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HORMONES INFLUENCING TUMOR PROGRESSION IN POSTMENOPAUSAL BREAST CANCER WOMEN

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ABSTRACT
This study systematically unfolds the various genetic and hormonal risks involved in breast cancer development in post-menopausal women who underwent reproductive surgeries. The breast cancer patients had higher hormonal levels (estrogen and progesterone). A 9.3 fold and 2.5 fold increased risk of breast cancer was found in women who had undergone reproductive surgeries like tubectomy and hysterectomy respectively before menopause. The PCR studies of the hormone regulating genes like *ESR1* and *PROGINS* showed that ESR1 *PvuII* genotypes had a 2.2 folds higher risk in heterozygous genotypes and 3 folds increase in risk for the combined genotypes of ESR1 *PvuII* (TC/CC). Whereas ESR1 *XbaI* genotypes showed 4.4 fold increase in heterozygous allele AG and the risk for homozygous mutant allele GG also increased, interestingly the homozygous mutants were conspicuously absent in controls. The correlation study for both ESR1 *PvuII* and *XbaI* genotypes, showed a significant association between increased hormone levels in tubectomised cases. Polymorphism in PROGINS showed heterozygous Pp genotypes were significantly associated with a calculated risk of 6.7 folds increase in breast cancer. Correlation studies of Pp genotypes showed significant association with reproductive surgeries and serum progesterone levels, which was higher. It is concluded that reproductive surgeries may modulate the estrogen and progesterone levels in women. This may lead to mutations in the hormone regulating genes like ESR1 and PROGINS. And the polymorphic nature of these genes in turn could lead to the significant increase in hormonal levels in post-menopausal breast cancer patients, further aggravating the disease.

KEYWORDS: Breast Cancer, Estrogen, Progesterone, Estrogen receptor-alpha (ESR1) gene and PROGINS.

INTRODUCTION
It has been well established that endogenous sex steroid hormones play an important role in the etiology of breast cancer in postmenopausal women.[1,2] Progesterone and estrogen are the two foremost important steroid hormones involved in normal breast development and formation of cancer.[3] In the breast, estrogens bind to estrogen receptors with high affinity, triggering DNA synthesis, cell division, and proliferation of the breast epithelial cells.[4,5] Proliferating cells are susceptible to genetic errors during DNA replication, which, if uncorrected, can lead to malignancies.[6] There are two types of estrogen receptors, the estrogen receptor-a (ER-a) and the estrogen receptor-b (ER-b).

Estrogen receptor-alpha, encoded by the ESR1 gene, has become the candidate genes in breast cancer association studies due to their significance in growth, development and progression of breast cancer.[7,8] The most important factors of risk for breast cancer are related to endogenous hormone levels and major reproductive events, suggesting that genes in the estrogen pathway may influence breast cancer risk. The estrogen receptor alpha (ESR1) is one of the most important mediators of hormonal response in estrogen penetrating tissues such as the breast.[9,10] ESR1 gene is localized on chromosome 6q24-q27, it extends more than 140 kb and includes eight exons. The most studied variants in this gene are the PvuII T-C dbSNP (rs2234693) and XbaI A-G, dbSNP (rs9340799) polymorphisms in intron 1, 397 and 351 base pair upstream of exon 2 respectively.[11] These variants have been implicated in gene expression by influencing transcription and have been frequently evaluated for their association with breast cancer. This gene encodes an estrogen receptor, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription.[12] To obtain a better understanding of the association of the PvuII and XbaI polymorphisms with breast cancer susceptibility, we evaluated these two variants of the ESR1 gene in a

sequence of breast cancer cases and normal age matched controls.

The influence of progesterone to the development and tumorigenesis is less unstated though the proliferative effects of estrogen on mammary gland are well documented. Confirmation from epidemiological studies has shown that early onset of menarche, nulliparity, a late first birth, reproductive status and late menopause are affected by the absence or presence of progesterone, thereby intensifying a woman's risk for breast cancer. Recently, data from epidemiological studies revealed that in postmenopausal women who use a combination of estrogens and progesterone the risk of breast cancer increases, when compared to women who used estrogens alone.[13,14] The steroid hormone, progesterone, is a key modulator of normal reproductive functions which consist of ovulation, uterine and mammary gland development.[15,16] The physiological properties of progesterone are facilitated by interaction of the hormone with two particular intracellular progesterone receptors designated as PR-A and PR-B. Progesterone receptors belong to member of a huge family of structurally associated gene products identified as the nuclear receptor superfamily of transcription factors. The nuclear receptors regulate gene transcription by discriminative binding to DNA regulatory sequences as well as by specific interactions with co-activator or co-repressor proteins to regulate the activity of the RNA polymerase complex.[17] The single-copy PR gene, situated on chromosome 11q22– 23, uses distinct promoters and translational start sites to generate two protein isoforms, isoform-A and isoform-B[18,19] that are identical except for an additional 165 amino acids present only in the NH2 terminus of isoform-B.[20,21] Though isoform-B shares many important structural domains with isoform-A, the two isoforms are functionally different transcription factors.[22] The two isoforms control the biological action of progesterone: isoform A, is capable to inhibit the activation of the estrogen receptors, and isoform B, has the ability to activate the estrogen receptors.[23] Numerous polymorphisms have been identified for this gene, among which a polymorphism named PROGINS, which arises due to the insertion of an Alu element into intron G between exons 7 and 8 of isoform A of the PR gene, results in an increase of 306 base pair gene product.[24] The physiological properties of progesterone are entirely reliant on the presence of the human PR, as proven in PRKO mouse.[16] The functional activity of both isoform-A and isoform-B were simultaneously ablated in the PRKO mouse, which showed that progesterone is necessary for the formation of ductal and alveolar structures during pregnancy. The experimental studies on PRKO mouse, revealed that removal of PR function results in a significant reduction in susceptibility to 7,12-dimethylbenz(a)anthracene-induced mammary tumors when used in the perspective of an established carcinogen induced mammary tumor model.[25] Studies on transgenic mice revealed that the mammary development was abnormal and characterized by excessive lateral ductal branching and inappropriate alveolar growth when it carried either an additional isoform-A or –B.[26]

Hence the aim of this study was to determine the influence of these two genes in post menopausal breast cancer women, and to evaluate their impact in women who had undergone reproductive surgeries before attaining post menopausal status in cases and controls.

MATERIALS AND METHODS

This breast cancer study is a hospital based case- control study conducted on South Indian population. All incident breast cancer patients were newly diagnosed during the study period.The procedures followed were in accordance with the ethical standards of the responsible committee of the Institutes/Hospitals, to participate in a face to face interview using astructured questionnaire. One hundred and fifty postmenopausal breast cancer patients and one hundred and fifty normal postmenopausal women were enrolled in this study. Breast cancer patients diagnosed by pathologists and oncologists on the basis of clinical examinations as well as pathological examinations were included in this study. The controls in our study were postmenopausal age matched women who did not have breast cancer or other diseases however some of them had undergone reproductive surgeries, which are listed in the tables-1&2 given in the results section.

Collection of samples

3-5 ml venous blood samples were collected in vacutainers from patients and controls, and centrifuged immediately for plasma separation. Biopsy samples were also collected from breast cancer patients for genomic studies.

Epidemiological data

The data was collected by interviewing the patients and controls by following the standard protocol. All patients were interviewed for recording their demographic factors like age, reproductive surgeries like tubectomy and hysterectomy and other confounding factors - like BMI, food habits, environmental exposures etc. These parameters have been documented by several investigators and in view of the available information we have not included these parameters in this paper.

Biochemical Estimation

Serum Hormonal levels (estrogen and progesterone) were determined by Enzyme linked immunoassay method with florescent detection Technique (ELFA) using mini-VIDAS assay kits (bioMerieuxVitek, Inc.).

Molecular studies and genotyping

Genotyping PvuII and XbaI Polymorphisms of ESR1 gene and PROGINS

Genomic DNA was isolated from the blood and biopsy samples by salting out method according to Alluri et al.[27] Determination of PvuII and XbaI Polymorphism.

To detect the PvuII and XbaI polymorphisms we used PCR and restriction fragment length polymorphism (RFLP) protocols according to van Meurs JB et al & Bergink AP et al.[28,29]

The target DNA was amplified by PCR. The reaction was performed in eppendorf Thermal Cycler. PvuII and XbaI genotypes were determined with PCR-RFLP method. Briefly, the primers for analysis were 5'CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3' (forward) and 5'TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3'(reverse). These primers generated a 1374 bp fragment, and contain a part of intron 1 and exon 2 of the ER-gene. The PCR products were digested by the PvuII and XbaI restriction endonucleases, respectively. The DNA fragments were then separated using 1.5% agarose gel and detected by ethidium bromide staining genotype) and XX (GG-genotype), signifying the absence of restriction sites, gave one 1374 bp fragment. cc, signifying the presence of PvuII restriction sites on both alleles, was digested into two fragments (936 bp and 438 bp). The xx genotype was revealed by XbaI digestion into two fragments (900 bp and 400 bp).

Determination of PROGINS Polymorphism

Briefly, the primers for PROGINS analysis were 5'-GGC AGA AAG CAA AAT AAA AAG A-3' (forward) and 5'-AAA GTA TTT TCT TGC TAA ATG TC-3' (reverse). The PCR reaction was carried out in a final volume of 25 ml, containing 1X buffer, 2.5 mM MgCl2, 0.1 mM of each dNTP, 50 nM of each primer, 1 U of Taq Polymerase, and 200 ng of DNA. Amplification was performed with an initial denaturation step at 95°C for 7 min, followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 50°C for 1 min, and extension at 72°C for 1 min and a final extension step at 72°C for 7 min. The amplification product was visualized in a 1% agarose gel under UV light. The PCR product presented a single band of 149 bp in the homozygous individuals without the mutation, designated as P1P1. The presence of one 149-bp and one 455-bp band indicated heterozygous individuals, who have one allele without the mutation and one allele with the mutation; these individuals were designated as P1P2.

Statistical analysis

All data was statistically analyzed. The patients characteristics and demographical factors were calculated using mean ± standard deviation. Genotyping experiments were presented as allelic frequencies and Genotype distribution with those expected from Hardy-Weinberg Equilibrium(HWE) were made using chi square test, and Values of P (two - tailed) less than 0.05 were considered statistically significant. Odds ratio, were calculated using MedCalc for Windows, version 7.4.1.0 (MedCalc Software, Mariakerke, Belgium).

RESULTS

Age Distribution of Breast Cancer Patients

Out of 300 post-menopausal women selected for our study, the age range of breast cancer patients was between 46-76 years. The mean average age at which breast cancer was generally identified was 61 years. Breast cancer patients were divided into 4 groups according to age at diagnosis; these were 41-50, 51-60, 61-70 and 70-80 years. Incidence of breast cancer cases was higher in the age group 50-60 years (52%) when compared to other age groups and the incidence was low in the age group 70-80 years (6%).

Age of Menarche in Breast Cancer Cases and Controls

The age of Menarche range for breast cancer patients and normal controls was found to be between 11-16 years. Breast cancer patients and controls were divided into 3 groups according to age of Menarche; 11-12, 13-14 and 15-16 years. Incidence of breast cancer cases was highest in the 13-14 (88.6%) menarche age group and the highest number of controls (92%) was also present in this age group of 13-14 years. The incidence in 11-12 years age group was 8.6% in breast cancer cases and 6% in controls. The incidence was very low in 15-16 menarche age groups. In our study the age of menarche was not significantly associated with breast cancer as there was not much difference between cases and controls.

Distribution of Breast Cancer Patients According To Estrogen (ER), Progesterone (PR) and Human Epithelial Growth Factor Receptor (HER2)Status

When we compared the percentage of receptor status it was found that the percentage of ER- PR- accounted for 46% of total breast cancer patients which was highest followed by ER+ PR+ 42% followed by ER+ PR– 10% and least being the EP-PR+ 2% We found in our study that HER2+(HER2 Positive) receptors in breast cancer patients accounted for about 30% of breast cancer patients and HER2-(HER negative) receptors in breast cancer patients were 70% of breast cancer patients. Incidence of HER2-ER-PR-(triple negative), in breast cancer were 28%.

Breast Cancer Patients as Per Tumor Grade Classification

This is a system used to classify cancer cells by the pathologist in terms of how abnormal the cells look upon histopathological examination of the tumor biopsies. In the present study, we found that grade III tumors were highest with a frequency of (46%), followed by grade II tumors (45.3%), grade IV (8%) and the least being grade I (0.6%).

Types of BreastCancer

In our study the incidence of Invasive ductal carcinoma (IDC) were highest up to 66% followed by Ductal carcinoma in situ (DCIS) 19.3%, Invasive lobular carcinoma (ILC) 12%, Lobular carcinoma insitu (LCIS)

2%, Mucelanious carcinoma (MC) 0.6% being the lowest.

Types of Breast Cancer Based On Family History

In our study we found sporadic breast cancers cases with highest frequency 66% followed by familial breast cancer cases 24% and hereditary cancers with lowest frequency 8%.

Incidences of Reproductive Surgeries like Tubectomy and Hysterectomy in Breast Cancer

In our study, we found that, out of 150 post-menopausal women with breast cancer 105 (70%) had undergone tubectomy before menopause and in controls out of 150 postmenopausal normal women 30 (20%) had undergone tubectomy before menopause and the chi-square p value was 0.0001, and the odds ratio was 9.3 demonstrating a significant association between tubectomy and breast cancer. Our results showed an increase in breast cancer risk among those women who had tubal sterilization before menopause (Table 1).

Among 150 breast cancer cases 27 (18%) post-menopausal women had under gone hysterectomy and in controls 12(8%) women had undergone hysterectomy and the chi square p = 0.01 and the odds ratio = 2.5 signifying an association between hysterectomy and breast cancer (Table 2).

Table 1: Frequency of Tubectomy in Breast cancer cases and controls.

CATEGORY	CASES N=150(%)	CONTROLS N=150(%)	95% CI	x2 TEST	ODDS RATIO	P-VALUE
TU Positive	105(70%)	30(20%)				
TU Negative	45(30%)	120(80%)	5.4-15.8	82.305	9.3	.0001

Significant P < 0.05, Non-Significant P > 0.05.

Table 2: Frequency of Hysterectomy in Breast cancer cases and controls.

CATEGORY	CASES N=150(%)	CONTROLS N=150(%)	95% CI	χ^2 test	Odds ratio	P-value
HY Positive	27(18%)	12(8%)				
HY Negative	123(82%)	138(92%)	1.2-5.1	6.631	2.52	.01

Significant P < 0.05, Non-Significant P > 0.05.

Determination of Estrogen and Progesterone Hormone Levels in Breast Cancer Patients and Controls

Estrogen and progesterone hormonal levels were estimated in 300 women including both breast cancer patients and controls in South Indian population. Normal value of serum estrogen levels in post- menopausal women was <21 pg/ml. Normal value of serum progesterone hormone levels in post-menopausal women was <0.41 ng /ml. We found that 60% BC patients had elevated levels of serum estrogens and in normal post-menopausal women taken as controls, 20% had elevated levels of serum estrogen levels and the chi square p = 0.0001 and the odds ratio = 6, specifying a significant association between breast cancer and elevated serum estrogen levels(Table 3). About 80% of BC patients had elevated serum progesterone levels and in normal women taken as controls only 22% had elevated progesterone levels and the chi-square p value = 0.0001 and the odds ratio = 14.1 indicating the association between elevated serum progesterone levels (Table 4 The percentage increase in both hormones (serum estrogen and progesterone) was 44%.

Table 3: Frequency of elevated serum estrogen levels in cases and matched controls.

CATEGORY	CASES N=150(%)	CONTROLS N=150(%)	95% CI	χ^2 test	Odds ratio	P-value
Elevated estrogen levels	90(60%)	30(20%)				
Normal estrogen levels	60(40%)	120(80%)	3.5-10	50	6	.0001

Significant P < 0.05, Non-Significant P > 0.05.

Table 4: Frequency of elevated serum progesterone in cases and matched controls.

CATEGORY	CASES N=150(%)	CONTROLS N=150(%)	95% CI	χ^2 test	Odds ratio	P-value
Elevated Progesterone levels	120(80%)	33(22%)				
Normal progesterone levels	30(20%)	117(78%)	8.13-24.7	108.17	14.1	.0001

Significant P < 0.05, Non-Significant P > 0.05.

In our study, we found that, out of 150 post-menopausal women with breast cancer 105 (70%) had undergone tubectomy before menopause and in controls out of 150 postmenopausal normal women 30 (20%) had undergone tubectomy before menopause and the chi-square p value was 0.0001, and the odds ratio was 9.3 demonstrating a significant association between tubectomy and breast cancer. Our results showed an increase in breast cancer

risk among those women who had tubal sterilization before menopause.

ER-alpha PvuII gene polymorphism was carryout by polymerase chain reaction and restriction fragment length polymorphism. Polymerase chain reaction amplified products (1374 bp) were visualized on Ethidium bromide containing agarose gel. PCR amplified products were digested with PvuII restriction enzyme. Digested alleles were separated on 1.5% agarose gel stained with Ethidium bromide. Three different combinations of fragment lengths were obtained in RFLP, the presence of PvuIIrestriction sites on both alleles, was digested in to two fragments (936bp and 438bp) signifying the presence of restriction sites, correspond to TT homozygous wild types, 1374 bp fragment representing CC homozygous mutants signifying the absence of restriction sites and a combination of three bands 936, 438 and 1374 bp represent heterozygous TC genotypes(Figure 1). Breast cancer patients showed 48% TT, 38% TC and 14% CC genotypes when compared to controls that had 74% TT, 26% TC, and 0% CC genotypes respectively.

Table 5: Distribution of T/C polymorphism in PvuII region of ESR1 gene among breast cancer cases and controls.

Genotype	CASES N=150(%)	CONTROLS N=150(%)	95% CI	χ^2 test	Odds ratio	P-Value
TT	72(48%)	111(74%)				
TC	57(38%)	39(26%)	1.36– 3.72	10.16	2.25	0.0016
CC	21(14%)	0(0%)	3.94 -1108	27.94	66.1	0.003
TC/CC	78(52%)	39(26%)	1.89 – 5.01	21.31	3.08	0.0001

p = < 0.05 (Significant), 95% Confidence Interval, χ2: Chi Square.

The above Table 5 shows the results for the T/C in PvuII polymorphism, both homozygous mutant CC and heterozygous mutant TC genotypes were more in cases when compared to controls. This analysis found a statistically significant variation among cases and controls.

The heterozygous mutant TC genotype were more in breast cancer cases (38%) when compared to normal controls (26%). The difference between cases and controls was statistically significant (OR 2.25, 95%CI 1.36-3.72, Chi-square 10.16, p-= 0.0016).

The homozygous mutant CC genotype was more in cases (14%) when compared to controls (0%). The difference between cases and controls was statistically significant (OR 66.1, 95%CI 3.94-1108, Chi-square 27.94, p = 0.003).

In our study, we found that the combined mutation both hetero and homozygous TC/CC between BC cases and controls was highly significant and the genotype showed 3.08 folds elevated risk for breast cancer (OR 3.08, 95%CI 1.89-5.01, Chi-square 21.31, p= 0.0001).

A second polymorphism A/G in XbaI region of ESR1 genotypes

PCR amplified products were digested with XbaI restriction enzyme. Digested alleles were separated on 1.5% agarose gel stained with Ethidium bromide. Three different combinations of fragment lengths were obtained in RFLP. The AA genotype signifying the presence of XbaI restriction sites on both alleles, was digested results in two fragments 981 and 393bp correspond to samples homozygous wild type, 1374bp fragment representing samples homozygous mutant signifying the absence of restriction sites and a combination of three bands 981, 393 and 1374 bp for heterozygous samples (Figure 1). Breast cancer patients showed 36% AA, 40% AG and 24% GG genotypes when compared to controls that had 80% AA, 20% AG, and 0% GG genotypes respectively.

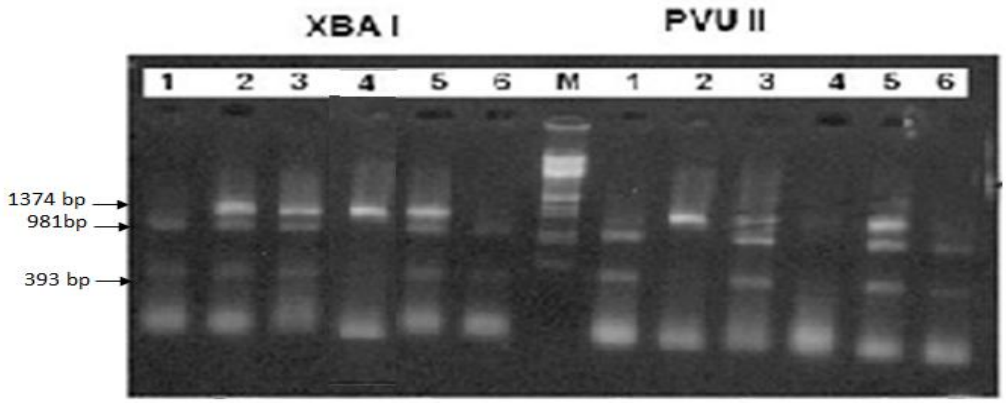

Figure 1: ESR1 PCR amplified products variation after XBA I and PVU II restriction enzyme digestion. Lane M = 100- marker; XBA I: Lane 2,3&5 heterozygous mutations (AG genotype); Lane 4 homozygous mutation (GG genotype); Lane 1&6 wild type (AA genotype). PVU II: Lane3 &5 heterozygous mutations (TC genotype); Lane 2 homozygous mutation (CC genotype); Lane 1&6 wild type (TT genotype).

Table 6: Distribution of A/G polymorphism in XbaI region of ESR1 genotypes among breast cancer cases and controls.

Genotype	CASES N=150	CONTROLS N=150	95% CI	χ^2 test	Odds ratio	P-Value
AA	54(36%)	120(80%)				
AG	60(40%)	30(20%)	2.58-7.65	30.6	4.44	0.0001
GG	36(24%)	0(0%)	9.7-2678	57.9	161	0.0004
AG/GG	96(64%	30(20%)	4.22-11.9	59.6	7.11	0.0001

p = < 0.05 (Significant), 95% Confidence Interval, χ^2: Chi Square.

The above Table 6 shows the results for the A/G in XbaI polymorphism, both the heterozygous genotypes AG and homozygous mutant genotype GG were more in patients when compared to controls, there was a significant variation in the distribution of AG and GG genotypes among patients and controls.

The heterozygous mutant AG genotype was more in breast cancer cases (40%) when compared to normal controls (20%). The difference between cases and controls was statistically significant (OR 4.44, 95%CI 2.58-7.65, Chi-square 30.6, p= 0.0001).

The homozygous mutant GG genotype was more in cases (24%) when compared to controls (0%). The difference between cases and controls was statistically significant (OR-161, 95%CI, 9.7-2678, Chi-square 57.9, p-= 0.0004).

In our study, we found that the dual mutant both hetero and homozygous AG/GG between BC cases and controls was significant and the genotype showed 7.11 fold higher risk for breast cancer (OR 7.11, 95%CI 4.22-11.9, Chi-square 59.6, p= 0.0001).

Further we analyzed the combined effects of the two polymorphisms PvuII T/C and XbaI A/G Polymorphism in ESR1 as shown in Table 3.9 From the combined genotype analysis of two polymorphisms in the ESR1 gene, it was seen that TT/AG, TT/GG, TC/AG, TC/GG, CC/AG, CC/GG pooled genotypes were more in patients when compared to controls and it was statistically significant. In our study, we found TT/AG showed 1.71 folds greater risk of breast cancer and the p = 0.001. The combined genotype for TT/GG showed 1.78 fold higher risk of breast cancer and the p = 0.0009, the combined genotypic effect for TC/AG showed 3.1 fold increase risk of breast cancer (p = 0.0001). The combined genotype for TC/GG exposed 4.37 fold greater risk (p = 0.0001). The CC/AG genotype showed 4.95 increased risk for BC (p = 0.0001). The CC/GG genotype also showed 210 folds increase risk for breast cancer (p = 0.0002). The combined genotype for TC/AA, CC/AA was not statistically significant (p= < 0.05).

The analysis of PROGINS was based on the PCR amplification of a fragment encompassing the 306-bp, which arises due to the insertion of an Alu element into intron G between exons 7 and 8 of isoform A. Polymerase chain reaction amplified products was visualized in 1% agarose gel under UV light. The PCR product presented a single band of 149 bp in the homozygous individuals without the mutation, designated as PP. The presence of one 149-bp and one 455-bp band indicated heterozygous individuals, who have one allele without the mutation and the other allele with the mutation designated as Pp (Figure 2). The individuals with mutations in both alleles with a single band of 455-bp designated as pp.

Breast cancer patients showed 70% PP, 30% Pp and 0% pp genotypes when compared to controls that had 94% PP, 6% Pp, and 0% pp genotypes respectively.

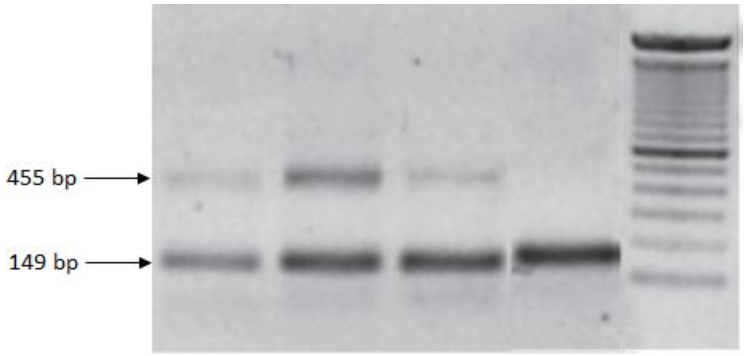

Figure 2: Progins PCR amplified product, Lane M marker; lanes 1, 2, and 3 heterozygous mutation (Pp genotype); Lane 4 wild sample (PP genotype).

Table 7: Distribution of PROGINS gene polymorphism in Breast Cancer patients and controls.

Genotype	CASES N=150(%)	CONTROLS N=150(%)	95% CI	χ^2 test	Odds ratio	P-Value
PP	105(70%)	141(94%)				
Pp	45(30%)	9(6%)	3.14-14.3	29.26	6.71	<0.0001
pp	0(0%)	0(0%)	.02-68.14	0.003	1.0	1.0

p = < 0.05 (Significant), 95% Confidence Interval, χ^2: Chi Square.

The table 7 shows the results for the PROGINS polymorphism, the heterozygous genotype Pp were more in patients when compared to controls. The heterozygous Pp genotype was more in breast cancer cases (30%) when compared to normal controls (6%). The difference between cases and controls was statistically significant (OR 6.71, 95%CI 3.14-14.3, Chi-square 29.26, p= 0.0001). The homozygous mutant pp was not found in both cases (0%) and controls (0%).

Table 8: ESR1 PvuII gene Polymorphism Correlation with Demographical Factors, Reproductive surgeries and Bio-chemical Factors.

Characteristics	Cases	Wild type TT	Heterozygous TC	Homozygous mutant CC	P-Value
	N=150	N=72 (48%)	N=57 (38%)	N=21 (14%)	
Age					
41-50	33(22%)	18(54.55%)	12(36.36%)	3(9.09%)	0.25
51-60	73(48.67%)	30(41.1%)	28(38.36%)	15(20.55%)	0.24
61-70	38(25.33%)	18(47.37%)	17(44.74%)	3(7.89%)	0.82
71-80	6(4%)	6(100%)	0(0%)	0(0%)	0.93
Age of Menarche					
11-12	13(8.67%)	8(61.54%)	4(30.77%)	1(7.69%)	0.31
13-14	133(88.67%)	64(48.12%)	49(36.84%)	20(15.04%)	0.93
15-16	4(2.67%)	0(0%)	4(100%)	0(0%)	0.14
Reproductive surgery Tubectomy					
Tubectomy positive	105(70%)	33(31.43%)	54(51.43%)	18(17.14%)	0.0001
Tubectomy negative	45(30%)	39(86.67%)	3(6.67%)	3(6.67%)	0.0001
Reproductive surgery Hysterectomy					
Hysterectomy positive	27(18%)	15(55.56%)	12(44.44%)	0(0%)	0.38
Hysterectomy negative	123(82%)	57(46.34%)	45(36.59%)	21(17.07%)	0.38
Estrogen hormone Levels					
<21.1(non-elevated)	60(40%)	60(100%)	0(0%)	0(0%)	0.0001
>21.1 (elevated)	90(60%)	12(13.33%)	57(63.33%)	21(23.33%)	0.0001
Progesterone hormone levels					
<0.42 (non-elevated)	27(18%)	21(77.78%)	3(11.11%)	3(11.11%)	0.001
>0.42 (elevated)	123(82%)	51(41.46%)	54(43.9%)	18(14.63%)	0.001
Estrogen Receptor status					
ER+/PR+	63(42%)	27(42.86%)	24(38.1%)	12(19.05%)	0.28
ER+/PR-	15(10%)	3(20%)	6(40%)	6(40%)	0.03
ER-/PR+	3(2%)	3(100%)	0(0%)	0(0%)	0.17
ER-/PR-	69(46%)	39(56.52%)	27(39.13%)	3(4.35%)	0.05
Her2 status					
Her2+	45(30%)	21(46.67%)	18(40%)	6(13.33%)	0.83
Her2-	105(70%)	51(48.57%)	39(37.14%)	15(14.29%)	0.83

Correlation of ESR1 PvuII T/C Gene Polymorphism with Demographical Factors, Reproductive Surgeries and Bio-Chemical Factors

In our study, the data presented in the above table 8 revealed the relationship between the ESR1PvuII (T/C) genotypes and its relation with demographical data of the breast cancer patients. The agegroup of the post-menopausal breast cancer patients 51-60 showed a high frequency (48.67), but we were unable to find the statistically significant association between genotype and the age groups. Age at menarche was also not statistically significant with genotypes. Reproductive surgery like tubectomy was significantly associated with genotypes. The tubectomy positive cases were significantly associated with hetero and homozygous mutant genotypes TC and CC (P=0.0001). Reproductive surgery like Hysterectomy was not statistically significant with the genotypes. In the present study, we found that the elevated estrogen levels were significantly linked to mutant genotypes (P=0.0001). The elevated progesterone levels were also significantly associated with the mutant genotype TC and CC (P=0.001). Genotypes were correlated with ER/PR status to find out the association with the polymorphism, it was observed that ER+PR- (P=0.03), ER-PR- (P=0.05) tumors in cases showed significant association withthe genotypes. We were unable to find any association between HER2 status and the genotypes.

Table 9: Correlation of ESR1 Xba I gene Polymorphism with Demographical Factors, Reproductive surgeries and Bio-chemical Factors.

Characteristics	Cases	Wild type AA	Heterozygous AG	Homozygous mutant GG	P-Value
	N=150	N=54 (36%)	N=60 (40%)	N=36 (24%)	
Age					
41-50	33(22%)	12(36.36%)	15(45.45%)	6(18.18%)	0.96
51-60	73(48.67%)	24(32.88%)	31(42.47%)	18(24.66%)	0.43
61-70	38(25.33%)	12(31.58%)	14(36.84%)	12(31.58%)	0.51
71-80	6(4%)	6(100%)	0(0%)	0(0%)	0.02
Age of Menarche					
11-12	13(8.67%)	8(61.54%)	3(23.08%)	2(15.38%)	0.06
13-14	133(88.67%)	46(34.59%)	54(40.6%)	33(24.81%)	0.31
15-16	4(2.67%)	0(0%)	3(75%)	1(25%)	0.26
Reproductive surgery Tubectomy					
Tubectomy positive	105(70%)	18(17.14%)	54(51.43%)	33(31.43%)	0.0001
Tubectomy negative	45(30%)	36(80%)	6(13.33%)	3(6.67%)	0.0001
Reproductive surgery Hysterectomy					
Hysterectomy positive	27(18%)	12(44.44%)	12(44.44%)	3(11.11%)	0.31
Hysterectomy negative	123(82%)	42(34.15%)	48(39.02%)	33(26.83%)	0.31
Estrogen hormone Levels					
<21.1(non-elevated)	60(40%)	54(90%)	6(10%)	0(0%)	0.0001
>21.1 (elevated)	90(60%)	0(0%)	54(60%)	36(40%)	0.0001
Progesterone hormone levels					
<0.42 (non-elevated)	27(18%)	18(66.67%)	6(22.22%)	3(11.11%)	0.0005
>0.42 (elevated)	123(82%)	36(29.27%)	54(43.9%)	33(26.83%)	0.0005
Estrogen Receptor status					
ER+/PR+	63(42%)	24(38.1%)	18(28.57%)	21(33.33%)	0.64
ER+/PR-	15(10%)	0(0%)	9(60%)	6(40%)	0.03
ER-/PR+	3(2%)	3(100%)	0(0%)	0(0%)	0.09
ER-/PR-	69(46%)	27(39.13%)	33(47.83%)	9(13.04%)	0.46
Her2 status					
Her2+	45(30%)	12(26.67%)	21(46.67%)	12(26.67%)	0.12
Her2-	105(70%)	42(40%)	39(37.14%)	24(22.86%)	0.12

Correlation of ESR1 Xba I Gene Polymorphism with Demographical Factors, Reproductive Surgeries and Bio-Chemical Factors

In the present study, (Table 9)we found that the age group between 51-60 showed a high frequency of cases (48.67%), whereas the age group 71-80 showed the least frequency of 6(4%) and all the 6 cases were homozygous wild AA whereas no cases were found with AG and GG genotype (P=0.02). Age at menarche was also not associated with genotypes. Reproductive surgeries like tubectomy were significantly associated with genotypes. The tubectomy positive cases were significantly associated with hetero and homozygous mutant genotype AG and GG (P=0.0001). Reproductive surgeries like Hysterectomy were not statistically associated with the genotypes. In the present study, we found that the elevated estrogen levels were significantly linked to AG and GG genotypes (P=0.0001). The elevated

progesterone levels were also significantly associated with the mutant genotype AG and GG (P=0.0005). With regards to the correlation with the receptor status, we found that the ER+PR- tumors in cases showed significant association with the genotypes, P=0.03. We were unable to find any association between HER2 status and the genotypes.

Table 10: Correlation of PROGINS gene Polymorphism with Demographical Factors, Reproductive surgeries and Bio-chemical Factors.

Characteristics	Cases	Wild type PP	Heterozygous Pp	Homozygous mutant pp	P-Value
	N=150	N=105 (70%)	N=45 (30%)	N=0 (0%)	
Age					
41-50	33(22%)	23(69.7%)	10(30.3%)	0(0%)	0.96
51-60	73(48.67%)	50(68.49%)	23(31.51%)	0(0%)	0.69
61-70	38(25.33%)	27(71.05%)	11(28.95%)	0(0%)	0.86
71-80	6(4%)	5(83.33%)	1(16.67%)	0(0%)	0.47
Age of Menarche					
11-12	13(8.67%)	11(84.62%)	2(15.38%)	0(0%)	0.24
13-14	133(88.67%)	91(68.42%)	42(31.58%)	0(0%)	0.24
15-16	4(2.67%)	3(75%)	1(25%)	0(0%)	0.82
Reproductive surgery Tubectomy					
Tubectomy positive	105(70%)	67(63.81%)	38(36.19%)	0(0%)	0.01
Tubectomy negative	45(30%)	38(84.44%)	7(15.56%)	0(0%)	0.01
Reproductive surgery Hysterectomy					
Hysterectomy positive	27(18%)	26(96.3%)	1(3.7%)	0(0%)	0.009
Hysterectomy negative	123(82%)	79(64.23%)	44(35.77%)	0(0%)	0.009
Estrogen hormone Levels					
<21.1(non-elevated)	60(40%)	47(78.33%)	13(21.67%)	0(0%)	0.07
>21.1 (elevated)	90(60%)	58(64.44%)	32(35.56%)	0(0%)	0.07
Progesterone hormone levels					
<0.42 (non-elevated)	27(18%)	24(88.89%)	3(11.11%)	0(0%)	0.02
>0.42 (elevated)	123(82%)	81(65.85%)	42(34.15%)	0(0%)	0.02
Estrogen Receptor status					
ER+/PR+	63(42%)	38(60.32%)	25(39.68%)	0(0%)	0.02
ER+/PR-	15(10%)	9(60%)	6(40%)	0(0%)	0.37
ER-/PR+	3(2%)	2(66.67%)	1(33.33%)	0(0%)	0.89
ER-/PR-	69(46%)	56(81.16%)	13(18.84%)	0(0%)	0.006
Her2 status					
Her2+	45(30%)	29(64.44%)	16(35.56%)	0(0%)	0.33
Her2-	105(70%)	76(72.38%)	29(27.62%)	0(0%)	0.33

Correlation of PROGINS Gene Polymorphism with Demographical Factors, Reproductive Surgeries and Bio-Chemical Factors

The data in the table 10 represents the relationship between the PROGINS genotypes (PP and Pp) and its relationship with the demographical factors of the breast cancer cases. The homozygous mutant (pp) genotype was not found in our study. The age group of the post-menopausal breast cancer patients 51-60 showed a high frequency (48.67%) of cases, but we were unable to find the statistically significant association between genotypes and the age groups. Age at menarche was also not statistically significant with genotypes. Reproductive surgery like tubectomy was significantly associated with heterozygous mutant genotype Pp (P=0.01). The Hysterectomy positive cases were statistically significant with the PP genotypes (P=0.009). We also found that the elevated estrogen levels were not significantly associated to genotypes (P=0.07). The elevated progesterone levels were significantly associated with the heterozygous genotype Pp (P=0.02). Genotypes were correlated with ER/PR status to find out the association with the polymorphism, it was observed that ER+PR- (P=0.03), ER-PR- (P=0.05) tumors in cases showed significant association with the genotype. We were unable to find any association between HER2 status and the genotypes.

DISCUSSION

Reproductive surgeries like tubectomy before menopause disturbs the normal hormonal balance in the body and initiates an increase in hormone levels. These are important risk factors for the prognosis of the breast cancer. Most of the research on breast cancer first reports the relative abundance of the biochemical entities like the serum hormonal levels, which appear to be of primary importance, as the serum elevated levels of estrogen and progesterone are a prerequisite for breast cancer. Also, there are numerous studies on genetic

polymorphism in oncogenes like *PTEN. P53, BRCA1, BRCA2*, to name a few[30,31] which are responsible for the disease. Some of the previous studies emphasizes that the polymorphism in the genes may elevate the levels of steroid hormones. In spite of several epidemiologic, clinical, genetic, and biochemical studies on breast cancer[32] there still exists unexplained phenomena where there is no clear evidence pointing the reason behind the elevated serum hormonal levels. In this paper, we presented for the first time that breast cancer involves complex interactions between endogenous hormones and genes involved in their regulation. In this study we could predict that development of breast cancer is a long process involving a step by step changes occurring in the reproductive age of a women's life cycle i.e. before menopause, though breast cancer may occur in the post-menopausal period when women become weaker due to aging process. The prolonged exposure to endogenous steroid hormones may have induced mutations in the genomic DNA.

The steps involved in the carcinogenesis of breast cancer in our study are as follows:
1) Documenting the reproductive surgeries like tubal sterilization or partial hysterectomy.
2) Determining the levels of serum steroid hormones like estrogen and progesterone.
3) Studies on Genetic polymorphism or over expression of genes.

Reproductive surgeries like Tubectomy are one of the strongest factors which enhance the risk of breast cancer. Tubectomy or partial hysterectomy before menopause may affect a women's breast cancer risk by altering her cumulative exposure to endogenous ovarian hormones. In the case of tubal sterilization, the fallopian tubes are cut or blocked and in partial hysterectomy the ovaries are left uncut and the uterus is removed, hence the focus of the hormone is more on the breast and less on other reproductive organs.[33] As a result, the steroid hormone affects the breast cells. The main organ producing the steroid hormones are the ovaries. Therefore, in case of tubectomy and partial hysterectomy the normal hormonal balance in the body get disturbed and the imbalance may lead to elevated levels of endogenous steroid hormones which may in turn leads to genetic polymorphism and can cause breast cancer.In our present study, we found that, the frequency of the tubal sterilization was very high in the breast cancer patients. Our results show that out of 150 post-menopausal women selected for our study, 105 (70%) had tubal sterilization before menopause (P = 0.0001) indicating a significant association between tubectomy and breast cancer. Whereas in normal controls out of 150 postmenopausal women only 30 (20%) were tubectomy positive. Hence the chances of the normal women who had under gone tubectomy have 9.3 fold increase risk of breast cancer.

Among the 150 cases, 27 (18%) women with hysterectomy had breast cancer and in normal controls 12(8%) had undergone hysterectomy (p=0.01). Hence the chances of the normal women who had undergone hysterectomy have 2.5 folds' increase in the risk of breast cancer.

Elevated levels of serum steroid hormones like estrogen and progesterone

It is an established fact that endogenous hormones play an important role in the etiology of breast cancer in postmenopausal women.[1,2] Physiologically, the human female breast is under the primary control of different hormones; the role of estrogen appears to be central. Estrogen exposure is an established major risk factor for breast cancer, in both pre and post-menopausal women.[34] Reproductive factors that increase a woman's lifetime exposure to estrogen increase the risk of breast cancer.[32]

Excessive reproductive hormone secretion is positively associated with breast cancer risk, especially in postmenopausal women as the peripheral production of estrogen by aromatization of androstenedione by adipose tissues is favored.[8,35] The conversion of androstenedione to estrone by adipose tissue provides the major source of estrogen in postmenopausal women and this mechanism explains the phenomenon that level of endogenous estrogen has higher breast cancer risk. [36]Progesterone also has been hypothesized to increase breast cancer risk. Progesterone levels appear to be a modest risk factor for postmenopausal breast cancer. Progesterone is a steroid hormone associated with the female reproductive process, and is critical for normal female development and growth. Steroid hormones are produce in females by ovaries and in males by testes, and in both genders by the adrenal glands and by conversion from other sex steroids in tissues such as liver or fat catalyzed by the specific enzymes.

Previous studies have reported that elevated levels of endogenous steroid hormones are known to have a profound influence on the etiology of cancer of the breast.[2,37] Our results demonstrated that the levels of serum estrogen and progesterone were elevated in breast cancer cases compared to normal women. The elevated serum estrogen levels were seen in 60% of patients whereas only 20% had elevated levels in controls (p=0.0001). The risk of breast cancer was 6 folds increased in women with elevated estrogen levels. The elevated serum progesterone levels in BC cases were 80% and only 22% had elevated levels in controls (p=0.0001). Hence the risk of breast cancer, calculated statistically was 14.1 fold increases.

Estrogen and progesterone receptors

ER and PR receptors play an important role in breast cancer because they are the binding sites of serum estrogen and progesterone hormones. In the present study, the percentage of receptor status(ER/PR), was as follows : ER^-/ PR^- accounted for 46% of total breast cancer patients which was highest followed by ER+/PR+

42% followed by ER+/PR$^-$ 10% and least being the EP$^-$ /PR+ 2%. These findings are in line with the findings reported[38,39] where a significant fraction of breast tumors expressed ER+/PR+ and ER- /PR- groups. 1-2% of the tumors are expected to carry ER+/PR- or ER- /PR+ receptors. It was reported that women who lack ER and PR receptor expression have been found to have 1.5- to 2-fold higher risk of mortality.[38,40] In this study, we report that HER2+ (HER2 Positive) receptors in breast cancer patients accounted for about 30% and HER2- (HER2 negative) receptors in breast cancer patients were 70%. Incidences of HER2-ER-PR- (triple negative) were 28%. The ER+ and PR+ are the binding sites of estrogen and progesterone hormones respectively hence they play a very important role in breast cancer prognosis. With more number of receptors, there are more chances of getting breast cancer. The women whose tumors are hormone receptor positive (ER+ and PR+) are often recommended endocrine therapy (e.g. Herceptin) as the first-line treatment, which blocks the receptors. But for ER- and PR- there is no alternate treatment except chemo-therapy (e.g. 5-FU, Gemcitabine) which has many side effects. Our study reports that in 27% ER- and 40% PR- breast cancer cases the levels of endogenous hormones were high and hence there was a possibility of the hormones to bind to other receptors in the absence of ER- PR- receptor. For the first time, we could emphasize that tumors binding sites of estrogens and progesterone may be the G protein-coupled receptor, GPR30 in ER- and PR- breast cancer cases. Hence, we suggest that, for ER- and PR- tumors the alternate treatment could be done by blocking the G protein- coupled receptors (GPCRs).

Genetic polymorphism

In breast, the endogenous hormones bind to the receptors with high affinity, triggering DNA synthesis, cell division, and proliferation of the breast epithelial cells.[4,5] Proliferating cells are susceptible to genetic errors during DNA replication, which in turn can lead to tumorigenesis.[41]

Estrogen receptor alpha /ESR1 397T/C (PvuII) polymorphism

In our study, we have seen that the elevated serum estrogen levels play a very important role and has been associated with genetic polymorphism in the T/C in PvuII region and A/G in Xbal-I region of *ESR1* gene. The allelic distribution of polymorphisms in *ESR1* gene was found to be significant in breast cancer cases compared with controls. Our study for *ESR1PvuII* genotype revealed that the risk of breast cancer was increased by 2.2 folds in heterozygous TC (p=0.001) and in homozygous mutant allele CC the odds ratio was 66.1 because there were no homozygous mutants in the controls (P=0.003). The individuals with combined genotypes (TC/CC) showed 3 folds augmented risk of breast malignancy (Odds ratios (OR: 3.08, 95% CI: 1.89-5.01, p=0.0001). *ESR1XbaI* genotype showed 4.4 fold increase in heterozygous allele AG (P=0.0001) and the risk for homozygous mutant allele GG was further increased as no homozygous mutants were present in the controls (p=0.0004). The individuals with combined genotypes (AG/GG) showed 7.1 folds augmented risk of breast cancer (OR: 7.1, 95% CI: 4.2-11.9, p=0.0001). In the correlation study, interestingly, the association of serum estrogen levels, serum progesterone levels and tubectomy was also found to be significantly associated with the genotypes TC and CC of *PvuII* region and the genotype AG and GG of the *XbaI*. Several case-control studies have been conducted over the past decade in different population groups like Chinese, Americans, Caucasian, etc.[2,9,42,43] but still there is variability among many studies. But our results indicated that the *PvuII* and *XbaI* polymorphism in *ESR1* gene were associated with increased risk of breast cancer, as well as disease progression, supporting our hypothesis that due to tubectomy before menopause, the serum estrogen concentration was disturbed leading to the increased levels of serum estrogens. It is evident that prolonged exposure to the elevated levels of estrogen may have induced polymorphism in the *ESR1* gene, as the receptors are the binding sites of the hormones and binding promotes gene expression. Hence high expression of this gene could be due to elevated estrogen levels which in turn relates to the risk of getting breast cancer due to these mutations in these genes.

Genetic Variations in PROGINS

In the present study, we evaluated the possible role of PROGINS with breast cancer, and found that the frequency of heterozygous allele Pp insertion polymorphism was higher in breast cancer cases 45(30%) when compared to controls (6%). We did not find any homozygous mutant alleles pp in our study group. But the homozygous wild PP genotypes were more in both cases 105 (70%) and in controls (94%). We found heterozygous polymorphism Pp genotypes to be significantly associated in the risk of breast cancer by 6.7 folds (p=0.0001). Further, correlation studies have shown that Pp genotype was observed in the cases where progesterone levels were higher than 0.62ng/ml when compared to the levels less than 0.62ng/ml in post-menopausal women and also higher in tubectomy positive cases. Higher levels of serum hormones levels might be responsible for the alu insertion polymorphism in some of the breast cancer cases. The PR gene polymorphism has been reported in breast and ovarian cancers.[24,44] (Liao J et al, 2015, Donaldson CJ et al, 2002). Wang-Gohrke et al[45] found an inverse association between breast cancer risk in women younger than 50 years and carriers of the PROGINS allele. McKenna, N. J et al[46] had shown an association with ovarian cancer with increased frequency of mutant allele in their study, D'Amora et al[47] also observed that PROGINS variants may influence cell proliferation, viability, and apoptosis in endometrial cell metabolism.

Elevated levels of progesterone are linked in post-menopausal breast cancer as progesterone is involved in

the regulation of extracellular matrix metalloproteinases, stimulating the expression of angiogenic factors and cell cycle-regulating factors. The inflammatory response to progesterone within the breast creates a risk of breast cancer (Michigan State University News-report, 2009). The physiological effects of progesterone are mediated by specific intracellular proteins known as PRA and PRB, their physiological roles differ due to their structural and functional properties. The PRA isoform of the progesterone receptor has been shown to repress estrogen receptor activation and the transcriptional activity of the PRB isoform. We hypothesize that the physiological function of the progesterone is concentrated mainly on the female reproductive system, however, due to the reproductive surgery like tubectomy the function of the progesterone gets altered and the action of the hormone is more on the breast cells. This may lead to increase levels of progesterone hormone which in turn may lead to overexpression of PR gene. This situation may induce alu insertion polymorphism in the PRA isoform due to strong progesterone action. The polymorphic PRA PROGINS allele has been shown to have increased transcriptional activity. Such a complex activation sequence offers several steps in which other regulatory mechanisms of the progesterone signaling pathway can be integrated. Hence, we speculate that our results support the view that the presence of the PRA isoform could lead to a greater mitogenic action of progesterone resulting in breast cancer.

SUMMARY AND CONCLUSIONS

This investigation was aimed to improve our understanding of the molecular link between endogenous steroid hormones and their involvement in modulating genetic parameters influencing/increasing the risk of breast cancer. From a new perspective our research has given us new insights that the initiation of breast cancer is a long and gradual process that involves multiple factors and their link to cancer progression. We tried to correlate the effect of reproductive surgeries before menopause like tubal ligation and partial hysterectomy to the potential risk of breast cancer. This has led to an understanding that, most of the hormonal mediated breast cancers are triggered by the elevated endogenous levels due to reproductive surgeries before menopause, thereby disturbing the normal hormonal balance in the body. It is suggested that this imbalance in hormone levels interact with hormone regulating genes to create genetic variations and over expressions ultimately leading to breast cancer progression. Our results clearly demonstrated a statistically significant association between tubal ligation and elevated serum hormones such as estrogen and progesterone and the polymorphism in hormone regulating genes like *ESR1*and*PROGINS.*

This is our novel approach and first report which emphases that elevated levels of steroid hormones influences the polymorphisms in the hormone regulating genes and not the polymorphisms in the genes which are causing elevated hormone levels leading to breast cancer as specified by some previous studies.

Genetic polymorphism plays an important role in tumorigenesis and cancers. Further, the polymorphism in the *ESR1* gene and *PROGINS* was significant and also estrogen and progesterone levels were elevated. This may be due to the fact that ER and PR are the binding sites of hormones and elevated levels of hormones, promotes the expressions of *ESR* and *PR* genes and thereby showing mutations in those genes and their involvement in breast cancer progression.

Another important finding in our study was that the G protein-coupled receptor or GPRs were the binding sites of estrogen and progesterone in ER- PR- breast cancer cases because serum estrogen and progesterone levels were elevated in breast cancer patients with negative estrogen and progesterone receptors.

In conclusion the results of our analyses provided evidence that the polymorphisms and gene expression studies in the hormone regulating genes like *ESR1, PR* were initiated by the elevated serum steroid hormones which are in turn influenced by the reproductive surgeries like tubal-ligation to substantially impact in breast carcinogenesis, this is being reported for the first time. Our study also cautions those normal women who have under gone tubal ligation before menopause have elevated serum steroid hormone levels and hence they are at the greater risk of developing cancer in the future. Our research can help in preventing breast cancer in normal women by bringing the awareness in the society about the bad effects of reproductive surgeries which leads to elevated steroid hormone levels and prone to causing breast cancer. It is suggested that alternative methods in sterilization should be adapted instead of surgeries like tubal ligation. Considering the role of progesterone in breast cancer proven by the epidemiological and biological studies, we postulated that the variations in the PR gene may be facilitating women towards breast cancer. A note of caution could be useful to young women undertaking reproductive surgeries.

Further, determining the steroid hormone levels in tubectomy positive patients should be considered as a new diagnostic tool. Also new drugs could be designed to target or block the hormonal receptors like G protein-coupled receptor or GPRs in ER- PR- breast cancer patients since we found increased hormone levels in these patients.

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REFERENCE

1. The Endogenous Hormones and Breast Cancer Collaborative Group. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst., 2002; 94: 606–616.
2. Onland-Moret NC, Kaaks R, van Noord PA. Urinary endogenous sex hormone levels and the risk of postmenopausal breast cancer. Br J Cancer, 2003; 88: 1394–1399.
3. Daniel C and Silberstein G. Postnatal Development of the Rodent Mammary Gland. New York: Plenum Press, 1987; 3–36.
4. Bernstein L, Press MF. Does estrogen receptor expression in normal breast tissuepredict breast cancer risk? J Natl Cancer Inst, 1998; 90: 5–7.
5. Gruber CJ, Tschugguel W, Schneeberger C, Huber JC. Production and actions of estrogens. N Engl J Med, 2002; 346: 340–352.
6. Feigelson HS, Henderson BE. Estrogens and breast cancer. Carcinogenesis, 1996; 17: 2279–2284.
7. Gustafsson JA and Warner M. Estrogen receptor beta in the breast: role in estrogen responsiveness and development of breast cancer. J Steroid Biochem Mol Biol, 2000; 74(5): 245-248.
8. Clemons M and Goss P. Estrogen and the risk of breast cancer. N. Engl. J. Med, 2001; 344(4): 276-285.
9. Shin A, Kang D, Nishio H, et al. Estrogen receptor alpha gene polymorphisms and breast cancer risk. Breast Cancer Res Treat, 2003; 80: 127–131.
10. Han W, Kang D, Lee K M et al. Full sequencing analysis of estrogen receptor-alpha gene polymorphism and its association with breast cancer risk. Anticancer Res, 2003; 23(6): 4703–4707.
11. Andersen T I, Heimdal K R, Skrede M, Tveit K, Berg K, Borresen A L. Oestrogen receptor (ESR) polymorphisms and breast cancer susceptibility. Hum Genet, 1994; 94(6): 665–670.
12. Greene G L, Gilna P, Waterfield M et al. Sequence and expression of human estrogen receptor complementary DNA. Science, 1986; 231: 1150-1154.
13. Colditz G, and Rosner B. Cumulative risk of breast cancer to age 70 years according to risk factor status: data from the Nurses' Health Study. Am J Epidemiol, 2000; 152: 950–964.
14. Ross R K, Paganini-Hill A, Wan P C, Pike M C. Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. J Natl Cancer Inst, 2000; 92(4): 328-32.
15. Clarke C L, Sutherland R L. Progestin regulation of cellular proliferation. Endocrine, 1990; 11: 266–300.
16. Lydon J P, DeMayo F J, Funk C R et al. Mice lacking progesterone receptors exhibit pleiotropic reproductive abnormalities. Genes and Development, 1995; 9: 2266–2278.
17. McKenna N J, O'Malley B W. Nuclear receptors, co regulators, ligands and selective receptor modulators: making sense of the patchwork quilt. Annals of the New York Academy of Sciences, 2001; 949: 3–5.
18. Kastner P, Krust A, Turcotte B et al. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J, 1990; 9: 1603–1614.
19. Conneely O, Kettelberger D, Tsai M J, Schrader W and O'Malley B. The chicken progesterone receptor A and B isoforms are products of an alternate translation initiation event. J. Biol Chem, 1989; 264: 14062–14064.
20. Wen D, You-Feng X, Mais D, Goldman M, and McDonnell D. The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells. Mol. Cell Biol, 1994; 14: 8356–8364.
21. Sartorius C, Melville M, Hovland A, Tung L, Takimoto G, and Horwitz K. A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Mol. Endocrinol, 1994; 8: 1347–1360.
22. Giangrande P, Kimbrel E, Edwards D, and McDonnell D. The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. Mol. Cell Biol, 2000; 20: 3102–3115.
23. Fabjani G, Tong D, Czerwenka K et al. Human progesterone receptor gene polymorphism PROGINS and risk for breast cancer in Austrian women. Breast Cancer Res Treat, 2002; 72: 131-7.
24. Donaldson C J, Crapanzano J P, Watson J C, Levine E A, Batzer M A. PROGINS Alu insertion and human genomic diversity. Mutat Res, 2002; 501: 137-41.
25. Lydon J, Ge G, Kittrell F, Medina D, and O'Malley B. Murine mammary gland carcinogenesis is critically dependent on progesterone receptor function. Cancer Res, 1999; 59: 4276–4284.
26. Shyamala G, Yang X, Cardiff RD, Dale E. Impact of progesterone receptor on cell-fate decisions during mammary gland development. Proc Natl Acad Sci USA, 2000; 97: 3044–3049.
27. Alluri RV, Mohan V, Komandur S, ChawdaK, ChaudhuriJR and HasanQ. MTHFR C677T gene

mutation as a risk factor for arterial stroke: A hospital based study. Eur. J. Neurol., 2005; 12: 40-44.
28. Van MeursJB, Schuit SC, Weel AE, et al. Association of 5'estrogen receptor alpha gene polymorphisms with bone mineral density, vertebral bone area and fracture risk. Hum MolGenet., 2003; 12: 1745–1754.
29. Bergink AP, van Meurs JB, Loughlin J, et al. Estrogen receptor alpha gene haplotype is associated with radiographic osteoarthritis of the knee in elderly men and women. Arthritis Rheum, 2003; 48: 1913–1922.
30. Liaw D, Marsh D J, Li J et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet, 1997; 16: 64-7.
31. Stratton M R, Rahman N. The emerging landscape of breast cancer susceptibility. Nat Genet, 2008; 40: 17-22.
32. Benson J R, Jatoi I, Keisch M, Esteva F J, Makris A, Jordan V C. Early breast cancer. Lancet, 2009; 373: 1463-79.
33. Fatima H, Jamil K, Raju GS. Evidence of Hormonal Regulation in Breast Cancer Postmenopausal Women with Reproductive Surgeries. Journal for Cancer. Photon, 2014; 112: 142-152.
34. Yager JD and Davidson NE. Estrogen carcinogenesis in breast cancer. New England Journal of Medicine, 2006; 354: 270-282.
35. Yue W, Berstein LM, Wang JP, et al. The potential role of estrogen in aromatase regulation in the breast. J Steroid Biochem Mol Biol, 2001; 79: 157.
36. Martin A M and Weber B L. Genetic and hormonal risk factors in breast cancer. J. Natl. Cancer Inst., 2000; 92(14): 1126-1135.
37. Vidyadhar B Bangal, Kunaal K Shinde, Satyajit P Gavhane, Rashmi K Singh. Breast Carcinoma in women - A Rising threat. International Journal of Biomedical and Advance Research, 2013; 4(2): 73-76.
38. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN et al. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinico-pathologic features and survival. Clin Med Res., 2009; 7(1-2): 4-13.
39. Jamil K, Kumar K, Fatima SH, Rabbani S, Kumar R. Clinical Studies on Hormonal Status in Breast Cancer and its Impact on Quality of Life (QOL). J Cancer Sci Ther, 2009; 1: 083-089.
40. Dunnwald LK, Rossing MA and Li CI. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. Breast Cancer Res, 2007; 9(1): R6.
41. Feigelson H S, Coetzee G A, Kolonel L N, Ross R K, Henderson B E. A polymorphism in the CYP17 gene increases the risk of breast cancer. Cancer Res, 1997; 57: 1063–1065.
42. Wedren S, Lovmar L, Humphreys K, et al. Oestrogen receptor alpha gene haplotype and postmenopausal breast cancer risk: a case control study. Breast Cancer Res, 2004; 6: R437–R449.
43. Modugno F et al. Association of estrogen receptor alpha polymorphism with breast cancer risk in older Caucasian women. Int J Cancer, 2005; 116(6): 984-991.
44. Liao J, Ding D, Sun C, Weng D, Meng L, Chen G, Ma D. Polymorphisms of progesterone receptor and ovarian cancer risk: a systemic review and meta-analysis. J Obstet Gynaecol Res., 2015; 41(2): 178-87.
45. Wang-Gohrke S, Chang-Claude J, Becher H, Kieback D, and Runnebaum I. Progesterone receptor gene polymorphism is associated with decreased risk for breast cancer by age 50. Cancer Res., 2000; 60: 2348–2350.
46. McKenna N J, Kieback D G, Carney D N et al. A germline TaqI restriction fragment length polymorphism in the progesterone receptor gene in ovarian carcinoma. Br. J. Cancer, 1995; 71: 451–455.
47. D'Amora P, Maciel T T, Tambellini R et al. Disrupted cell cycle control in cultured endometrial cells from patients with endometriosis harboring the progesterone receptor polymorphism PROGINS. Am J Pathol, 2009; 175: 215-24.