta (°C)	product size
rs3813865	outer 1 F : 5' TGA TGT TGG TTG GGC ATC TA 3'	54		499 bp
	outer 1 R : 5' CCTCGA GGT GAG AAC TGA CA 3'	55	54	
	inner 1 F : 5' CTC ACC CCA CCA AAG CCT AC 3' (C allele)	58		303 bp
	inner 1 R : 5' CCA CAG ACT GAA ATT GAA CCC 3' (G allele)	54		236 bp
rs2070672	outer 2 F : 5' CCATTCATGTGGCAGGTGGTG 3'	61		455 bp
	outer 2 R : 5' CCA ATG CCC TCT TGC TAC TC GTC TA 3'	63	58	
	inner 2 F : 5' TGG AGT TCC CCG TTG TCG AG 3' (G allele)	62.4		277 bp
	inner 2 R : 5' GTCC TGC CCT TTG GCA CTC GT 3' (A allele)	59.3		218 bp
rs3813867	outer 3 F : 5' GAGCCAGTCGAGTCTACATTG 3'	55		281 bp
	outer 3 R : 5' CAATCCAGCCAAATCACTTGTGG 3'	56.8	56	
	inner 3 F : 5' CCCTTCTTGGTTCAGGAGTGG 3' (G allele)	57.6		185 bp
	inner 3 R : 5' TGCTGCACCTAACACTGGAG 3' (C allele)	57.3		136 bp

Restriction fragment length polymorphism (*RFLP*) was performed to examine rs3813867 by using RsaI restriction enzyme. This enzyme complement to the wild type of rs2031920 which was in complete linkage disequilibrium to rs3813867 (Hayashi et al., 2001). The template for *RFLP* was a *PCR* product containing rs2031920, and it was amplified using forward primer as outer primer of rs3813867 (Table 2) and reverse primer : 5' TTCATTCTGTCTTCTAACTGGC 3'. A 5 µl *PCR* product was mixed with 5 unit of RsaI (Gene Mark Biolab. Co. Ltd., Taichung, Taiwan), and incubated overnight in 37°C waterbath. The RsaI enzyme should cleaves the 416 bp product into 357 bp and 63 bp for homozygote wild type, and observed in 3% agarose gel electrophoresis against set of *DNA* ladder of 100-1000bp (1st Base, Singapore).

Results and Discussion

Optimization of tetra primer Amplification Refractory Mutation System Polymerase Chain Reaction (*ARMS PCR*) was applied by using different ratios of outer and inner primer concentration in 1:1, 1:2, 1:4, 1:8 and 1:10 with gradients of annealing temperature were at 54°C, 56°C and 58°C. Tetra primer *ARMS PCR* optimization for rs3813865 detection was shown in Figure 2. The final ratio for rs3813865 detection was 1:2 for outer and inner primer concentrations, and the annealing temperature was 54°C. These optimum condition was determined by the appearance of the thickest *DNA* fragment at determined size, while minimizing unspecific fragment. Optimum condition of tetra primer *ARMS PCR* to detect three *SNPs* in this study were successfully determined (Figure 3). Detection of rs2070672 was optimum in annealing temperature of 58°C with outer and inner primers ratio of 1:2. The best condition for tetra primer *ARMS PCR* of rs3813867 detection was in annealing temperature of 56°C using outer and inner primer ratio of 1:3.

Previous study found that reducing outer primer

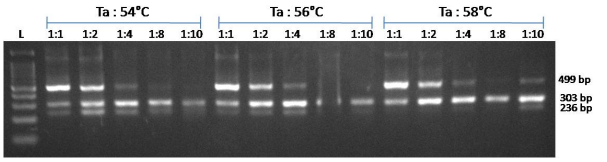

Figure 2. Tetra primer ARMS PCR optimization for rs3813865 detection with different outer and inner PCR primer ratios (1:1, 1:2, 1:4, 1:8, 1;10) and different annealing temperatures. The optimum PCR condition with was outer and inner primer ratio of 1:2 and annealing temperature of 54°C (L: DNA ladder 100-1500 bp, Ta: annealing temperature)

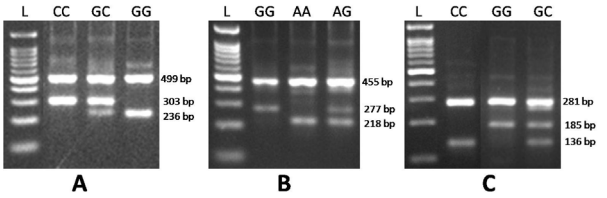

Figure 3. Tetra primer ARMS PCR for (A) rs3813865, (B) rs2070672, (C) rs3813867 detection.. (A) genotype variation of rs3813865 determined by control fragment (499 bp), specific fragment of C allele (303 bp) and G allele (236 bp); (B) genotype variation of rs2070672 determined by control fragment (455 bp), specific fragment of G allele (277 bp) and A allele (218 bp); (C) genotype variation of rs3813867 determined by control fragment (281 bp) and specific fragment of G allele (185 bp) and C allele (136 bp) (L: DNA Ladder 100-1500 bp)

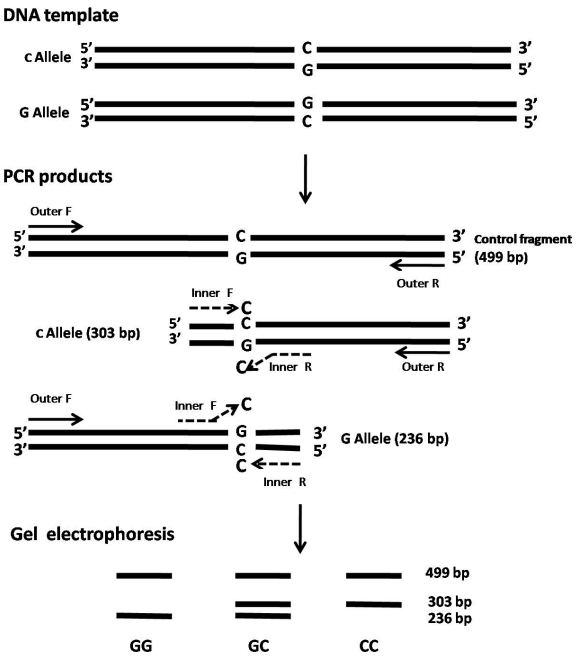

Figure 1. Illustration of the tetra primer ARMS PCR adapted from Ye et al. (2001) for detecting rs3813865 using primer set in table 2. The outer primer produces the control fragment (499 bp). The two allele specific bands are generated by using two pair of primers. A pair of primers (outer forward and inner reverse) produce a band representing G allele (236 bp), while another pair of primers (inner forward and outer reverse) produce a band representing C allele (303 bp). The differentiation of two allele specific bands in length, allows them to be determined by 3% gel electrophoresis

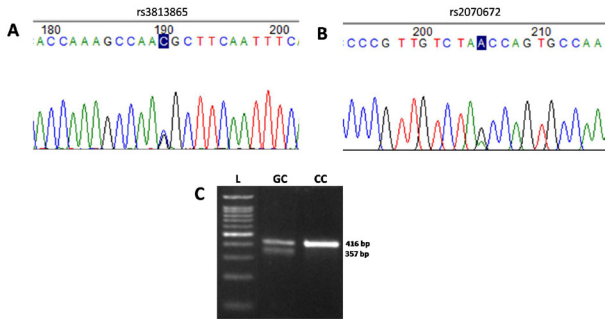

Figure 4. Validation assay to confirm tetra primer ARMS PCR result by using sequencing (A and B) and Restriction Fragment Length Polymorphism (RFLP) (C). A and B. Sequencing result of rs3813865 and rs2070672, both showed heterozygote genotype, GC and AG respectively. C. RFLP using RsaI restriction enzyme confirms the heterozygote GC (observed 416 bp, 357 bp and 59 bp), and homozygote CC (observed 416 bp) of rs3813867. Both validation methods were in concordance with genotype showed by the tetra primer ARMS PCR (data not shown)

concentration until its ratio between inner primer was 1:10 would enhance the allele-specific fragments without lacking of control fragment (Ye et al., 2001). This finding was different from our result that the control fragment would inconsistent when ratio between outer and inner primer concentration were higher than 1:4 (Figure 2). A touchdown program (5°C lower annealing temperature compared to average melting temperature) of tetra primer *ARMS PCR* reaction showed the most stable product as described by Ye et al. (2001). However, it was not replicated in the study by Medrano & de Oliveira (2014) as well as in this study, we observed that optimum annealing temperature were close to the average melting temperature of each primer set.

Therefore, based on our results, we suggest that designing primers is the most critical part of optimizing tetra primer *ARMS PCR*. Considering that the distribution of *CYP2E1* polymorphisms may differ from one ethnical background to another, the parallel use of NCBI (http://www.ncbi.nlm.nih.gov/*SNP*), with HapMap (http://www.hapmap.ncbi.nlm.nih.gov), or in our case the Singapore Genome Variation Project (http://www.statgen.nus.edu.sg) that cover Malay-based genotypes should be applied. Primer1 (http://primer1.soton.ac.uk/public_html/primer1.html) helped to design inner and outer primers, that equipped with suggested inner primer melting temperature and inner primer mismatch. By using Primer1, inner primer mismatch at position -2 from the 3' *DNA* terminus was provided to increase specific allele detection (Ye et al., 2001; Collins and Ke, 2012).

The mutant and wild type allele were distinguished by the fragment size of tetra primer *ARMS PCR* product. The size of *DNA* fragment produced by simultaneous amplification initiated by outer primers (produced control fragment) and cooperation between outer primer with inner primers (produced allele specific fragment), should be able to be observed in the 3% of agarose gel electrophoresis. The product of tetra primer *ARMS PCR* specific for rs3813865 contained 499 bp as a control fragment, 303 bp as C allele (mutant), and 236 bp as G allele (wild type) ; for rs2070672 contained 455 bp as a control fragment, 277 bp as G allele (mutant), and 218 bp as A allele (wild type); rs3813867 contained 281 bp as a control fragment, 185 bp as G allele (wild type), and 136 bp as C allele (mutant). In this report, we suggest that the *DNA* fragments between two specific allele could clearly separated if they have minimum 50 bp. For instance, if the two *DNA* fragments had only 20 bp differences, they could not been observed easily by using 3% agarose gel electrophoresis (data not shown).

Tetra primer *ARMS PCR* result require a validation assay to confirm its specifity. The detection of rs3813865 and rs2070672 by tetra primer *ARMS PCR* were confirmed with *DNA* sequencing (Figure 4A and 4B). The tetra primer *ARMS PCR* for rs3813867 detection was confirmed with *RFLP* (Figure 4C). Previous study also reported a consistent tetra primer *ARMS PCR* result with standard method for *SNP* or mutation detection, i.e. sequencing and *RFLP* (Akhlawat et al., 2014; Honardoost et al., 2014; Medrano and de Oliveira, 2014; Ye et al., 2001).

We conclude that tetra primer ARMS-*PCR* is a robust, simple and relatively economic method for genotyping *SNPs* in *CYP2E1* locus. When added with plate-based heat-block on *PCR* machine, this genotyping offers useful method for large scale of *SNP*-based association study.

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