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Original Article

Nested ARMS-qPCR is a fast and cost-saving method for single nucleotide polymorphism genotyping in clinical service

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Abstract: Single Nucleotide Polymorphism (SNP), not only has been used in genetic research widely, but also has magnificent clinical significance. Scientists have developed many methods to detect SNP, but few of which are available for clinic. Amplification refractory mutation system-quantitative PCR (ARMS-qPCR) has been used for detection of mutations, but its application in SNPs in genome has not been discussed well yet. Here, we genotyped three novel SNPs, NM_020630.4 (RET): c.135 G>A, NM_020630.4 (RET): c.2307 T>G and NM_018206.4 (VPS35): c.1648-24 T>C, in human genome with nested ARMS-qPCR. Nested ARMS-qPCR was accurate and available for the three SNPs. Compared with PCR-sequencing, the golden standard, nested ARMS-qPCR had no significant difference in SNP genotyping (P>0.05). We also found out the alleles and genotype frequency of three novel SNPs with nested ARMS-qPCR. Nowadays, qPCR is a conventional technique in molecular diagnosis lab, and 7500 Real time PCR System (Applied Biosystems) has been approved by authority in clinical usage. With the characteristics of accuracy, rapidity and ease of operation, ARMS-qPCR has a bright future in clinical screening of SNP.

Keywords: Single nucleotide polymorphism, ARMS-qPCR, RET, VPS35, genotyping

Introduction

In genetics, mutation and polymorphism have some differences. A mutation is defined as a permanent change in the nucleotide sequence, while a polymorphism is defined as a variant with a frequency above 1% [1]. Polymorphisms have many kinds, in which Single Nucleotide Polymorphism (SNP) is a kind of DNA polymorphism caused by single nucleotide variation, including transition, transversion, deletion and inversion in a given and specified genetic location. SNPs are distributed widely in genome with large quantities. According to location, SNPs are divided to 5' near gene, 5' untranslated region (UTR), intron, coding sequence (CDS), splice site (donor and acceptor), 3' UTR and 3' near gene, in NCBI/SNP database.

SNP may affect gene function or not. Some SNPs may have no function, such as some 5' near gene SNPs, intron SNPs as well as synonymous CDS SNPs. However, Some SNPs do have important functions, which change protein's function and are associated with disease. For example, methylene-tetrahydrofolate reductase (MTHFR), a key enzyme in folate metabolism, catalyses the reduction of 5, 10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate, the dominant circulating form of folate and carbon donor for the remethylation of homocysteine to methionine. NM_005957.4 (MTHFR): c.665C>T (p.Ala222Val) polymorphism, NCBI/ClinVar 3520, is susceptibility to cardiovascular diseases, diabetes, neural tube defects, and several malignant tumors, such as malignant lymphoma, ovarian cancer, gastric cancer, and colorectal cancer [2]. Therefore, detection of SNPs has important clinical significance.

Scientists have developed many methods to detect SNP. Several common methods for SNPs

Table 1. Primers for PCR-sequencing and nested ARMS-qPCR

Name	Sequence (5'-3')	Note
RET Exon2-2F	CACCATCCCTCACTCACTTCC	PCR-sequencing and nested PCR
RET Exon2-2R	CTGTGATAAGGGCGGCTTGA	
RET Exon2-WT1F[a]	AGCTGTATGTGGACCAGGTG	ARMS-qPCR, "T" indicate C>T
RET Exon2-MU1F[b]	AGCTGTATGTGGACCAGGTA	
RET Exon2-WT1R	GTTGTTCTCATGCAGCCGTG	
RET Exon13-2F	GTCTTTGCAGGCCTCTCTGT	PCR-sequencing and nested PCR
RET Exon13-2R	GGAAAGTGACCACTCAGCCC	
RET Exon13-WT1F	ACGCCTCCCCGAGTGAGCCT	ARMS-qPCR, "C" indicate T>C
RET Exon13-MU1F	ACGCCTCCCCGAGTGAGCCG	
RET Exon13-WT1R	ACCCTGCAGCTGGCCTTACC	
VPS35 Exon14-1F	AGGAAAATGACAGGAAAACTGTGC	PCR-sequencing and nested PCR
VPS35 Exon14-1R	CACCATGCCTGCAAACAAGC	
VPS35 Exon14-WT1F	ACAAGTTAATTTTGAATTTAAGGTAATT	ARMS-qPCR, "T" indicate C>T
VPS35 Exon14-MU1F	ACAAGTTAATTTTGAATTTAAGGTAATC	
VPS35 Exon14-WT1R	AATTCTGCCAGCTCTGCTTT	

[a]WT indicates the allele which is same as that in NCBI nucleotide database. [b]MU indicates the allele which is different from that in NCBI nucleotide database.

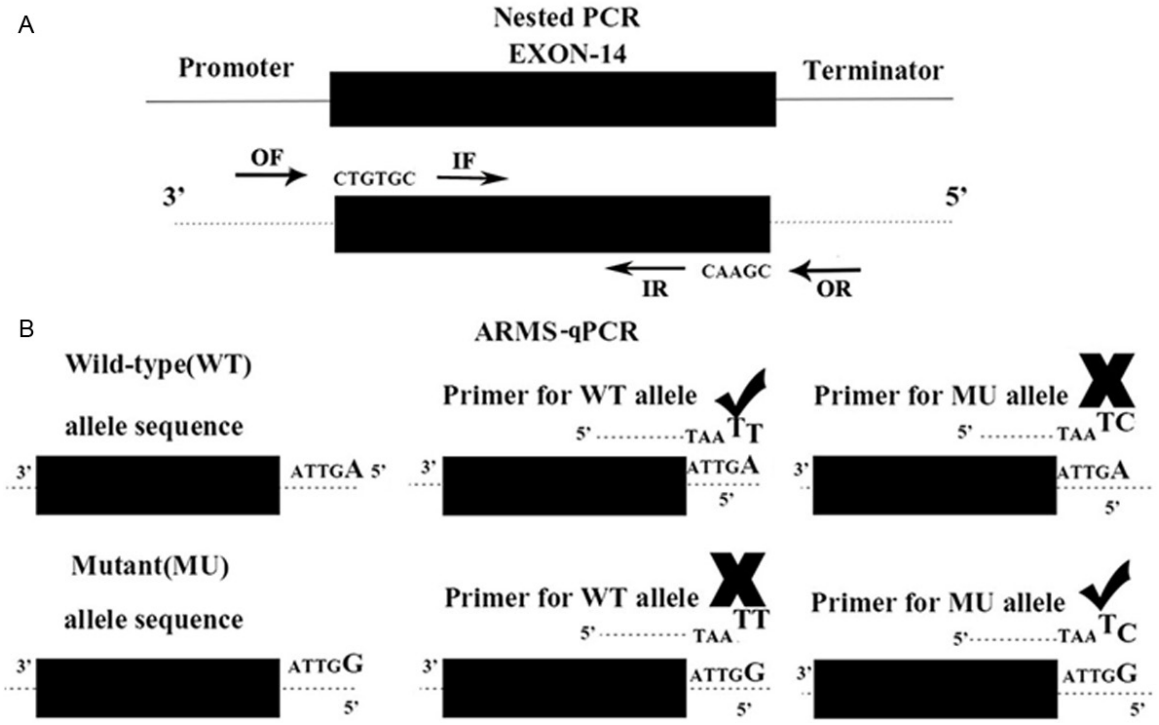


Figure 1. The principle of nested ARMS-qPCR. ARMS-qPCR is carried out as nested PCR. A. In the first step of nested PCR, DNA fragments of exon including SNP is amplified from the genome by outer primers, OF and OR. In the second step of nested PCR, using DNA fragments amplified in the first step of nested PCR as templates, SNP is genotyped by ARMS-qPCR with specially designed primers, IF and IR. B. The forward primer of ARMS-qPCR is the key to genotype SNP. The 3' end of forward primer for WT is complementary to WT allele of SNP, but not complementary to MU allele of SNP. Therefore, it can amplify WT/WT easily, but is hard to amplify MU/MU. Similarly, the 3' end of forward primer for MU is complementary to MU allele of SNP, but not complementary to WT allele of SNP. Thus, it can amplify MU/MU easily, but is hard to amplify WT/WT. However, both of forward primer for WT and MU can amplify WT/MU easily.

genotyping are single-strand conformation polymorphism (SSCP) [3, 4], PCR-restriction fragment length polymorphism (PCR-RFLP) [5, 6], molecular beacons, Taqman probe, denatur-

Table 2. Three novel SNPs identified by PCR-sequencing and nested ARMS-qPCR

SNP	Chromosome	Genomic position	mRNA position	Amino acid position	Function
RET c.135 G>A	10	NC_018921.2: g.43635178 G>A	NM_020630.4: c.135 G>A	NP_065681.1: p.45 Ala=	synonymous
RET c.2307 T>G	10	NC_018921.2: g.43653047 T>G	NM_020630.4: c.2307 T>G	NP_065681.1: p.769 Leu=	synonymous
VPS35 c.1648-24 T>C	16	NC_018927.2: g.48104087 T>C	NM_018206.4: c.1648-24 T>C	none	intron

ing high performance liquid chromatography (DHPLC), PCR-sequencing [7] and DNA chip [8, 9]. However, these methods are either expensive or time-consuming.

Amplification refractory mutation system-quantitative PCR (ARMS-qPCR) was firstly used as a tool to detect and quantify heteroplasmic mutant mitochondrial DNA [10]. Later, ARMS-qPCR was used as a tool to detect human mutation [11, 12] and hepatitis B virus mutation [13]. However, in the field of genetic polymorphism, ARMS-qPCR has not been valuated as a tool for SNP genotyping well till now. In this work, we evaluated nested ARMS-qPCR, which combined nested PCR with ARMS-qPCR, as a SNP genotyping method by identifying three novel SNPs, two in RET and one in VPS35. Compared with other SNP genotyping methods, nested ARMS-qPCR has characteristics of accuracy, rapidity as well as low cost.

Materials and methods

Healthy human group

The healthy group consisted of 136 people. They were recruited from people taking part in a health-screening survey at health examination center of Xiangya Hospital. Male and female ratio was even. Approximately 2 ml of peripheral blood samples were collected in tubes containing anticoagulant (EDTA-K_2). The experiments were conducted in accordance with the Declaration of Helsinki (2013). The informed consent was obtained from all people.

Extraction of DNA from peripheral blood

DNA samples were extracted using Phenol/chloroform method. Briefly, red blood cells (RBC) were lysed by freezing and thawing cycle. White blood cells (WBC) were lysed in nucleus lysis buffer with proteinase K and SDS. Samples were extracted by Phenol/chloroform. Isopropanol was used to precipitate DNA. DNA was dissolved in TE (10 mM Tris• HCl, 1 mM EDTA, pH8.0).

PCR-sequencing

RET Exon2, Exon13 and VPS35 Exon14 were amplified by PCR, with primer pairs RET Exon 2-2 F/R, RET Exon 13-2 F/R and VPS35 Exon 14-1 F/R, separately (**Table 1**). PCR was carried out in a total volume of 20 μL, containing 30 ng of DNA template (0.3 μL), 300 pmol/L primers (0.3 μL), and 10 μL PrimeSTAR HS Premix (TakaRa), with ddH_2O added to a final volume of 20 μL. PCR program: 98°C 5 min; 98°C 10 sec, 60°C 5 sec, 72°C 1 min, 40 cycles; 72°C 5 min; 4°C store. PCR product and primers were sent to Genscript (Nanjing, China) for sequencing.

Nested ARMS-qPCR

ARMS-qPCR was carried out as nested PCR (**Figure 1**). Step one, RET Exon2, Exon13 and VPS35 Exon14 were amplified by PCR. Step two, qPCR was carried out in a total volume of 20 μL, containing 0.3 μL of PCR product in step one as template (1:100 dilution before adding), 0.3 μL 300 pmol/L primers (**Table 1**), and 10 μL Maxima SYBR Green qPCR Master mix (Thermo Scientific), with ddH_2O added to a final volume of 20 μL. qPCR program: 50°C 2 min; 95°C 10 min; 95°C 15 sec, 55-60°C 30 sec, 72°C 30 sec, 40 cycles; 95°C 15 sec; 60°C 1 min; 95°C 30 sec; 60°C 15 sec, implemented in 7500 Real time PCR System (Applied Biosystems).

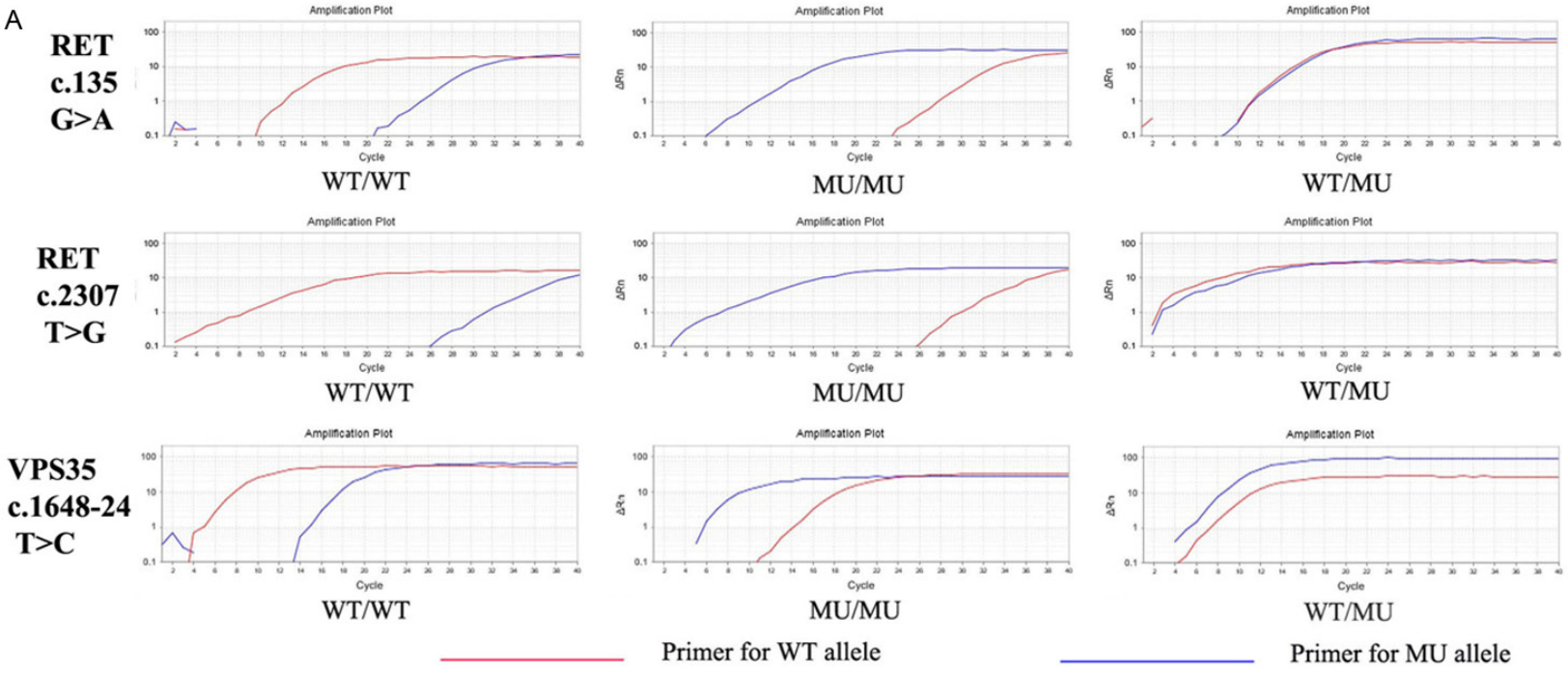
Statistical analysis

Paired t-test was used to compare the threshold cycle (Ct) value between wild type (WT) and mutant (MU) primer (**Table 1**). Chi-square (X^2) test was used to compare the genotype frequency between PCR-sequencing and ARMS-qPCR. Statistical analysis and graphs drawing were done with GraphPad Prism 6.0.

Results

Nested ARMS-qPCR is available for SNP genotyping

In the genetic research of pheochromocytomas (PCC) and Parkinson's disease (PD), we found 3


A
RET c.135 G>A
RET c.2307 T>G
VPS35 c.1648-24 T>C
Amplification Plot
Cycle
WT/WT
MU/MU
WT/MU
Primer for WT allele
Primer for MU allele
B
RET c.135 G>A
RET c.2307 T>G
VPS35 c.1648-24 T>C
MU/MU WT/WT WT/MU
bp MK PW PM PW PM PW PM
2000
1500
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750
500
250
100

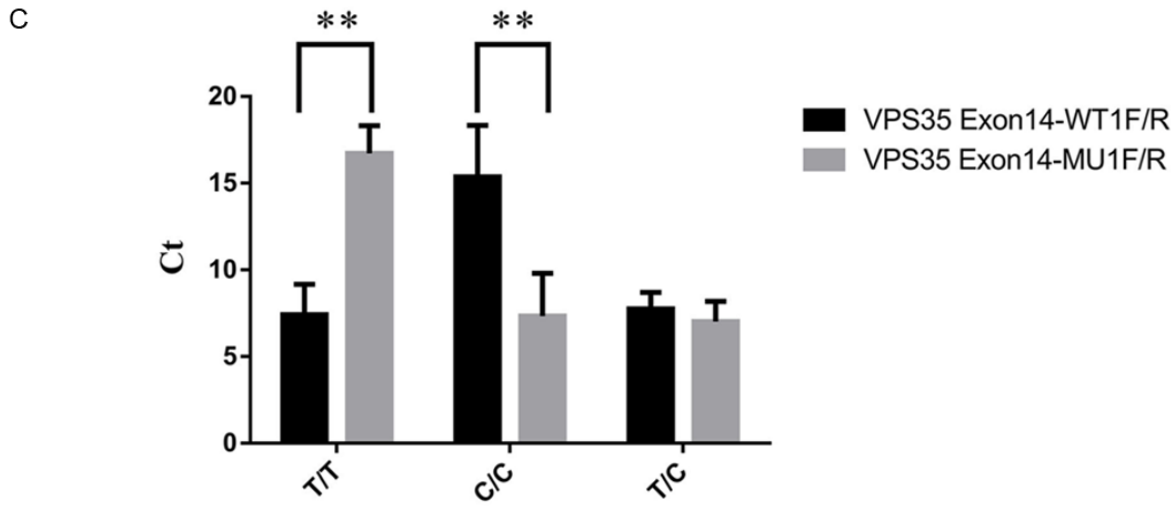

Figure 2. Nested ARMS-qPCR is available for genotyping three novel SNPs. A. Three novel SNPs can be genotyped by amplification plots of nested ARMS-qPCR. Every genotype of SNP is amplified in two tubes, one tube with primers for WT allele, and the other tube with primers for MU allele. Thus, every genotype of SNP gets two amplification plots. It is interesting that the pattern of two amplification plots is different in three genotypes of SNP. B. Three novel SNPs can be genotyped by agarose gel electrophoresis of nested ARMS-qPCR. Similarly, the pattern of agarose gel electrophoresis is different in three genotypes of SNP. MK indicates DL2000 DNA marker. PW indicates the primers for WT allele, and PM indicates the primers for MU allele. C. Comparison of nested ARMS-qPCR's Ct values between primers of VPS35 Exon14-WT1F/R and primers of VPS35 Exon14-MU1F/R in VPS35 c.1648-24 T>C. Paired t-test, $^{**}P<0.01$.

Table 3. Comparison of genotype frequency between nested ARMS-qPCR and PCR-sequencing (X^2 test)

Loci	Method	WT/WT	WT/MU	MU/MU	Total	X^2	*P* value
RET c.135 G>A	nested ARMS-qPCR	8	3	3	14	0.786	0.675
	PCR-sequencing	6	5	3	14		
	total	14	8	6	28		
RET c.2307 T>G	nested ARMS-qPCR	17	15	11	43	0.503	0.778
	PCR-sequencing	16	18	9	43		
	total	33	33	20	86		
VPS35 c.1648-24 T>C	nested ARMS-qPCR	6	28	22	56	4.223	0.121
	PCR-sequencing	14	21	21	56		
	total	20	49	43	112		

Table 4. Allele and genotype frequency of three novel SNPs measured by nested ARMS-qPCR

SNP	Chromosome Sample Count	Alleles (count/frequency)		Genotypes (count/frequency)		
		WT	MU	WT/WT	WT/MU	MU/MU
RET c.135 G>A	80	93 (58.13%)	67 (41.88%)	32 (40.00%)	29 (36.25%)	19 (23.75%)
RET c.2307 T>G	81	92 (56.80%)	70 (43.20%)	28 (34.57%)	36 (44.44%)	17 (20.99%)
VPS35 c.1648-24 T>C	92	75 (40.76%)	109 (59.24%)	6 (6.52%)	63 (68.48%)	23 (25%)

novel SNPs, two in RET associated with PCC and one in VPS35 associated with PD (**Table 2**). The three SNPs were NM_020630.4 (RET): c.135 G>A, NM_020630.4 (RET): c.2307 T>G and NM_018206.4 (VPS35): c.1648-24 T>C, which showed as RET c.135 G>A, RET c.2307 T>G and VPS35 c.1648-24 T>C respectively in follow-up article concisely. All three SNPs were assayed by nested ARMS-qPCR with primers for WT allele (WT1F) or primers for MU allele (MU1F) separately.

On one hand, we can discriminate three genotypes of SNP by observing amplification curve of nested ARMS-qPCR. For WT/WT, the amplification curves of WT1F all emerged much earlier than those of MU1F. However, for MU/MU, the amplification curves of WT1F all emerged much later than those of MU1F. If for WT/MU, the amplification curves of WT1F and MU1F all emerged almost simultaneously (**Figure 2A**). Meanwhile, the results of agarose gel electrophoresis were consistent with those of amplification plot (**Figure 2B**). On the other hand, Ct values of nested ARMS-qPCR can also be used for genotyping. For example, Ct values of VPS35 c.1648-24 T>C were treated with statistics. For WT/WT, the Ct value of WT1F was 7.43±1.75, but Ct value of MU1F was 16.72±1.60. The Difference between them reached -9.29±1.56, significantly in paired t-test (P=1.80E-04). For MU/MU, the Ct value of WT1F was 15.38±2.96, but Ct value of MU1F was 7.35±2.46. The Difference between them was 8.03±2.25 and significant (P=3.87E-10). However, for WT/MU, the difference of Ct value between WT1F and MU1F did not reach significant level, P=0.053 (**Figure 2C**). In conclusion, nested ARMS-qPCR was powerful tool to genotype SNP.

Nested ARMS-qPCR is as accurate as PCR-sequencing

To further verify the validity of nested ARMS-qPCR in SNP genotyping, the three SNPs had been screened with PCR-sequencing, the golden method for SNP genotyping. Furthermore, genotype frequency of nested ARMS-qPCR and PCR-sequencing had been analyzed by X^2 test. As for the three SNPs, genotype frequency between nested ARMS-qPCR and PCR-sequencing all had no significant difference (P>0.05). In detail, *P* value of RET c.135 G>A was 0.675, *P* value of RET c.2307 T>G was 0.778, and *P* value of VPS35 c.1648-24 T>C was 0.121 in X^2 test (**Table 3**). As a result, nested ARMS-qPCR was as an accurate SNP genotyping method as PCR-sequencing.

Allele and genotype frequency of three novel SNPs

Finally, it was with nested ARMS-qPCR that allele and genotype frequency of three novel

SNPs had been obtained (**Table 4**). Since some samples were fail to be extracted DNA, the "Chromosome Sample Count" was less than 136, the number of total peripheral blood samples. The minor allele frequency (MAF) of the three novel SNPs was as follow: A was 0.4188 in RET c.135 G>A; G was 0.4320 in RET c.2307 T>G; T was 0.4076 in VPS35 c.1648-24 T>C. Therefore, all three SNPs were polymorphism with MAF more than 1%. All these data would be submitted to NCBI/SNP so that researchers could get them freely.

Discussion

ARMS-qPCR for SNP may be implemented as several strategies, including one-step ARMS-qPCR, Taqman ARMS-qPCR, and nested ARMS-qPCR. One-step ARMS-qPCR means SNP is genotyped by amplifying with a pair of primers directly. Though one-step ARMS-qPCR is the most convenience strategy, design of its primers has limitation. In one-step ARMS-qPCR, 3'-end nucleotide of one primer, forward or reverse primer, has to be located in the SNP site, which makes optimized primers hard to design. In Taqman ARMS-qPCR strategy, Taqman probe is added to the ARMS-qPCR system to improve specialty, but Taqman probe is too expensive for some researchers to afford. However, nested ARMS-qPCR can not only get more optimized primers than one-step ARMS-qPCR, but also be cheaper than Taqman ARMS-qPCR.

In nested ARMS-qPCR, we adapt two-step strategy, nested-PCR and ARMS-qPCR. Nested-PCR amplifies the fragments containing SNP, which act as the templates in ARMS-qPCR. The significances of nested-PCR, the first step PCR, have two. First, DNA fragments including SNP can be amplified successfully and specifically as primers of nested-PCR can be optimized by software of primer design without the restriction that the 3'-end nucleotide of one primer has to be located in the SNP site. Second, ARMS-qPCR, the second step PCR, can also be carried out successfully and specifically since nested-PCR provided so large quantity of templates for ARMS-qPCR that interfering of genomic DNA can be neglected. In conclusion, SNP can be genotyped by nested ARMS-qPCR successfully.

ARMS-qPCR has the characteristics of accuracy, efficiency, rapidity and low cost. Moreover, qPCR and 7500 Real time PCR System (Applied Biosystems) have been used widely in clinic. Therefore, ARMS-qPCR has a bright future in clinical diagnosis of SNPs linked to disease and drug usage. A famous SNP is NCBI/Rs671, NM_000690.3 (ALDH2): c.1510G>A (p.Glu504-Lys), which is associated with acute alcohol sensitivity [14], susceptibility to hangover [15], efficacy of sublingual nitroglycerin [16], esophageal cancer [17], liver cancer [18], etc. Another example is some SNPs in CYP2C19 associated with drug response to Mephenytoin [19] and Proguanil [20].

Though the amount of SNPs in NCBI/SNP is enormous, there are still some SNPs have not been identified by people. This work is the best example. Furthermore, some data of SNPs in NCBI/SNP are not verified by authoritative institution, and different ethnics may have different SNP genotypes. Therefore, researchers should be very careful in using these data. For example, at the beginning of the project, we wanted to choose NCBI/SNP Rs34687100, NM_018206.4 (VPS35): c.1805T>A, p.Val602-Asp, as the target of ARMS-qPCR. In AGI_ASP population, genotypes frequency of Rs34687100 are A/A=0.941, A/T=0.059. However, we sequenced 80 Chinese people, all genotypes of whom were A/A. At last, we had to give up Rs34687100 as the target of ARMS-qPCR.

Disclosure of conflict of interest

None.

Abbreviations

SNP, Single Nucleotide Polymorphism; UTR, untranslated regions; CDS, coding sequence; GWAS, genome-wide association study; SSCP, single-strand conformation polymorphism; RFLP, restriction fragment length polymorphism; DHPLC, denaturing high performance liquid chromatography; ARMS-qPCR, amplification refractory mutation system-quantitative PCR; WT, wild type; MU, mutant; Ct, threshold cycle; MAF, minor allele frequency.

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