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RESISTIN GENE −420 C/G POLYMORPHISM: POSSIBLE ASSOCIATION WITH ITSEXPRESSION AND CLINICOPATHOLOGY CHARACTERISTICS IN BREAST CANCER PATIENTS

Mohan reddy.N[1], Kalyan Kumar.Ch[1], Kaiser Jamil *[1] and Lakshmi Narasu. M[2]

[1]Bhagwan Mahavir Medical Research Center,
Department of Genetics, Hyderabad -04, A.P, India.
[2]Institute of Science & Technology, JNT University, Hyderabad.A.P, India.
*Corresponding author: E-mail: kaiser.jamil @gmail.com, Tel: +919849706385; fax +91-4066631500

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ABSTRACT:

To explore the relationship between resistin gene −420 C/G polymorphism and breast cancer in south India, We genotyped 154 patients with breast cancer and 142 healthy control subjects, using a PCR-RFLP method. We have also analyzed the gene expression of resistingene in 30 breast carcinoma tumor and corresponding control tissues. Gene expression analyses were performed at mRNA level by RT-PCR. The frequencies of RETN C4-20C, C-420G and G-420G were 83.5%, 17.8%, and 1.4% in the breast cancer group and 89.5%, 4.0% and 0.0% in the control group. The results of our study indicate that the−420 C/G genotype showed 2.6 folds increased risk of breast cancer (p = 0.05).Whereas individuals with joint genotypes (CG/GG) showed 1.79 folds increased risk of breast cancer. In our study, we have also clearly found significantly higher expression in breast cancer tissues in comparison with control tissues (P =0.002). In conclusion, our results suggest that, resistin gene −420 C/G polymorphism is significantly associated with risk of breast cancer obese women.However, there was a significant association between resistin expression levels and −420 C/G polymorphism in the patients. −420 C/G polymorphism may play a role in inducing breast cancer risk by altering the expression level of the resistin gene.

Key Words: Resistin, Polymorphism, Gene expression, Obesity, Breast cancer.

I. INTRODUCTION

Breast cancer begins in the any part of breast, caused by abnormal cells growth and division. The adipose tissue is metabolically and immunologically active, which may provide the link between obesity and breast cancer. The adipose tissue able to secretes many proteins and hormones known as “adipokines” [1], which include adipocytokines (leptin, adiponectin, and resistin). It has been demonstrated that adipocyte-secreted factors can directly promote mammary tumorigenesis through induction of antiapoptotic transcriptional programs and protooncogene stabilization [2]. Recently our findings suggest that adipocytokineses, leptin

(Gln2548Arg) and adiponectin (+45T/G and 276G/T) gene polymorphisms may be useful biomarkers for breast cancer in obese women in Indian population [3, 4].

Resistin gene is located on chromosome 19p13.3 and 12.5 kDa protein, mainly expressed by macrophages in humans [5]. As an important adipose-derived hormone, resistin may provide novel insights into the mechanisms of cancer progression, regression and persistence. [6]. Various Single Nucleotide Polymorphisms (SNPs) have been identified in the resistin gene. One of the most frequently studied polymorphisms, RETN −420C >G (rs1862513), was reported to be associated with the regulation of RETN gene expression and serum resistin level [7-9]. Several studies have also associated the RETN −420C >G polymorphism with arteriosclerosis, coronary artery disease [10] and cerebrovascular disease [11]. To our knowledge, no data have been published on the association of the RETN −420C >G polymorphism with the risk of Breast cancer and genetic polymorphisms of the resistin gene in south Indian population have not yet been studied. Therefore, in this study, we aimed to investigate another adipocytokines gene SNP −420 C >G (rs1862513), in RETN gene and their possible association with breast cancer susceptibility in obese women. We also analyzed gene expression profiles of RETN in both primary breast tumoral tissue and corresponding normal tissue.

II. PATIENTS AND METHODS

2.1. Study population

Breast cancer patients were assessed by pathologists on the basis of clinical examinations as well as mammography and clinical pathological examinations. The breast cancer study is a Hospital-based case-control study conducted in South Indian population. All incident breast cancer cases were newly diagnosed during the study period. Ethical Committee approved the study for the benefit of humans in general. 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 a structured questionnaire.

2.2. Selection criteria

Senior pathologists confirmed all diagnoses. We interviewed and collected the data about the patient demographic factors; collected the information on age, menopausal status, smoking, usual alcohol intake, BMI and previous cancer diagnoses. Participants were also asked about their family history of cancer, and the clinical information in these cases was obtained from medical records like tumor size, Grade, Axillary nodes, and whether they were receiving chemotherapy, hormonal therapy or radiotherapy. Patients were recruited following certain inclusion and exclusion criteria, which were determined before the beginning of the study.

2.3. Inclusion and exclusion criteria

Patients included in the study were women with breast cancer who were found to have positive (tumour-involved) axillary lymph nodes. Patients must have adequate blood counts, and adequate kidney and liver function.

2.4. Sample collection

Based on the above criteria, a total of 154 breast cancer patients and 142 age-matched controls were enrolled in the study. Sampling was done from two major Hospitals in Hyderabad, Andhra Pradesh (India) between the period 2008 to 2012. Those were BhagwanMahavir Hospital and Research Centre, Hyderabad, India and MNJ Cancer Hospital, Hyderabad, India.

2.5. Collection of biopsy and blood samples

Tumor breast tissues (biopsies) were collected in normal saline from the pathology lab after diagnosis. The tissues were, immediately transferred and stored at -80°C till further processing was done. The tumor samples obtained were of various tumor sizes and diagnosed mainly as invasive Ductal Carcinoma

(IDLS). About 3 ml Blood samples were collected from healthy women (Voluntarily) by venipuncture. These samples were used as controls, in various experiments.

2.6. Collection of demographic factors and clinical data

All patients were interviewed for recording their demographic factors. Clinical records for ER/PR status were obtained from medical records of the patients. Similarly we could record some demographic parameters of control subjects. Wherever possible, this data was correlated to obtain meaningful information to determine the risk factors of the disease. We also determined the significance of all the parameters for statistical evaluations.

2.7. Genotyping methodology

Genomic DNA was isolated by the salting-out method from the tissue samples of the cases and peripheral blood samples of the control group. The quantity of recovered DNA was determined spectrophotometrically. Polymerase Chain Reaction (PCR)-based Restriction Fragment Length Polymorphism (RFLP) was done to identify the Resistin gene −420(C>G) (rs1862513) polymorphism.

2.8. Genotyping of the RETN −420C/G Polymorphism

The following primers were used, forward primer 5'- TGT CAT TCT CAC CCA GAG ACA-3' and reverse primer 5'- TGG GCT CAG CTA ACC AAA TC-3' (Bioserve Biotechnologies, Hyderabad, India). PCR was performed in 20 ml reaction mixture containing 100 ng of genomic DNA template, 2 ml10X PCR buffer, 0.8 mMdNTP, 2.5 mM MgCl2, 0.5 mM primers, and 1U of Taq DNA polymerase (Bioserve, India). The reaction condition employed were initial denaturation at 95^0C for 5 min, followed by 35 step cycles of denaturation at 95^0C for 30 s, annealing 59^0C for 30 s, and extension72^0C for 30 s followed by a terminal extension time of 10 min. Ten ml of PCR product was digested with BbsI restriction enzyme (Fermentas) for 16 h at 37^0C.

2.9. RNA Extraction and cDNA Synthesis

The samples were thawed and homogenized using ominiTHq electric homogenizer (Inkarp) Total RNA was extracted from biopsy of breast tumors and corresponding non-tumor tissues using the RNAeasy Mini Kit (Qiagen, Germany). Total RNA was extracted from peripheral blood using a PAXgene blood RNA kit (Qiagen, Germany), According to the manufacturer's instructions. RNA was digested with DNase I (Invitrogen). The concentration and purity of extracting RNA were determined by measuring the absorbance at 260 nm and 280 nm. All samples whose concentration was a minimum of 100ng/µl and 260/280 between 1.8 to 2 were included in the study. Alternately the samples were run on agarose to check for the integrity. Reverse transcription was performed in a personal Master cycler (Bio-Rad CFX 96), using 1µg of total RNA in the presence of Random Hexamer (50ng/µl) and Reverse Transcriptase (50 U/µl) in a total volume of 20 µl, including also: 10 × TaqMan RT Buffer, $MgCl_2$ solution (25 mM), dNTPs mixture (10 mM), an RNAse Inhibitor (20 U/µl) and nuclease-free water. The reaction mixture was incubated for 10 minutes at 25°C, 60 minutes at 42°C, heated for 5 minutes to 95°C and then at 4°C for a minimum of 2 minutes. The resulting cDNA was stored at –20 ° C until further use.

2.10. Gene Expression Analysis

In assessing the relative gene expression on the SYBR chemistry of, *RETN gene*, real time - polymerase chain reaction (qRT-PCR) was performed, with1 µl of cDNA, 12.5 µl SYBR Green, and with specific primers F: 5'-CCAGCCATCAGCCATGAGGGT-3' and R: 5'- GGAGCCCTTTCTGAATCCGCA-3' Which was synthesized at Bio serve Biotechnologies Ltd. (Hyderabad, India). Primers were designed in different exons for all the genes to avoid

amplification from contaminating DNA. A three-step PCR assay was standardized using a Bio-Rad thermo cycler and carried out with an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. A final extension at 72°C for 5 min was carried out. Each sample was run in triplicates and each PCR experiment included one non-template control wells. A melt curve was also added to the PCR program for confirming a single intact PCR product without any non-specific amplification. An NTC was also added with all the experimental setups. Gene expression levels were normalized to the expression of the housekeeping gene GAPDH. In addition to SYBR green fluorescent detection, the amplified products were also resolved on 1.8% agarose gels and visualized by staining with ethidium bromide. In the present study, increased mRNA expression was defined as ≥ 2. 0 folds, "normal" expression was ranging from 0.5001 to 1.9999 folds and decreased mRNA expression was ≤0. 5 folds The data collection step was performed at a temperature that was 3°C lower than the melting temperature of each amplicon and higher than that of primer-dimers of each primer pair. This cycle was followed by a melting curve analysis, ranging from 55°C to 95°C, with temperature increasing steps of 0.5°C every 10 s. Baseline and threshold values were automatically determined for all plates using the Bio-Rad iQ5 Software 2.0.

2.11. Statistical analysis

Genotyping experiments are 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).

III. RESULTS

3.1. Clinical Characteristics of Study Subjects

A total of 154 breast cancer patients and 142 age matched controls formed our study group. The distribution of biological characteristics and selected risk factors of the case controls are presented in table 1. The age range for breast cancer patients was 20-70 years. The mean age at which breast cancer was generally identified was 49.87 years. Breast cancer patients were divided into 5 groups according to age at diagnosis; these were 21-30, 31-40, 41-50, 51-60 and 61-70 years. Incidence of breast cancer cases and a control group was higher in the age groups 41-50 (32% and 28%) years when compared to other age groups, and the incidence was very low in the age group 61-70 years. The frequency of overweight and obese patients (25%, and 55%) and control (35%, 54%) showed a high Body Mass Index (BMI). Age at menarche of breast cancer patients showed that about 54% of them had attained menarche between 13 -14 years, while 12% attained it by 15-16 years. This is similar to what was observed in the normal population. Depending on the menopausal status, breast cancer patients and controls were also categorized into premenopausal (48% and 58%). and postmenopausal groups (52% and 42%). It was observed the majority of sporadic breast cancer cases were higher in postmenopausal group when compared to premenopausal group.

3.2. Genotyping of the RETN −420C/G Polymorphism

*RETN*C420G Gene polymorphisms were analyzed in DNA obtained from Tissue samples of 154 Breast Cancer patients and compared to 142 healthy age matched women volunteers blood samples. *RETN*C420G Polymorphism was analyzed by PCR –RFLP and the PCR product (534bp) was digested withBbsI restriction enzyme. The DNA fragments were then separated using 2 % agarose gel and detected by ethidium bromide staining. The digestion products were

then resolved on a 2.5% agarose gel containing ethidium bromide. The homozygous wild −420 CC genotype was identified by two bands (327bp and 207bp), the heterozygous mutant −420 CG genotype produced three bands (534bp, 327bp and 207bp), and homozygous mutant −420 GG genotype remained uncleaved (534bp) (Figure 1). Frequencies of CC, CG, and GG genotypes were 60%(n=93), 35%(n=54), 5%(n=7) in the breast cancer cases and 73%(n-104), 25%(n=36), 2%(n=2) in the controls respectively . Table 2 shows results for the *RETN* C420G Polymorphism, CG, and GG genotypes showed higher frequency in breast cancer patients than controls. From *RETN* Genotyping Analysis, it was observed that there was significant difference in the distribution of CC genotype between cases (60%) and controls (73%). GG genotype was observed only in 5% of breast cancer cases and it was present only 2% in the control group, while the distribution of CG genotype was higher in breast cancer cases (35%) when compared to controls (25%). Odds ratios (OR: 1.59, 95% CI: 0.96-2.62) and p values (0.05). We next analyzed the joint effects of the two genotypes; Both CG/GG genotype was higher in breast cases 40% and 27% in controls. However individuals with joint genotypes showed 1.79 folds increased risk of breast cancer (Odds ratios (OR: 1.79, 95% CI: 1.09-2.93 and p values (0.01)).

3.3. Correlation of demographic factors and genotype frequencies of −420(C>G) polymorphism

Distribution of RETN gene −420(C>G)polymorphism and its relation with clinicopathological data of the patients are shown in table-3. In correlation of the genotype with clinical data, patients with age group between, 21-30 (56%), 31-40 (71%) and 31-40 (62%) showed high frequency of CC genotype than CG genotype. In patients with BMI >30 showed high frequency of CC and CG genotype and it is statistically significant (p=0.0001). RETN−420(C>G)polymorphism showed a significant association with postmenopausal status,er/pr positive and Her-2 neu positive (p=0.0001)

3.4. Expression of *RETN gene* in breast cancer tumoral tissue and corresponding normal tissue

The Relative gene expression patterns of *RETN* gene were quantified in 30 breast cancer tumor samples and corresponding blood samples of the same individual by real time PCR. Gene expression levels were shown ratio between *RETN* geneand the reference gene *GAPDH* to correct for the variation in the amount of RNA. The mRNA expression of the investigated genes of breast cancer tissue and blood samples was reported in Tables 4 and depicted in Figures 2. In this study, *RETNmRNA* levels showed up regulation in 16 of 30 (54%) casesin tumoral tissues (range 2.20 to 8.15) and0 of 30 (100%) cases in normal tissues. The increased expression of the *RETN* was significantly greater (P = 0.002) in the tumoral tissue when compared with normal tissues.

IV. DISCUSSION

Obesity results from an accumulation of adipose tissue, and the subsequent alteration in adipokine secretion might contribute to an increased cancer risk.Leptin and adiponectin has been shown to induce proliferation, survival and anchorage-independent growth. Although a role for adipokines such as leptinand adiponectin in obese breast cancer patients have been partially demonstrated [12, 13]. The role of resistin in affecting breast cancer has never been studied in obese breast cancer patients.Resistin is the most recently described adipocyte-derived peptide, and it was initially suggested that it plays a role in the development of insulin resistance and obesity. To our knowledge, this is the first study to examine the association between the RETN polymorphism and the risk of breast cancer in obese women. We herein investigated whether the RETN promoter

polymorphism could be associated with the development of breast cancer in obese women. In this study, we analyzed the RETN -420C>G SNP in obese breast cancer patients. To our knowledge the present study is the first to report the association between RETN -420C>G SNP and breast cancer risk in south Indian patients. Genotyping distribution for the SNP 420C>G in patients and controls differ significantly, suggesting that the polymorphism may modify the risk for breast cancer. The data of the present study showed that heterozygous genotype had more than 2.8 folds higher risk breast cancer than the carriers of the normal genotype. However individuals with joint genotypes (CG.GG) showed 1.79 folds increased risk of breast cancer (Odds ratios (OR: 1.79, 95% CI: 1.09-2.93 and p values (0.01)). Our findings suggests a potential role for 420C>G SNP in the genetic predisposition to breast cancer disease among south India. It has been reported that the RETN variant could gain the ability to bind the Sp1/3 transcription factor, which might markedly enhance RETN gene promoter activity and increase transcription of the RETN gene [9]. Previous studies have also demonstrated that the RETN −420□G allele was associated with higher expression of resistin mRNA and resistin levels in humans [7,8].

Further we also estimated the impact of the RETN polymorphism -420C>G on the clinical parameters in breast cancer patients.In our study, there was statistically significant association between 420C>G polymorphism and expression of estrogen and progesterone receptors, HER-2 expression. According to menopausal status both pre and post-menopausal women with heterozygous genotype significantly associated with breast cancer risk. We suggest that 420C>G genotype may also increase the risk of early onset breast cancer before age 40. According to BMI status, obese women with CG genotype had a higher statistically significant risk of developing breast cancer, in contrast Mattevi et al. [12] found resistin gene polymorphism (-420C>G) was associated with body mass index (BMI) and reported that women carrying G-allele had lower BMI.

Further we analysed Gene expression profiling in patients with 420C>G SNP. Gene expression is an important tool to evaluate genetic heterogeneity in carcinoma and is useful to develop expression based classification for different type of cancer. RETN gene expression is associated with biological effects of aberrant cells proliferation.In this study, we have analyzed the gene expression of RETN gene in 30 breast carcinoma tumor and corresponding control tissue gene expression analyses performed at mRNA level by RT-PCR. In our study, we have clearly found significantly higher expression of RETN in breast cancer tissues in comparison with control tissues.We suggest that the expression of resistin mRNA was reported to be highest in subjects with the RETN −420□CG genotype. Our results suggest that resistinmight be another adipokine that increases insulin resistance related malignant risk. Our data support the hypothesis that the resistin molecule may play a role in the progression of breast cancer. Previously three case-control studies that were conducted separately in China [13], Korea [14] and Taiwan [15] found that high levels of resistin were associated with increases in breast cancer risk.

V. CONCLUSION

In conclusion, our results suggest that, resistin gene −420 C/G polymorphism is significantly associated with risk of breast cancer obese women.However, there was a significant association between resistin expression levels and −420 C/G polymorphism in the patients. −420 C/G polymorphism may play a role in inducing breast cancer risk by altering the expression level of the resistin gene. Further investigations are needed to provide novel insights into the potential role of resistin as a mediator of obesity in cancer

development, which may enhance our understanding of tumor development.

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TABLES AND FIGURES:

Table: 1 Demographic characteristics of patient and control individuals

Characteristics	Cases N=154(%)	Controls N=142(%)
Age of Diagnosis		
21-30	16(10%)	32(23%)
31-40	38(25%)	36(25%)
41-50	50(32%)	40(28%)

51-60	38(25%)	20(14%)
61-70	12(8%)	14(10%)
Body mass index(BMI) status		
Normal weight >18.50 to <24.99	30(19%)	16(11%)
Overweight >25 to <29.99	40(26%)	50(35%)
Obese >30.00	84(55%)	76(54%)
Age at Menarche		
<12	52(34%)	38(27%)
13-14	84(54%)	78(55%)
15-16	18(12%)	24(17%)
>17	0(0%)	2(1%)
Menopausal Status		
Post-Menopausal	80(52%)	60(42%)
Pre-Menopausal	74(48%)	82(58%)

Table: 2 Distribution of Genotype frequencies of Resistin gene −420(C>G) (rs1862513) Polymorphism in obese breast cancer cases and controls.

Genotype	Cases N=154(%)	Controls N=142(%)	95% CI	χ2 test	Odds ratio	P-Value
CC	93(60%)	104(73%)	0.34 to 0.91	4.91	0.55	0.01
CG	54(35%)	36(25%)	0.96 to 2.62	2.85	1.59	0.05
GG	7(5%)	2(2%)	0.68 to16.32	1.51	3.33	0.13
CG/GG	61(40%)	38(27%)	1.09 to 2.93	4.91	1.79	0.01

Table: 3 Correlation of breast cancer patients demographic factors and Resistin gene −420(C>G) (rs1862513) genotype frequencies

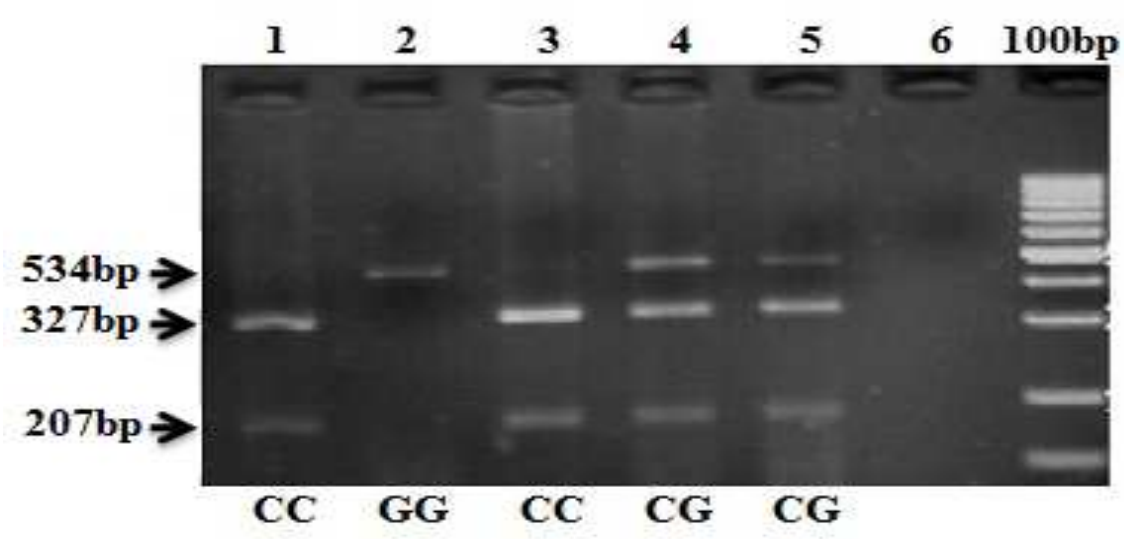
Characteristics	Cases N=154(%)	Wild type CC N=93(%)	Heterozygous CG N=54 (%)	Homo mutant GG N=7(%)	P-Value
Age of Diagnosis					
21-30	16(10%)	9(56%)	6(38%)	1(6%)	0.01
31-40	38(25%)	27(71%)	11(29%)	0(0%)	0.0004
41-50	50(32%)	31(62%)	18(36%)	1(2%)	0.01
51-60	38(25%)	20(53%)	15(39%)	3(8%)	0.25
61-70	12(8%)	6(50%)	4(33%)	2(17%)	0.41
Body mass index(BMI) status					
Normal weight >18.50 to <24.99	30(19%)	15 (50%)	13(43.33%)	2(6.66%)	0.60
Overweight >25 to <29.99	40(26%)	19(48%)	19(47%)	2(5%)	1.00
Obese >30.00	84(55%)	59 (70%)	22 (26%)	3(4%)	<0.0001
Age at Menarche					
11-12	52(34%)	31(60%)	19(36%)	2(4%)	0.01
13-14	84(54%)	52(62%)	28(33%)	4(5%)	0.0003
15-16	18(12%)	10(56%)	7(39%)	1(5%)	0.31

Menopausal Status					
Post Menopausal	80(%)	50(63%)	25(31%)	5(6%)	0.0001
Pre Menopausal	74(%)	43(58%)	29(39%)	2(3%)	0.02
Estrogen Receptor status					
ER+ /PR+	48(31%)	33(69%)	13(27%)	2(4%)	0.0001
ER+/PR-	10(7%)	6(60%)	3(30%)	1(10%)	0.18
ER-/PR+	6(4%)	5(83%)	0(0%)	1(17%)	0.30
ER-/PR-	90(58%)	49(55%)	38(42%)	3(3%)	0.10
Her2 status					
Her2+	135(88%)	81(60%)	48(36%)	6(4%)	0.0001
Her2-	19(12%)	12(63%)	6(32%)	1(5%)	0.05
Chemotherapy					
5FU,Adriamycin,Endoxane	108(70%)	68(63%)	37(34%)	3(3%)	<0.0001
Hormonal therapy	11(7%)	5(46%)	4(36%)	2(18%)	0.66
RD	28(18%)	16(57%)	11(39%)	1(4%)	0.18
No Data	7(5%)	4(57%)	2(29%)	1(14%)	0.28

Table: 4 RT-PCR analyses of RETN mRNA expression in Breast Cancer Patients

RETN Expression	Tumor	Normal	P- Value
Altered Expression	16(54%)	0(0%)	0.002
Normal Expression	14((36%)	30(100%)	

Figure 1: Direct Visualization of PCR-RFLP of Resistin −420(C>G) Genotype by ethidium bromide staining 534bp PCR products were digested by BbsI restriction enzyme



Lanes1 and 3: Homozygous wild CC (327bp and 207bp)
Lane 2:Homozygous mutant GG (534bp)
Lanes 4 and 5:Heterozygous mutant CG (534bp, 327bp and 207bp)
Lane 7: 100 bp Ladder

Figure 2. A) ResistinMelt Curve and **B)** GAPDH Melt Curve