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Development of Cost-effective Tetra-primer Amplification Refractory Mutation System (T-ARMS) PCR for the Detection of *miR-146a* gene rs2910164 C/G Polymorphism in Breast Cancer

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Abstract

Evaluation of a single nucleotide polymorphism (SNP) and single nucleotide mutation in cancer patients and other diseases is crucial for genotypic characterization in order to select therapy and genetic counseling. The study aim is to develop and optimization for the detection of *miR-146a* gene rs2910164 C/G polymorphism in breast cancer. Tetra-primer Amplification Refractory Mutation System (T-ARMS) PCR was applied on 60 individuals to determine the genotype and allele frequencies. Genotype frequencies of *miR-146a* gene rs2910164 C/G polymorphism were homozygous CC 65%; heterozygous CG 6.65%; homozygous GG 28.35%; allele C and G frequencies were 68.5% and 31.5% respectively. These were in Hardy–Weinberg's equilibrium; p=0.18.

Our study provides a simple rapid and a cost-effective T-ARMS-PCR molecular technique that could be used for the detection allele specific DNA polymorphisms and mutations in certain diseases and normal population at large scale.

Keywords: miRNA-146a; Breast cancer T-ARMS-PCR; Single Nucleotide Polymorphism (SNP)

Introduction

Breast cancer is one of the leading cause of death among women world over and the second most common malignancy after lung cancer [1]. Multifarious patho-physiology of breast cancer as both environmental and genetic factors is involved in its causation. Nowadays its early detection options have improved thanks to the latest technology many new innovative techniques have been developed but still need to develop the low cost methods that could be applied in developing countries.

Single nucleotide polymorphisms (SNP) of microRNAs (miRNA) genes involved in cell cycle [2] that express the products in the form of microRNAs and proteins that bind to their corresponding 3'untranslated region of target mRNAs [3] to regulate are extremely crucial for both normal and abnormal biological processes [4]. The polymorphism as homozygous or heterozygous of miRNA genes may lead to the causation of cancer, metastasis and drug resistance [5]. At the DNA level 99.5% of it is identical in the general population, only 1% of single nucleotide polymorphism is present within the population an if these SNPs present in the coding region of genes, they can disrupt the structure and functions of proteins that can result the onset of disease processes [6].

Genotyping techniques are all based on the principles of a few basic technologies such as PCR-based methods, hybridization technologies, fragment length polymorphism analysis and Sequencing. The issues associated with these genotyping techniques are time consuming and costly [7]. Newton et al. in 1989 [8] and Shu et al. modified the PCR to detect the wild and mutant alleles [9]. Thus, the method employed to detect allele specific products should be trust worthy, which can detect large number of samples fast enough with minimal of cost.

In our present study, we investigated SNP rs2910164 (C/G) of *miR-146a* gene on human chromosome 5q33.3 which is associated with different types of cancers including breast cancer [10,11]. Different studies so far conducted have shown susceptibility of this polymorphism to cancer and other studies have refuted the findings of any type of association of this SNP with cancers like breast, bladder and kidney in Asian and white people [12].

In this pilot study tetra-primer T-ARMS-PCR was developed and optimized the protocol for amplification of both alleles in a single reaction to conclude the sample as homozygous or

heterozygous simultaneously. Then it was carried out in case control study to evaluate the clinical role ofmiR146a rs2910164 (C/G) genotype and allele frequencies in the susceptible and diagnosed breast cancer as a risk marker [13-16].

Materials and Methods

Sample selection and DNA extraction

Control sample of 60 individuals collected for the case control study were used for the pilot project. The study was approved by the institutional Ethical Review Committee (ERC) of medical faculty Riphah International University-Islamabad Pakistan (ref# Riphah/IIMC/ERC/16/016). The consent, study protocol quaternary forms were approved by institutional ERC. The study was conducted at Multidisciplinary Research Lab, Islamic International Medical College, Riphah International University-Pakistan in collaboration with Genetics Resource Centre. Written informed consent was obtained from every individual before drawing the blood with the respective institutional permission (ref # 12655). Genomic DNA was isolated from the blood samples according to standard chelex method and the extracted DNA was stored at -20°C before amplification. The quantification and purity of extracted DNA samples was done by measuring the absorbance at 260 nm and 280 nm (A260/A280) on NanoDrop2000c spectrophotometer (Thermo Fisher Scientific).

Primer designing and T-ARMS-PCR amplification

Allele specific primers with different lengths were designed manually and checked by BLAST tool- NCBI. Amplified DNA fragments for genotypes CC (allele-C) and GG (allele-G) are 290 bp; 203 bp and internal control 445 bp that can be easily separated by 6% poly acrylamide gel electrophoresis (PAGE). The complete contents of single nucleotide polymorphism 2910164 are available at the public website: http://www.ncbi.nlm.nih.gov/ SNPCATGGGTTGTGTCAGTGTCAGACCT[C/G]TGAAATTCAGTTCTTCAGCTGGGAT Chromosome: 5:160485411; Gene: *MIR146A* HGVS: NC_000005.10:g.160485411C>G (**Figure 1**). The sequences of the primers are given in the **Table 1**.

Allele specific T-ARMS-PCR amplification

Genotype for SNP *miR-146a* rs2910164 C/G was amplified by T-ARMS PCR in a thermal cycler (major sciences USA). The PCR reaction mixture of 25 ul in a 0.2 ml PCR tube containing 1 ul approximately 100 ng/ul of DNA template, master mix (12.5 mM $MgCl_2$) 4 µl (5X FIREPol (R) Master Mix- Solis BioDyne), 1 ul primer mix (each primer of 10 pmol/ul) and upto 19 ul water (PCR grade). PCR reaction was run according to the following conditions; holding time 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 minutes. The amplified products of each reaction were separated on 6% PAGE stained by 0.1% silver nitrate to visualize the bands.

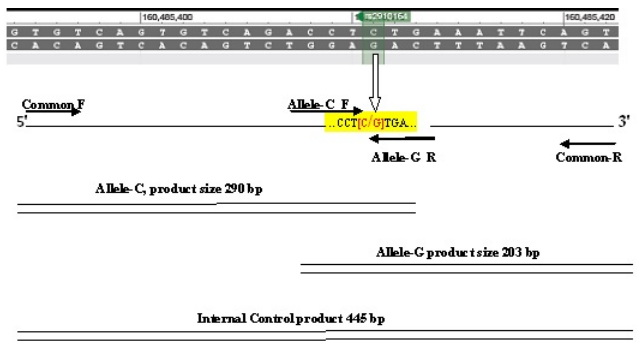

Figure 1 Schematic diagram of tetra-primers for the three amplified products used in T-ARMS-PCR. Common forward (CF) and common reverse (CR) primer set give the amplified product of 445 bp as an internal control. Allele-C F and CR primer set make the product size of 290 bp for allele-C and allele-G R and C F primer pair gives a 203 bp product for allele G. Common F: Common Forward (CF); Common R: Common Reverse (CR); Allele-C F: Allele-C Forward; Allele-G R: Allele-G Forward.

Table 1 Sequences of primers.

SNP	Primer Set/Sequence	Product size in bp
miR-146a (rs2910164)	Primer-F (Allele-C) 5'-tccatgggttgtgtcagtgtcagagctc-3'	290
	Common Primer-R 5'-gagtagcagcagcagcaagagagactt-3'	
	Common Primer-F 5'-tagacctggtactaggaagcagctgcat-3'	203
	Primer-R (Allele-G) 5'-atatcccagctgaagaactgaattacac-3'	
	Common primers R and F	445

Results

miR-146a rs2910164 C/G polymorphism of 60 control samples was done for genotypic and allelic frequencies are shown in the **Table 2**. Genotype frequencies were 65% for CC, 6.65% for GC and 28.35% for GG respectively. Allele frequencies were 68.35% and 31.65% for C and G respectively, and these were in Hardy–Weinberg's equilibrium (p=0.18). Findings of genetic variations of *miR-146a* rs2910164 polymorphism on specific locus of chromosome: 5:160485411 by allele specific T-ARMS-PCR containing the four primers (two

alleles specific and two common primers) in a single PCR-tube for one sample.

Table 2 Genotype and allele frequency of *miR-146a*rs2910164 (C/G polymorphism).

miRNA Gene	SNP	Genotype	Genotype frequency N= 60 (%)	Allele	Allele frequency n=120 (%)
146a	rs2910164 (C/G)	GG	17 (28.35)	G	38 (31.65)
		CG	4 (6.65)	C	82 (68.35)
		CC	39 (65)	-	-

Primer pair of allele-C (forward) and common reverse primers amplify the allele C and; primer pair allele-G (reverse) and common forward amplify the allele G that showed the heterozygous sample (heterozygous CG genotype). The three bands for alleles C, G and common internal control (amplified fragment of common forward and common reverse) were visualized on PAGE on a single lane. In homozygous GG genotype pattern allele-G (reverse) and common forward amplify the product of allele-G and common fragment resulted two bands appeared in **Figure 2**. Primers set of allele-C (forward) and common reverse amplify the allele C and; common forward and common reverse generated the allele C and control DNA fragments showing the two bands in **Figure 3**.

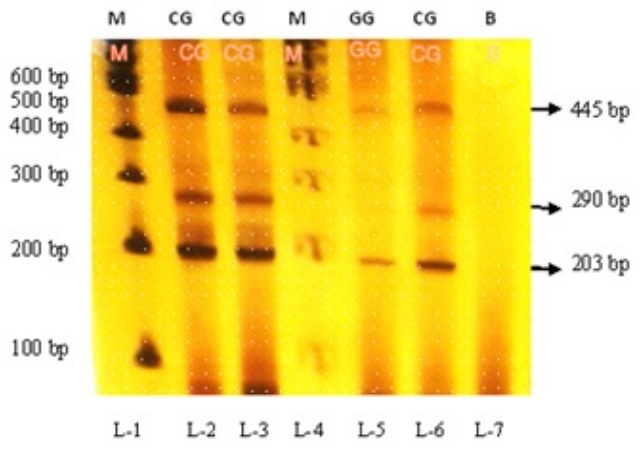

Figure 2 Bands pattern of amplified products on *Polyacrylamide gel electrophoresis* (*PAGE*) using Tetra-arms refractory mutation system-polymerase chain reaction (T-ARMS-PCR) assay for the detection of *miR-146a* rs2910164 C/G polymorphism.

Discussion

Different technologies like probe based microarray, real, direct sequencing, restriction fragment length polymorphism (RFLP) cleavage enzyme based methods [13] for single nucleotide polymorphisms (SNPs) or mutations and multiple genotypes identification by MALDI-TOF mass-spectrometry [14,15] that are expensive, time consuming, laborious and sophisticated instruments required. In addition expensive chemicals, equipments and difficult multi-step operation of post-PCR are needed. Conventionally, allele specific PCR method had been developed for detection of SNPs or point mutations in order to demonstrate the genotypes in separate PCR-tubes [16] and real-time PCR can be used which is quite costly [17].

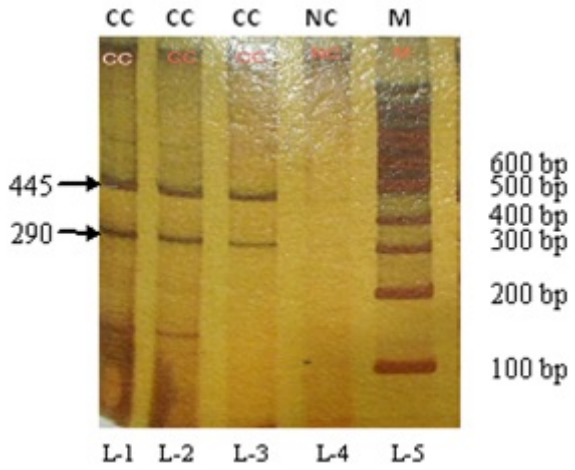

Figure 3 6% PAGE electrophoresis pattern of selected known homozygous*miR-146a* rs2910164 C/C polymorphism.

In our study, we develop the cheap T-ARMS-PCR method for *miR 146a* gene rs2910164 C/G polymorphism to find the zygosity as homozygous/heterozygous in a single PCR reaction containing manually designed four primers two allele specific and two common that ultimate reduces the expenses, additional special chemical, labor, contamination and miss-loading the allele of one sample with other. The unique feature of tetra-primers set is to make the DNA fragment resulted by the amplification by common forward and common reverse pair in each PCR-reaction that could be used as a internal procedural control and to confirm the genotype by sequencing. The primer designing strategy of pilot study and optimization of T-ARMS-PCR then applied for case control study in breast cancer susceptible genes.

Conclusion

The newly developed optimized allele specific T-ARMS-PCR is the simplest, less-time consuming and cost-effective method

in a single PCR-tube for detection of both alleles simultaneously that could be used for the measurement of alleles and genotype polymorphisms at a particular gene locus in order to diagnose and evaluate the treatment response in multi-factorial diseases in different ethnicity populations.

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Disclosure and Conflict of Interest

None

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