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ROLE OF ARE2 AND ARE3 PROMOTER REGION OF PSA IN FEMALE BREAST CANCER PATIENTS

Pavithra Venkateshaiah[1], Sathisha Tumkur Gururaja[2], Kasturi Kondapalli[1*], Jeevan Amos Seelam[1], Nasreen Sulthana[1] and KRS Sambasiva Roa[1]

[1]Department of Biotechnology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.
[2]Department of Biochemistry, Sri Siddhartha Medical College, Tumkur, Karnataka, India.

***Corresponding Author: Kasturi Kondapalli**
Department of Biotechnology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

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ABSTRACT
Direct sequencing analysis of ARE2 and ARE3 region of PSA gene in 50 patient and 50 age- matched control samples was carried out in the present study. The T>C substitution was found in both cases and controls with a varied percentage i.e., 53.4% and 46% controls. This indicates the change is a common polymorphism reported in ARE-2 region of the PSA gene in evaluated number of samples. According to the pilot study the DNA variation T>C may not be playing a role in breast cancer. Multiple numbers of insertions were reported in patient samples when compared to controls in ARE2 region, these sequence alterations were not present in control samples analysed. All these insertions were found in the amplicon ARE2 of patient samples (8%) which may impact the regulation of transcription of PSA gene from this region. Conversely, to validate the definitive role of ARE2 region affecting expression of PSA gene in breast cancer large sample size studies are needed. All the samples analysed for ARE3 region did not report any DNA sequence changes in controls as well as cases indicative of its insignificant role in breast cancer.

KEYWORDS: Prostate Specific Antigen, Androgen Response Element, Gene Polymorphism, ARE2, ARE3, Breast Cancer.

INTRODUCTION

Gene Locus of PSA

Gene locus of PSA is located on chromosome 19q13.3to q13. 4, which has a region of 261558bp.[1] PSA is coded, by 6Kbp length of DNA[2] and belongs to the Human kallikrein family and is also called hK3.[3] It forms a large cluster of serine proteases in the human genome and has 15 tandemly localized genes and has no interventions from any genes. Expression of PSA gene in female breast tissue is controlled by steroid hormones like androgens and progestin.[4]

PSA Gene expression in breast cancer

Gene of PSA encodes 240 amino acid glycoprotein serine protease. PSA is present as ~33kDa monomer and with α1anti chymotrypsin as 100kDa and as an 800kDa with α2 macroglobulin complex in the serum. In recent times enhanced ultrasensitive methods and RNA assays have revealed that PSA is not absolutely produced in the human prostate gland alone, but is also produced in the breast, ovary, liver, kidney adrenal, and parotid glands.[5&6] Diamandis *et al.,* was the first to discover immunoreactivity and mRNA expression of PSA in breast cancer. Seminal PSA and breast PSA molecular weight and mRNA sequence are found to be identical. Molecular mechanism of PSA in breast cancer have been poorly understood. Studies have shown that steroid hormones regulate PSA gene. Androgen receptor (AR) regulates wild type PSA gene by mediating transcription by binding to a cognate sequence on the PSA proximal promoter at positions -154 (ARE1) and --394 (ARE2). [7] Additionally at position -4200, ARE3 is located and is called distal enhancer.[8] The proximal and the distal enhancers of the PSA gene act together in a joint manner and are essential for optimal expression of the gene.[9] Reports have suggested that polymorphism of the PSA promoter ARE1 region has G/A substitution.[10]

In breast cell lines T-47D and BT-474, PSA production is induced by androgen, progesterone, mineral corticoids and glucocorticoids. Studies by Magklara *et al.,* have suggested that comparative levels of specific co-activators/co-repressors may differently change AR transcriptional activity in the promoter/enhancer region of KLK3 (PSA) of different breast cancer cell lines. DNA sequencing of PSA gene in breast tumors, have shown no mutations in the coding region of PSA gene. However, multiple mutations/polymorphisms have been detected in the promoter and enhancer region, which may change the regulation of the gene by steroid hormones

disturbing the PSA expression level.[11,12,13] It is well-known that expression of PSA is androgen regulated. [14] Studies suggest PSA might work as a growth factor or a regulator of growth factors and marker of endogenous hormone balance involving androgens, progesterone and estrogens.[15]

Binding of transcription factors to the DNA sequences inside a 5.8kB segment upstream of the coding region regulates gene expression of PSA. PSA gene expression is up regulated by a 15 base nucleotide sequence which helps in binding of steroid to androgen receptor. Three types of such response elements have been recognized ARE-I at −170 to −156 and ARE-II at −394 to −380, these two are within several hundred bases of the transcription start off site and a third ARE-III at -4200 bases further upstream.[8]

The recognition of single nucleotide substitutions, insertions and deletions in the PSA gene promoter increased the possibility that a change in PSA expression may depend on the particular sequences present in these response elements. Association to cancer tendency and prognosis was recommended by the amount of these evident mutations in prostate and breast carcinoma cell lines and in breast tumors.

The present study was undertaken to find polymorphisms at PSA promoter ARE2 and ARE3 region in the female breast cancer patients of South India.

AIM

Gene polymorphic studies on PSA gene promoters ARE2 and ARE3 region in female breast cancer patients and age matched controls.

MATERIALS AND METHODS

Materials

A case-control study in female patients with breast cancer was conducted. Serum samples were collected from Manipal super specialty hospital and City Cancer center situated in Vijayawada, India. Ethical clearance was approved for study in accordance with the ethical standards of the institutional ethics committee on human experimentation and with the revised Helsinki Declaration before starting the study. The written informed consent was taken from both patients and controls. Inclusion criteria: Newly diagnosed female patients with breast cancer in the age group of 30-60 years attending the Oncology clinic were included in the study. These cases were selected irrespective of the type and stage of the disease. The diagnosis was established based on clinical, radiological (mammography) and histo-pathological features. Exclusion criteria: The patients who have undergone or undergoing treatment for breast cancer had been excluded. Patients who have undergone surgery for breast cancer in the past have also been excluded. The patients or controls suffering from co-morbid conditions which affect serum levels of factors included in the present study and other malignancies were excluded from the study. Controls: The age matched control subjects were drawn from apparently healthy women attending master health check at the super specialty hospital. Collection of blood: 1ml of venous blood was drawn from median cubital/ basilica vein, into BD purple capped EDTA vacutainers under aseptic conditions, from all the participants of the study, stored under-70^{0}C and DNA extraction, PCR and gene sequencing was carried out as required.

Methods

Sequence analysis of 2nd Androgen Response Element (ARE2) and 3rd Androgen Response Element (ARE3) region of PSA gene.

DNA sequence analysis requires following important steps which are as follows:

DNA isolation: DNA isolation was carried out from blood by a rapid, non-enzymatic method by salting out cellular proteins.

Quantification of extracted DNA: DNA isolated from whole blood was quantified using electrophoresis technique on 1% agarose gel.

Polymerase Chain Reaction of extracted DNA of ARE2 and ARE3 gene to evaluate gene polymorphisms: After mixing all the contents, mentioned in table 1 PCR tubes were kept in thermal cycler and a 3 step PCR was carried out. Thermal profile used is mentioned in table 2. PCR amplification of ARE2 and ARE3 from blood DNA samples was carried out using specific oligonucleotide primers. Primer used is mentioned below. A detail of primers synthesis is mentioned in table 3. Table 4 gives details of primers and PCR conditions used for selected genes**.**

Table 1: Protocol: **For making a 50 µl PCR reaction**

PCR Mix	
Chemical	**Amount**
Distilled Water	36.5µl
10x buffer	5 µl
$MgCl_2$	4 µl (2mM)
dNTP's	1 µl (10mM/ µl)
Forward primer	1 µl (10pmol/ µl)
Reverse primer	1 µl (10pmol/ µl)
Template DNA	1 µl (~ 50ng)
Taq polymerase	0.5 µl (2.5U)

Table 2: Thermal profile used

Step	Temperature	Time
Initial Denaturation	94^{0}C	5min
Denaturation	94^{0}C	30sec
Annealing of primers	53^{0}C	30sec
Extension of Primers	72^{0}C	45sec
No of Cycles	35	
Final Extension	72^{0}C	5 Min

Primers used

ARE 2FP 5' TCTAGTTTCTGGTCTCAGAG 3'
ARE 2RP, 5' GAGAGCTAGCACTTGCTGTT 3'

And

ARE 3 FP 5' ACCTGAGATTAGGAATCCTC 3'
ARE 3 RP 5' CAGGCATCCTTGCAAGATG 3'

Table 3: primer synthesis profile

Oligo name	Sequence 5^{l} -3^{l}	Yield (OD)	Yield (µg)	Yield (nmol)	Vol for 100 pmol/µl	Tm (^{o}C)	GC Con tent	Synt hesis scale	MW (g/mol)
ARE2 FP	TCTAGTTTCTGGTCTCAGAG	11.2	329	53.8	538	55.3	45%	0.01µMol	6113
ARE2 RP	GAGAGCTAGCACTTGCTGTT	10.6	300	48.8	488	57.3	50%	0.01µMol	6148
ARE3 FP	ACCTGAGATTAGGAATCCTC	11.1	304	49.8	498	55.3	45%	0.01µMol	6100
ARE3 RP	CAGGCATCCTTGCAAGATG	10.9	302	52	520	56.7	52.6%	0.01µMol	5812

Table 4: Details of the primers and PCR conditions used for selected genes

GENE	PRIMER	Annealing Temp	$MgCl_2$	PCR product size
ARE 2	F: 5'-TCTAGTTTCTGGTCTCAGAG-3' R: 5'-GAGAGCTAGCACTTGCTGTT-3'	59^{O}C	2.0 mM	250 bp
ARE3	F: 5'-ACCTGAGATTAGGAATCCTC-3' R: 5'-CAGGCATCCTTGCAAGATG-3'	60^{O}C	2.0 mM	290 bp

Quantification of PCR product: After PCR, the products were checked by 2% agarose gel electrophoresis.

Purification of PCR products for Sequencing: In general, need about 10ng of purified PCR product per 100 bp of length. The purpose of this protocol is to remove unused primers and dNTP's from the PCR. 2-4 µl, was loaded onto an agarose gel for 10 min. To roughly quantify recovery. DNA of a known concentration was used as a standard.

DNA sequencing: DNA sequencing with a Taq-dye deoxy terminator– cycle sequencing kit (Applied Biosystems) was done using an automated ABI 3730 sequencer. DNA sequencing is one of the most important platforms for the study of biological systems today. Sequence determination is most commonly performed using di-deoxy chain termination technology.

The PCR products of ARE 2 & ARE 3 about 10 µl (50-100ng) for each of the blood DNA samples of cases was sequenced. The DNA samples of the cases and control subjects were sequenced to detect respective mutations. Sequencing was done using forward as well as reverse primers.

The sequences were analyzed by NCBI, BLAST software program to identify mutations. Reference sequences were taken from National Centre for Biotechnology Information of NIH and ENSEMBL of Sanger's Institute. The novel DNA sequence changes were confirmed by search against databases like SNPper and HapMap.[16,17,18]

RESULTS

The case control study was conducted on 50 female patients with breast cancer and 50 female controls with a mean age of 47.2±8.14 years and 46.8±8.4 years respectively. Direct sequencing analysis of ARE2 and ARE3 region of PSA gene in 50 patients and 50 age matched control samples was carried out in the present study. Statistical analysis was done using a z - test.

Figure 1 Shows gel picture of isolated DNA from blood samples of breast cancer patients and controls. Figure 2 shows gel picture of PCR products of ARE-2 and ARE-3 obtained after amplification with the primer set mentioned in Table 4.

On DNA sequencing six types of variation was identified in the ARE-2 region and no variations were detected in the ARE-3 region of patient group as well as in controls. Results are represented in table 5. Figure 3- 8 shows chromatograms of variation shown in table 5. The DNA sequence in the samples was evaluated with the reference sequence from NCBI database (Accession number U37672).

The DNA sequence variants found in patient group were T>C substitution in the ARE-2 region. In the control group, the variations identified were same T>C substitution and these changes are found to be overlapping with the changes found in cases.

The other variations detected are insertion of poly G tract, CAATCC insertion, single nucleotide T insertion, and insertion of oligo-nucleotide GTGGGTTACAATCCCCAATCCTTCATACAAACCC CCCC were detected in patient samples. And the detection of insertion of oligo-nucleotide GGGCT in the controls in the same region has been noted.

Statistical analysis

The T>C substitution was found in both cases and controls with a varied percentage, i.e. 54% (27/50) patients and 46% (23/50) controls. The difference was found to be insignificant with p = 0.42372 (z-test). This indicates the change was a common polymorphism reported in the ARE-2 region of the PSA gene in a evaluated number of samples. According to the pilot

study the DNA variation T>C may not be playing a role in breast cancer.

Multiple number of insertions were reported in patient samples when compared to controls in ARE2 region, which included a poly G tract (N=1), CAATCC (N=1), Insertion GTGGGTTACAATCCCCAATCCTTCATACAAACCC CCCC (N=1) and single nucleotide T insertion (N=2). These sequence alterations were not present in control samples analyzed. All these insertions were found in the amplicon ARE2 of patient samples (8%) which may impact the regulation of transcription of PSA gene from this region. However, to confirm the definitive role of ARE2 region affecting expression of PSA gene in breast cancer large sample size studies are needed. In the present study detection of insertion of oligo-nucleotide *GGGCT* in the controls in the same region has to be noted. All the samples analyzed for ARE3 region did not report any DNA sequence changes in controls as well as cases indicative of its insignificant role in breast cancer.

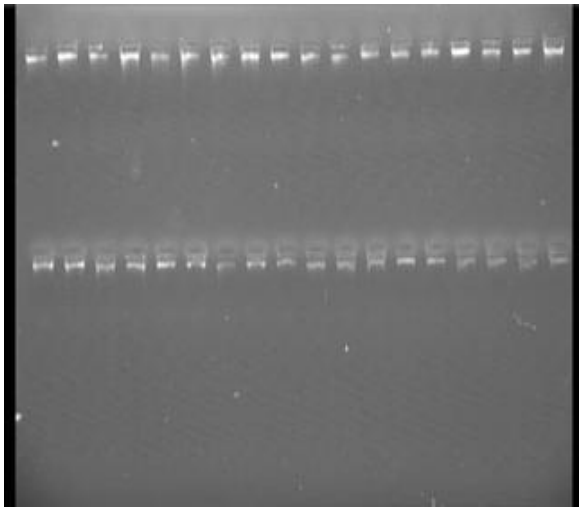

Figure1: showing agarose gel picture of isolated DNA from blood samples.

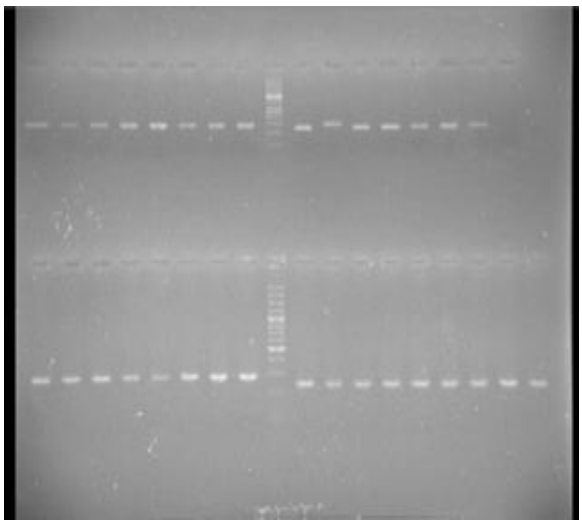

Figure 2: Ethidium bromide stained 2% agarose gel showing ARE2 and ARE3 PCR products:

Top wells: Lane 1-8 ARE-3 PCR product,
Lane 9 100 bp DNA ladder
Lane 10 -16 ARE-3 PCR product,
Lane 11 ARE-2 PCR product
Down wells: Lane 1-8 ARE-3 PCR product,
Lane 9 100 bp DNA ladder
Lane 10 -18 ARE-2 PCR products

Table 5: showing the DNA sequence variants identified in ARE-2 and ARE-3 amplicon: P-patient #; C-control #;

Gene	Primer	No. of samples analysed	No. of cases with sequence variation detected	Nature of sequence variation	No. of controls analysed	No of controls with sequence variation detected	Nature of sequence variation
ARE-2	Reverse and forward	50	P1, P4, P5, P7, P9, P12, P15,P17,P20,P22,P26,P28,P32,P33,P35,P37, P40,P41,P43,P11,P13,P45,P49,P50,P31,P46, P30 N=27	T>C: substitution	50	C4, C6, C9, C13,C17, C20,C21, C28,C30, C34,C38,C40,C42, C47, C49,C50,C24,C29, C33,C37, C41, C25, C39 N=23	T>C: substitution
			P2 N=1	Insertion *CAATCC*		C7 N=1	Insertion *GGGCT*
			P6 N=1	Insertion *poly G tract*			
			P2, P9 N=2	Insertion T			
			P3 N=1	Insertion GTGGGTTACAATC CCCAATCCTTCAT ACAAACCCCCCC			
ARE-3	Reverse and forward	50	no changes	no changes	50	no changes	no changes

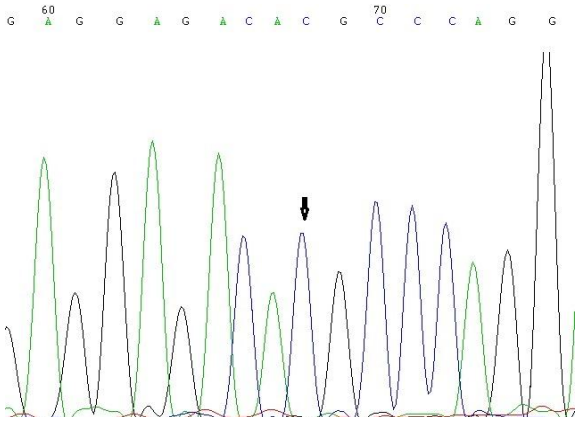

Figure3: Chromatogram shows T>C substitution found in cases as well as controls (Table 5).

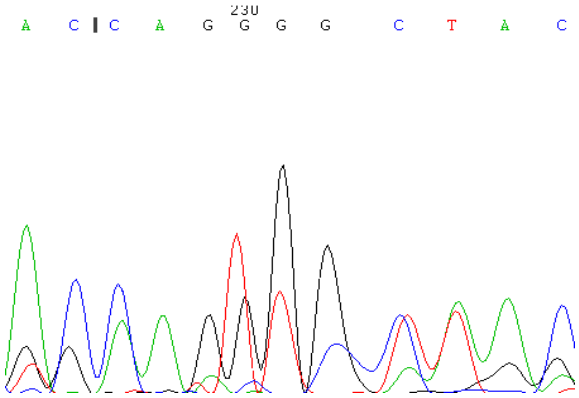

Figure 4: Insertion GGGCT in control sample

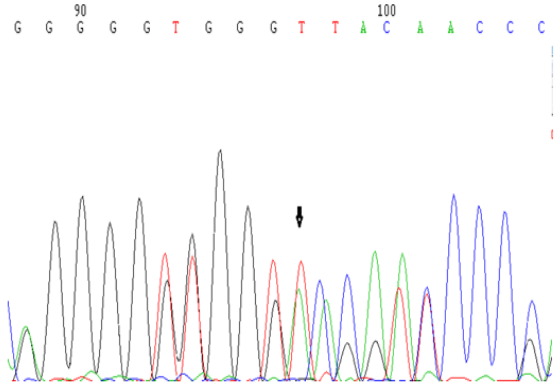

Figure 5: Insertion T in patient

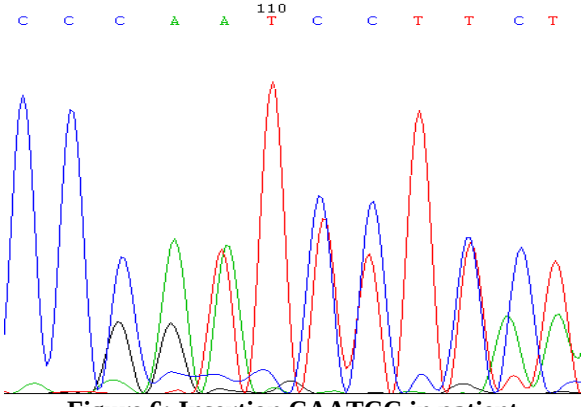

Figure 6: Insertion CAATCC in patient

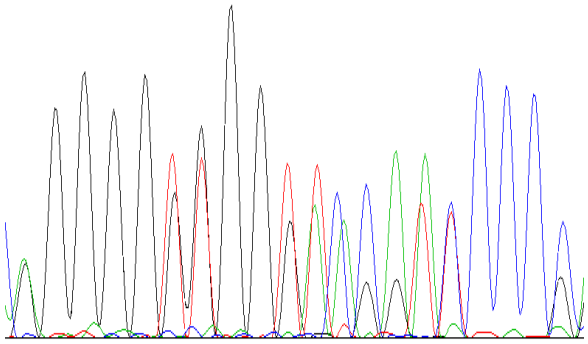

Figure 7: Insertion GTGGGTTACAATCCCCAATCCTTCATACAAAC CCCCCC

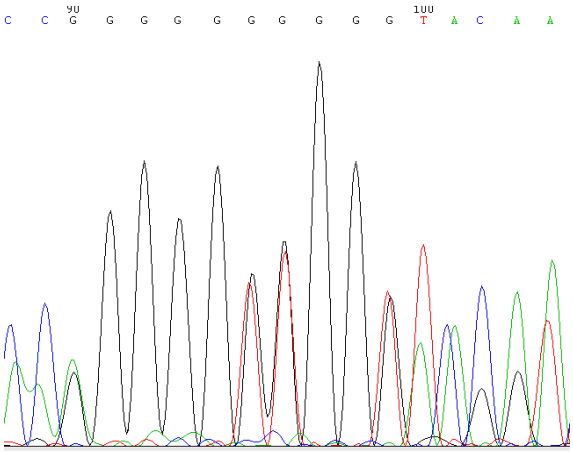

Figure8: Insertion poly G tract patient

DISCUSSION

Studies have shown PSA is not only produced by prostate alone, but also produced in the breast, ovary, liver, kidney, adrenal, and parotid glands. Diamandis *et al;*. were the first to discover immunoreactivity and mRNA expression of PSA in breast cancer. However, seminal PSA and breast PSA molecular weight and mRNA sequence are found to be identical PSA gene changes in breast cancer has been poorly understood.[2]

Studies have shown that steroid hormones regulate PSA gene. Androgen receptor (AR) regulates wild type PSA gene by mediating transcription by binding to a cognate sequence on the PSA proximal promoter.[9] Binding of transcription factors to the DNA sequences inside a 5.8kB segment upstream of the coding region regulates gene expression of PSA. A 15 base nucleotide sequence which mediates elevated affinity binding of androgen receptor complexed with its similar steroid ligand up regulates PSA gene expression. Three types of such response elements have been recognized ARE-I from −170 to −156 and ARE-II from −394 to −380, these two are within several hundred bases of the transcription start off a site and a third ARE-III about 4200 bases further upstream. Element (ARE3) is located at the distal enhancer.[8]

The proximal and distal enhancers of the PSA genes interact in a cooperative manner and are required for optimal expression of the gene. studies on ARE1 polymorphism in breast cancer have suggested that polymorphism of the PSA promoter ARE1 region has G/A substitution.[10] Other AREs of PSA genes have been evaluated in the present study, i.e., ARE2 and ARE3.

From direct sequencing analysis six types of variation were identified in the ARE-2 region. The DNA sequence variations found in patient group as well as a control group is T>C substitution in the ARE-2 region, these changes are found to be overlapping. The T>C substitution was found in both cases and controls with a varied percentage, i.e. 54% patients and 46% controls. The difference was found to be insignificant. This indicates the change was a common polymorphism reported in the ARE-2 region of the PSA gene in a evaluated number of samples. According to the pilot study the DNA variation T>C may not be playing a role in breast cancer.

The other variations detected are insertion of poly G tract, CAATCC insertion, single nucleotide T insertion, and insertion of oligo-nucleotide GTGGGTTACAATCCCCAATCCTTCATACAAACCC CCCC were detected in patient samples. These sequence alterations were not present in control samples analyzed. All these insertions were found in the amplicon ARE2 of patient samples (8%) which may impact the regulation of transcription of PSA gene from this region. However, to confirm the definitive role of ARE2 region affecting expression of PSA gene in breast cancer, large sample size studies are needed.

All the samples analyzed for ARE3 region did not report any DNA sequence changes in controls as well as cases indicative of its insignificant role in breast cancer.

CONCLUSION

The main purpose of the gene polymorphism study was to identify the variations in ARE2 and ARE3 promoter region of the PSA gene and to check whether this polymorphism has any effect in expression of PSA in breast cancer patient and whether these variations can be used to diagnose breast cancer risk at gene level, but according to the pilot study carried out one can conclude that ARE3 has no significant role in breast cancer, on the other hand, even though ARE2 region showed polymorphism in patients with breast cancer the variation observed were insignificant and percentage of the few variations observed was very low to conclude its role in breast cancer. Therefore, to substantiate the definitive function of ARE2 region affecting expression of PSA gene in breast cancer, large sample size studies are needed. ARE2 could be one of the molecular markers to identify females having risk of developing breast cancer before the onset of breast cancer.

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