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TETRA-PRIMER ARMS-PCR AS AN EFFICIENT ALTERNATIVE FOR SNPS DETECTION IN MOLECULAR DIAGNOSTIC: A COMPARISON STUDY

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ABSTRACT

Objective: The aim of this research was to compare the efficiency of the tetra-primer amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) with restriction enzyme for detection of SNP anomalies in breast cancer blood samples. **Method**: one hundred breast cancer patients were analyzed for SNP mutation using tetra-primer ARMS-PCR technique and restriction enzyme (Pst1), to identify allele distribution of *LCN2* on exon one a SNP rs11556770 (G/T). In this case, a single nucleotide transforms the amino acid from Glutamine, Gln (CAG), to Histidine, His (CAT). **Result**: Both techniques, tetra-primer amplification refractory mutation system–polymerase chain reaction (ARMS–PCR) and restriction enzyme, yielded normal (non-pathogenic) genotype pattern for the same SNP of sets samples, wherein tetra-primer ARMS PCR was 444bp, 156b, While restriction enzyme (PstI) three band 261bp, 135bp, 48bp. Nevertheless, both results estimate a normal allele for both techniques. **Conclusion**: Tetra-primer arms PCR method attained high specificity and sensitivity, along with the fact that this technique is more affordable, it can therefore be concluded that this method is an efficient and convenient option for detection of SNP anomalies for diagnostic purposes.

KEYWORDS: T-ARMS-PCR, breast cancer, LCN2, Pst1, SNP.

2. INTRODUCTION

Breast cancer is one of the most widespread and severe malignancies that affect women.[1] In Saudi Arabia, breast cancer constitutes 28.7% of all cancers in women.[2] Previous studies in Saudi Arabia indicate that the incidence of breast cancer was expected to increase by 350% in 2025.[3]

Recent studies have implicated lipocalin 2 (*LCN2*), a member of the lipocalin family, in leukemia and other solid tumors. The lipocalin family is a large group of small-secreted glycoproteins involved in binding and transportation of small lipophilic molecules. The physiologic ligand for *LCN2* is still unknown. In breast cancer, a high molecular weight complex consisting of *LCN2* and matrix metalloproteinase-9 (MMP-9) was observed in patient urine samples. Elevated *LCN2* expression was shown to associate with decreased survival rate in breast cancer patients. Furthermore, Increased *LCN2* expression was found to be associated with increased tumor cell motility.[4]

Up to recent studies, counting single nucleotide polymorphisms (SNPs) is a relatively approachable analysis and can be used as a diagnostic tool for detecting variability in individuals susceptible to cancer.[5] For the purification of restriction endonucleases, an abbreviated method was created. This method utilizes phosphocellulose and hydroxylapatite chromatography and outcomes in enzymes of adequate purity to allow their use in DNA sequencing, molecular cloning, and physical mapping.[6] In current study, to investigate the frequency of the *lipocalin 2* alleles, DNA extracted from blood samples, PCR product digestion with *PstI* restriction enzyme for detection of *lipocalin* alleles 2.

Tetra-primers amplification refractory mutation system–polymerase chain reaction (ARMS–PCR) is one of many methods developed to detect the genotype (**figure 1**). It is a straightforward and economical method of genotyping single-polymerase polymorphisms (SNPs) compared to contemporary genotyping tools such as restriction enzyme.[7] The method utilizes primers in a single PCR followed by gel electrophoresis, while the restriction enzyme method requires longer time, tools and reagents.[8]

The mismatches in the techniques are observed in the three termini of the alleles-specific primers where four

primers are used to determine the genotype. The genotyping process in tetra-primer ARMS–PCR is done in a single reaction using two distinct annealing temperatures (Tm), a high temperature in initial cycles and a lower one in latter cycles using touch down PCR. Touchdown PCR is associated with optimizing PCRs without having to optimize and/ or redesign the primers.[9]

As the tetra-primer ARMS–PCR reaction begins, two allele-specific primers (inner primers) amplify regions that compromise the mutation. As the production of outer primers fragments continues, it acts as a template to the inner primers, which result in the production of allele-specific fragments. The distinction of the two allele-specific fragments can be done by detecting their distinct sizes in an agarose gel after placing them in different distance from the polymorphic nucleotide (**figure 1**).[10]

The[11] research designed specific tetra-primer ARMS–PCR assays for 6 mutations in which the results showed that all the mutations were easily detected with the specific and multiplex tetra-primer ARMS–PCR. Comparatively, the[5] research had similar genotyping results as well. Previous study in Saudi Arabia indicate that tetra ARMS-PCR method was an effective detection for genotyping of (BRCA1 c.4132G>A) and (BRCA2 c.1385A>).[12]

The objective of this study was to compare the efficacy of the tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) with the restriction enzyme for detecting SNP anomalies in blood specimens of breast cancer.

3. MATERIALS AND METHODS

3.1 Human subjects

Among 200 women aged between 18 years and 80 years from Jeddah, a case-control study was conducted; 50% of whom were controlled and 50% were patients with breast cancer. Each of them gave written informed consent to their participation in the study on age, positive family history of cancer, consumption of contraceptive pills, significant psychological distress and early detection of tumors by mammography screening.

3.2 Sample collection

Participant blood samples were taken from University Hospital King Abdulaziz and Medical Reference Clinics in Jeddah city. Biomedical ethics unit approved the study at the University of King Abdulaziz No. (2/36/40591).

3.3 Extraction of DNA

Using ReliaPrepTM Blood gDNAMiniprep System kits, genomic DNA was extracted. It was taken from the whole blood and stored in the EDTA coated tubes Lavender top tube, Franklin Lakes, NJ, USA.

3.4 Amplification for Lipocalin 2 gene

PCR used the GoTaq ® Green Master Mix to amplify the gene of Lipocalin 2. First step modeling the PCR system by determination the tetra-primer ARMS-PCR primers Tm, in this research was set 60°C and 56°C. Touchdown PCR is a common method using a cycling program to adjust the temperature of the annealing step. Furthermore, the program used has four major steps:

Step 1: denaturation at 95°C for 2 min for one cycle.
Step 2: 15 cycles for denaturation at 95°C for 45 seconds(s), annealing at 60°C for 45s and extension for 1 min at 72°C.
Step 3: 20 cycles for denaturation at 95°C for 45s, 1 minute extension at 72°C and 45s annealing at 56°C.
Step 4: Extension for a cycle at 72°C for 5 minutes.[13]

The results of the PCR were confirmed by analyzing 3µl of the PCR product in 2% (w/v) agarose gel, the rest of the PCR product stored at -20C (**Table 1**).

3.5 Genotyping of *Lipocalin 2 gene*

The resulting DNA fragments had a length of 444 bp. procedure determined the genotypes for this SNP. They were prepared as follows: 10µl of PCR product, 16.3µl of sterile, deionized water, 0.2µl of 100X BSA, 2µl of 10X RE buffer, and mixed by pipetting in a labeled clean and dry Eppendorf tubes. Finally, 0.5µl of restriction enzyme (***PstI***) were added. The tubes were incubated at 37℃ for 2 hours. After that, the genotypes were resolved after running it on 2% (w/v) agarose gels electrophoresis.

In normal case the resulting for restriction enzyme (*PstI*) three band 261bp, 135bp, 48bp and for mutation are two bands 243, 162 whereas in tetra-primer ARMS PCR primers technique for normal 444bp, 156b and for mutation 444bp, 327bp.

3.6 Ethical approval and consent to participate

The ethical Committee and unit of biomedical ethics approved the study at King Abdulaziz University (No.2//ـ36/40591). The study was ethically approved, and participants consent was take to participate in the study.

3.7 Statistical analysis

The Statistical Package of Social Sciences (SPSS version 20) (SPSS Inc., Chicago, IL, U.S.A) has been used. Data was presented as mean +/- standard deviation or number (percentage) wherever appropriate. The continuous variables were made between two groups using unpaired sample t-test and between more than two groups using One-Way ANOVA (LSD) test and also between categorized data using Chi-Square test. The level of significance was α=0.05.

4. RESULT AND DISCUSSION

Molecular diagnostics is a part of laboratory medicine that relies on the detection of individual biological molecules and help distinguishing between tumors of diverse histologic origin. Furthermore, novel cancer therapeutic approaches rely on comprehensive detection of molecular markers in malignant tumors. The invention

of PCR led to a remarkable advancement in clinical genetic testing both on gene and protein levels. Molecular-based techniques, such as SNPs analysis, are non-invasive and require relatively simple instrumentation, consuming very small amounts of biological sample. Currently, identification of individuals with hereditary cancer is a routine practice in clinical oncology. Additionally, based on the detection of molecular characteristics of tumor tissues or other biologic parameters of malignancy, the most effective treatment can be specifically chosen and implemented.

Novel applications are developed each year for tumor diagnosis that are becoming standard approaches, including serum, protein and gene expression analyses and mutation analysis is now routinely applied for hereditary cancer predictive and prognostic value.[14] Screening and detection of breast cancer, as well as other forms of malignancies, at their earlier spares the patient the financial burden of expensive therapies.[15] Molecular personalized diagnostics is becoming the first option for oncologic potential prognostic information and curing options for patients with numerous cancer disorders in order to maximize treatment benefit and minimize unnecessary resistance and toxicity.

The NIH Consensus Conference outlined the definition of a prognostic molecular factor. These factors have to meet certain criteria: they have to provide significant prognostic value, which has been validated by clinical testing. The determination of the factor must be feasible, reproducible, and accessible and has quality control. Clinicians must readily interpret the results; the measurement of the marker must not consume tissue needed for other tests, particularly histopathologic evaluation.[16,17,18]

In this research, a comprehensive comparison between two techniques; tetra-primer ARMS-PCR and restriction enzyme, for analyzing SNPs mutation via identifying allele distribution of *LCN2*. Both techniques yielded the same diagnostic outcome; a normal non-pathogenic genotype pattern for the same set of samples. However, resulting bands were different in number and lengths for the two methods.

Genotypes for *LCN2* gene by tetra-primer ARMS PCR primers technique are illustrated in (**figure 2**). Lines showed genotyping in the patients group, a double band of 444 and 156bp showed the presence of homozygous allele G that is the normal gene type.

Genotypes for *LCN2* gene as mentioned in (**figure 3**) by Restriction enzyme (*PstI*), resulted in three bands of 261, 135 and 48bp. Lines showed genotyping in the patients group, which has also shown the presence of homozygous allele G and the normal gene type.

The standard method of restriction endonuclease *PSt1* digestion resulted in three bands of different lengths that are a characteristic of the normal pattern of the gene, 261, 135 and 48bp, which indicated a nonpathogenic case. This result is a natural outcome of occurrence of two cutting points at the DNA sequence. Whereas, in the case of tetra-primer ARMS-PCR for the same samples, only two bands, 444 and 156bp, resulted where only one detecting point is present in the case of normal gene sequence.

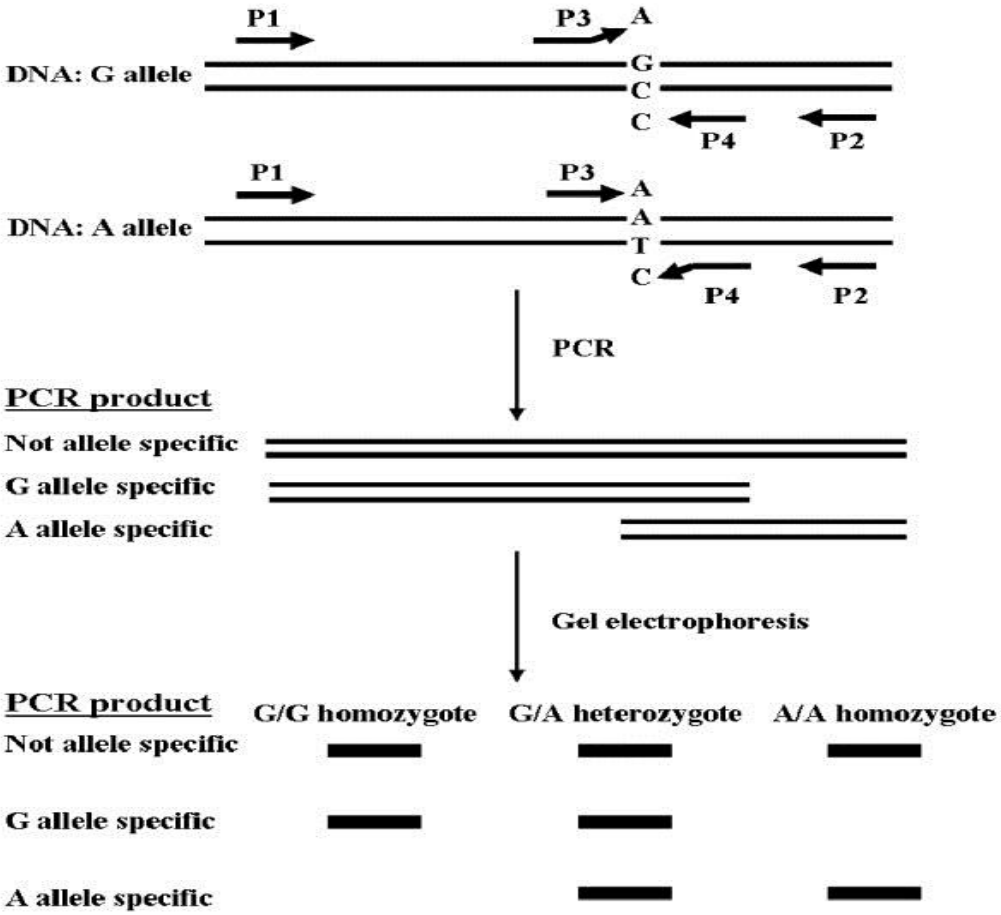

Figure 1: Tetra-primer ARMS PCR [10]

Table 1: Primer designing by blast software.

System	Primer sequence (5' –3')	Allele	Tm(°C)	LENGTH	Amplicon (bp)	CG%
Forward outer primer (F1)	5'CTTCCTCGGCCCTGAAATCAT -3'	-	61.3 ºC	21	444	**52.38**
Reverse inner primer (R2)	5'-TGG TTG TCC TGG AAG TTC TG**C**-3'	G	61.3ºC	21	156	**52.38**
Forward inner primer (F3)	5'-GAGCAAGGTCCCTCTGCA**T**-3'	T	59.5 ºC	19	327	**57.89**
Reverse outer primer (R4)	5'- CAT CTG TGC AGG GGG ACT3'	-	58.4 ºC	18	-	**61.11**

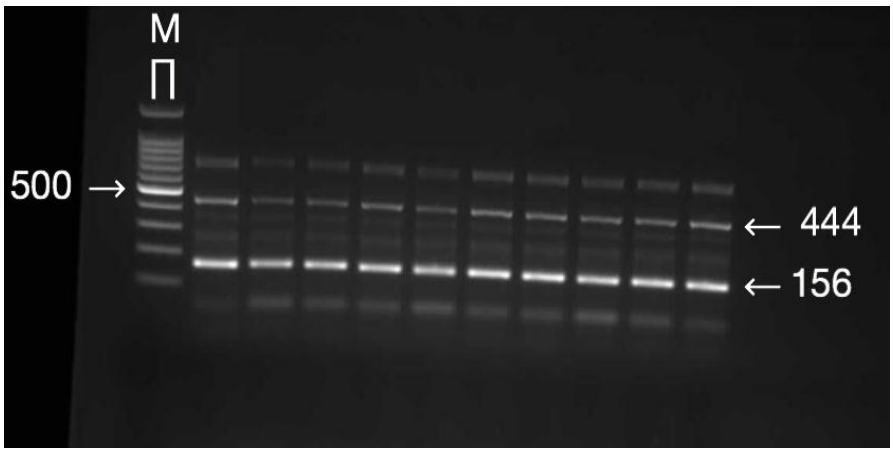

Figure 2: Photograph of a 2% (W/V) agarose gel showing the result of amplification of human exon 1 in LCN2 gene by tetra-primer ARMS PCR primers.

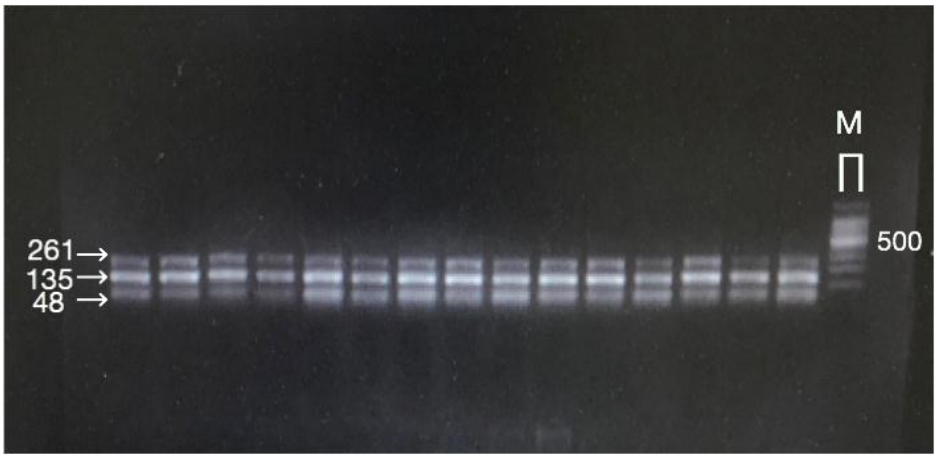

Figure 3: Photograph of a 2% (W/V) agarose gel showing the result of amplification of human exon 1 in *LCN2* gene by Restriction enzyme.

5. CONCLUSION

Although tetra-primer ARMS-PCR is a relatively simple and economical method, once the melting temperature is optimized, it has proven to be an efficient and precise approach for genotyping of SNPs without the need of any further requirements other that standard primer design and conventional PCR equipment. Furthermore, it is less time-consuming and more affordable alternative than restriction endonuclease genotyping.

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