Association of CASC16 variant rs4784227 with breast cancer risk in Pakistan

Sidra Muhyuddin[1*], Humaira Arshad[2], Zubair Azhar Nomi[2], Saima Noreen[2], Komal Arshad[3], Tabinda Afzal[4], Saima Riaz[5], Warda Sarwar[5], Sumreen Sarwar[5], Mah Noor[6], Iqra Irshad[6], Samra Abbas[7]

1*. Department of Zoology, Faculty of Veterinary and Animal Sciences, Muhammad Nawaz Sharif University of Agriculture, Multan, Pakistan

2. Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad

2. Department of Animal and Dairy Sciences, Faculty of Veterinary and Animal Sciences, Muhammad Nawaz Sharif University of Agriculture Multan, Pakistan

2,4. Department of Zoology, Faculty of Veterinary and Animal Sciences, Muhammad Nawaz Sharif University of Agriculture, Multan

3,6. Department of Pharmaceutics, Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan

5. Department of Chemistry, The Women University of Multan, Pakistan

5. Department of Chemistry, Government College Women University Faisalabad, Pakistan

5. Department of Pharmacy, Physiology and Pharmacology, University of Agriculture Faisalabad

6. Department of Microbiology and Molecular Genetics, The women University Multan

7. Department of Chemistry, University of Management and Technology Sialkot, Pakistan

Corresponding Author: Sidra Muhyuddin…Lecturer

ORCID: https://orcid.org/0009-0006-8086-1688

Abstract

Breast cancer is characterized by uncontrolled growth of malignant breast tissue cells and is a heterogeneous disease. The most common types are invasive ductal and lobular carcinoma. Annually, about 1,000,000 new cases arise worldwide, with an increasing prevalence in Asia. In Pakistan, approximately 51.7 out of 100,000 women are affected. This study investigates the association of the CASC16-rs4784227 polymorphism with breast cancer, analyzing both genetic and non-genetic risk factors. The study included 115 subjects (70 patients, 45 controls) from southern Punjab. Significant non-genetic factors included age, family history, menarche age, menopausal status, breastfeeding, BMI, and diet. Estrogen receptor (ER), progesterone receptor (PR), and HER2 status were also examined with 55.71% ER-positive, 62.86% PR-positive, and 54.29% HER2-negative cases. The most common cancer type was ductal carcinoma and stage 3 was the most prevalent. Tetra arms PCR was used for genotypic analysis which showed an association between SNP rs4784227-CASC16 and breast cancer in the Pakistani population.

Key words: Breast cancer, Invasive ductal carcinoma, Cancer- Susceptibility Candidate 16 gene (CASC16), Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2)

INTRODUCTION

Cancer is a global public health issue, with about 10 million cases and 6 million deaths annually. It occurs when normal cell division is disrupted and cases are expected to rise, especially in developing regions where over 70% of cancer deaths occur. By 2050, developing countries may account for two-thirds of global cancer cases. Types of cancer include carcinoma, lymphoma, sarcoma, and leukemia, each affecting different body tissues. Gene mutations disrupt cell growth, causing invasive cancer cells that may spread. **Breast cancer (BC)** is the most common cancer in women globally and the second leading cause of cancer-related deaths. Its incidence is rising especially in lower-income countries with increasing death rates. In developed regions urban Caucasian women are at the highest risk while BC rates are lower in Africa and Asia. However, lifestyle changes have led to a rise in BC cases in Asia, including Pakistan, where it is the most common cancer among women partly due to genetic factors linked to family marriages. Accurate classification, such as ductal and lobular carcinoma, is crucial for effective treatment. **Hormonal risk factors** for breast cancer (BC) are linked to prolonged estrogen exposure, such as early menarche, late menopause and frequent menstrual cycles. Reducing menstrual cycles through exercise and extended lactation can lower BC risk. Estrogen receptor mutations also contribute to the heightened risk. (Zheng et al., 2003; Bernstein et al., 1987). **Non-hormonal risk factors** also play a significant role in breast cancer (BC) risk. Alcohol consumption disrupts estrogen metabolism and increases cancer likelihood while high-fat diets, red meat and caffeine also raise risk. A diet rich in fruits, vegetables, calcium and vitamin D helps lower BC risk. Other factors include smoking, viral infections (HPV, EBV) and environmental pollutants but regular physical activity and a strong immune system can mitigate some risks. (Dumitrescu & Shields, 2005; Brody et al., 2007). **Genetic risk factors** play a significant role in the onset of breast cancer (BC), with hereditary cases accounting for 10–14% of all BCs. Family history provides important insights into diagnosis and treatment, as familial BC often involves genetic heterogeneity. About 30% of familial cancer cases result from mutations in specific genes responsible for DNA repair, such as BRCA1, BRCA2, ATM, TP53, and CHEK2. Studies of high-penetrance, low-frequency mutations in these genes, particularly BRCA1 and BRCA2, explain approximately 25% of familial BC risk, while 5% of overall BC cases are linked to these mutations. Additionally, genome-wide association studies have identified

several low-penetrance, high-frequency genetic variants, such as TOX3, FGFR2, and MAP3K1 that are associated with increased BC risk, contributing to the remaining 75% of susceptibility. **High Penetrance BC Susceptibility Genes:** The **BRCA1 gene**, located on chromosome 17q21, is a major high-penetrance gene linked to breast cancer (BC) susceptibility. Mutations in BRCA1 account for 15%-45% of hereditary BC cases, leading to a 60%-80% lifetime risk in female carriers. These mutations are associated with early onset, bilateral BC, and increased risks of ovarian, colon, and prostate cancers. Common mutations like 185delAG and 5382insC contribute to BRCA1-related BC cases, making up 2%-5% of familial BC cases. Mutations in the **BRCA2 gene** carry a 60%-85% breast cancer (BC) risk, with a 6% risk in male carriers, and also increase the likelihood of cancers in the pancreas, gallbladder, and prostate. Founder mutations like 6174delT in Ashkenazi Jews and 999del5 in Icelandic and Finnish populations contribute to varying BC risks. Additionally, the ATM gene, linked to ataxia-telangiectasia (AT), raises BC risk by 3.3-3.9 times, with higher risks seen in mothers of AT patients in Scandinavia. **Low Penetrance BC Susceptibility Genes:** Low-penetrance breast cancer (BC) susceptibility genes, or modifier genes, are common polymorphic genes that slightly increase BC risk. While each variant poses a minor risk, combined effects can exceed that of high-penetrance genes. These genes are involved in pathways like DNA repair, detoxification, and immune processes. Examples include the P450 gene (CYP1A1), linked to estrogen metabolism, and the androgen receptor gene, associated with androgen signaling and smoking risks. High-penetrance genes like p53 (Li-Fraumeni syndrome), STK11/LKB1 (Peutz-Jeghers syndrome), and PTEN (Cowden syndrome) also play critical roles in BC susceptibility. **Gene CASC16 and SNP rs4784227:** Cancer- Susceptibility Candidate 16 gene (CASC16) also termed LOC643714is a kind of long non-protein coding RNA and located at chromosomes 16q12.1. CASC16 gene has higher expression in breast cancer cells as compared with normal cells. **Rs4784227-CASC16** as single nucleotide polymorphisms can influence the FOXA1 DNA binding sequence which are situated at the 16q and may increase the FOXA1-binding ability to the promoter of TOX3 genes. A member of the high mobility group of proteins that modifies chromatin structure is recognised as TOX3. For the change of TOX3 expression, the progesterone receptors, oestrogen receptor and positive lymph nodes are related with this gene. Thisincreased level of Ki67 the subtype of basal tumour is related with the low level of TOX3 expression. The PR , ER positive and positive lymph nodes in the tumour and normal tissue collection are correlated with the high expression of mRNA (O'Flaherty and Kaye, 2003). TOX3 and

LOC643714 The TOX3 and LOC643714 genes are linked to estrogen receptor (ER) positive and negative breast cancer (BC) subtypes, with single nucleotide polymorphisms (SNPs) like rs4784227-CASC16 altering FOXA1's ability to regulate TOX3 expression. This interaction affects BC susceptibility and luminal cell differentiation, highlighting the role of genetic variation in cancer risk, particularly in populations from Southern China, Europe, and Korea.

MATERIALS AND METHODS

In order to evaluate association of selected SNP CASC16-rs4784227 with probability of BC, research work was carried out in Zoology laboratory at The Women University Multan (Punjab) Pakistan. **Sample and data collection:** From NISHTAR hospital (MINAR) Multan, blood samples of BC patients werecollected. The collection was completed in the duration of 6 months from October 2019 to March 2020. Total 190 blood samples were collected out of which 145 were BC patients and 45 were healthy controls. 3- 5ml blood was drawn from each patient and immediately poured into EDTA vials and mixed to prevent it from clotting. Subsequently, these EDTA vials were freezed until further processing.

Patient inclusion and exclusion criteria: Females of any age were included and males were excluded as cases and cancerfree females of any age as controls.

DNA isolation: Salting out technique was used for DNA extraction. (Helms, 1990).

Procedure of DNA isolation:

The following steps were followed in DNA extraction:

1. Added equal volume of buffer A and blood, double volume of cold water. (Made master mix in sterile glassware) vortexed the tubes six to eight times and left for 10 minutes on ice.
2. Tubes were spun in Temperature controlled centrifuge machine (Model: Universal 320R, Hettich, Zentrifugen) at (3500rpm is 2205XG) for fifteen minutes at 4°C. Wasted the supernatant into 10% bleach solution from opposite side of pellet when the centrifuge finished, in the 2ml buffer A and 6ml water re-suspended the pellet (for 30 seconds vortex at medium speed in master mix 2). For fifteen minutes spun at (3500rpm) at 4°C. Discarded the supernatant and colour of the pellet was white creamy.
3. In the pellet added 5ml of Buffer B and 500µl of 10% SDS. Vortex vigorously for

60 seconds to re-suspend the pellet, added proteinase K solution (50µl).

4. Left to incubate for two hours at 60˚C in an incubator. Left the samples for 10 minutes on ice after taken out from incubator. Solution became cloudy when added 4ml of 5.3M NaCl solution.
5. For 20 minutes at (4500rpm is 3645XG) at 4°C spun the samples immediately. In the15ml tube poured off the supernatant. Re-centrifuged at (4500rpm is 3645XG) for twenty minutes at 4°C.
6. Into 50ml falcon tube immediately removed the supernatant. Cold isopropanol was added equal to the volume of supernatant. To precipitate DNA inverted 5 to 6 times.
7. Wide bore yellow tip was used to remove DNA and transferred into 1ml of 70% ethanol present into falcon tube. For 10 minutes centrifuged the samples at maximum speed and the supernatant discarded immediately. Position of DNA in the tube was marked. At 37˚C for 15-20 minutes allowed the DNA to dry. In 300µl low TE DNA was re-suspended. At room temperature left them to re-dissolve for overnight.
8. The remaining nucleases inactivated by heating the DNA in a water bath for one hour at 70°C.
9. After that frozen the DNA at -4°C.

Agarose gel Electrophoresis of DNA analysis: The extracted DNA was analyzed by gel electrophoresis. There are following stepsthat were followed in gel electrophoresis:

1. For gel electrophoresis first of all made 1% agarose gel. To make 1% agarose gel took 0.2g agarose and dissolved it into 20ml 1xTAE buffer to make a solution. After that heated the solution in the oven for 15 to 20 seconds until solution got transparent when solution became transparent removed out the flask from oven and added 5µl ethidium bromide in the flask.

2. For gel electrophoresis first of all made 1% agarose gel. To make 1% agarose gel took 0.2g agarose and dissolved it into 20ml 1xTAE buffer to make a solution. After that heated the solution in the oven for 15 to 20 seconds until solution got transparent when solution became transparent removed out the flask from oven and added 5µl ethidium bromide in the flask.

3. Set the gel caster and applied the combs after that poured the transparent agarose solution into the gel caster and let it to polymerize about half an hour.
4. After half an hour when the gel was completely polymerized, removed the comb carefully and put the gel caster into electrophoresis unit. Filled the electrophoresis unit with 1xTAE buffer until the gel was completely immersed in the 1xTAE buffer.
5. Took 2µl dye on the cellophane paper and 5µl DNA sample mixed them together and the samples were laden into the wells of the gel by using pipette.
6. Covered the lid of electrophoresis unit and connected the electrodes with it. The anode pole was of red colour and cathode pole was of black. The DNA moved from cathode to anode.
7. Connected the unit of electrophoresis with power source and ran the gel at 90 voltage for 15 to 20 minutes when the 75-80% dye line away from the gel border. After that increased the voltage to 100V and again ran the gel for 10 to 15 minutes.
8. Carefully removed the gel from the gel box after switched off the power and disconnected the electrodes.
9. Visualized the gel in the UV transilluminator.

Spectrophotometric analysis: DNA's quality and quantity measured through spectrophotometer by measuring optical density (OD)/absorbance at (260nm) and (280nm). Optical density on (260nm) showed DNA quality while ratio of OD at 260nm OD at 280nm illustrated the quality of DNA. The quality of DNA was good if the ratio greater than 1.7. The quality of DNA was bad if the ratio was less than 1.7.

Genotypic analysis by tetra ARMS PCR: Genotypic analysis of rs4784227-CASC16 SNP was determined by tetra ARMS PCR by using G-Storm Thermocycler (model# GS4822). For the tetra ARMS PCR the primers that were used, are given below in the table.

Table I: Primer sequences of rs4784227 gene CASC16 used for ARMS-PCR genotyping

SNP	Primers	Sequences	Length	PCR products	Reference
rs4784227	**FIP (T allele)**	AAAAGTCCCAATTTGTAGTGTTTGaT	26	322	Tajbakhsh *et al.*, 2019
	RIP (C allele)	AATGGAGTATTTACATCACAATAATgG	27	297	
	FOP	ACTGACCCCTTTAGACACGG	20	495	
	ROP	AGGGCTTCAACACAGTCAGTTC	21		

Procedure of tetra ARMS PCR

For tetra ARMS PCR master mix was prepared by mixing the PCR buffer, $MgCl_2$, dNTPs, Taq DNA polymerase, injection water, forward inner primer, reverse inner primer, forward outer primer and reverse outer primer to make final volume of 8µl. In each tube added 2µl of DNA and 18µl of PCR master mix, after that spun the PCR tubes in micro centrifuge for just few seconds to settle down the solution in the PCR tubes and then put the PCR tubes in the Thermocycler.

Table II: Components of PCR

Components of PCR	Final Concentration	Volume (µl)
10X Buffer	1X	2
25mM $MgCl_2$	2.5mM	2
2.5mM dNTPs	200µM	1.6
10µM Primer (FOP)	10µM	1
10µM Primer (FIP)	10µM	1
10µM Primer (RIP)	10µM	1
10µM Primer (ROP)	10µM	1
Taq DNA polymerase	1.25U/µl	0.25
Template DNA	100ng	2
H_2O		8.15

PCR cycling conditions

PCR amplifications for rs4784227-CASC16 was carried out in a 20µl final volume. The tetra ARMS-PCR conditions for rs4784227 were as follows: initial denaturation at 94°C for five minutes, after that 35 cycles including denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, an extension at 72°C for 30 seconds followed by 72°C for 7 minutes as the final extension step and finally held at 4°C. These steps are showing in the figure 6.

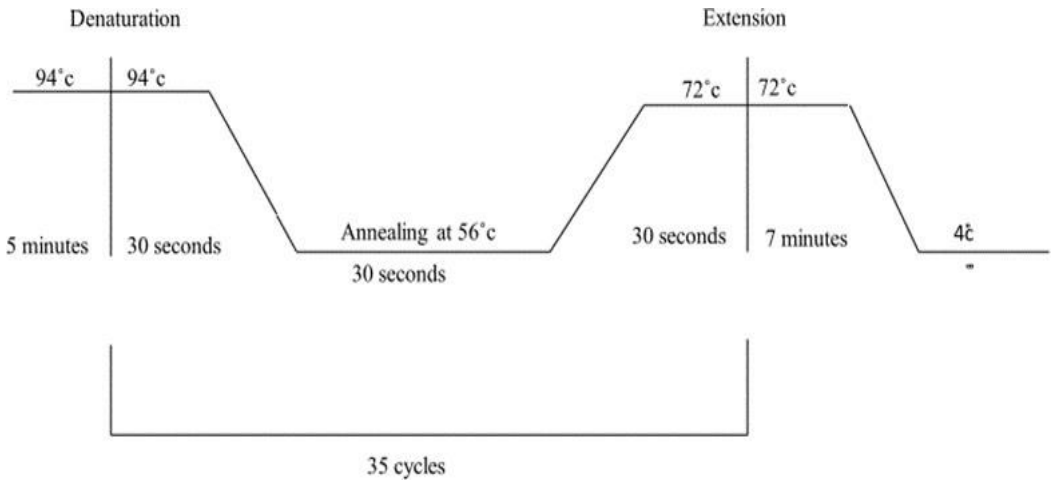

Figure 1. PCR amplification conditions

Analysis of PCR products by gel electrophoresis:

To analyze PCR products, gel electrophoresis was performed using 1.5% agarose gel. A mixture of 1.5g agarose powder and 80ml 1X TAE buffer was heated, cooled, and combined with 10µl ethidium bromide. After pouring the solution into a gel caster and allowing it to polymerize, the gel was placed in an electrophoresis unit filled with 1X TAE buffer. Samples with loading dye and 1KB ladder were loaded, and the gel was run at 90V for 10 minutes, then at 100V for 20-25 minutes. Bands were visualized under a UV transilluminator.

RESULTS

Statistical analysis for association of genotypic and allelic frequency of polymorphism rs4784227 by using SPSS software version 20 with BC patients and controls. P-value ≤0.05 was considered to be statistically significant.

Table III: Association of demographic factors of cases and controls

Risk factor	Categories	Patients	Percentage	Controls	Percentage	P-value
Age	A (24-33)	10	14.28	15	33.33	0.003
	B (34-43)	22	31.42	22	48.88	
	C (44-53)	25	35.71	6	13.33	
	D (54-63)	10	14.28	2	4.44	
	E (64-73)	3	4.28	0	0	
Family history of BC	Positive	25	35.71	3	6.66	0.000
	Negative	45	64.28	42	93.33	
Family history of any cancer	Positive	26	37.14	7	15.55	0.013
	Negative	44	62.85	38	84.44	
Menarche age	≤13	34	48.57	10	22.22	0.005
	≥13	36	51.42	35	77.77	
Menopause age	Premenopausal	25	35.71	31	68.88	0.001
	Postmenopausal	45	64.28	14	31.11	
Marital status	Married	56	80	6	13.33	0.357
	Un-married	14	20	39	86.66	
Breast feeding	Yes	36	51.42	37	82.22	0.001
	No	34	48.57	8	17.77	
Any disease	Yes	28	40	15	33.33	0.471
	No	42	60	30	66.66	
Smoking	Smoker	11	15.71	2	4.44	0.063
	Non-smoker	59	84.28	43	95.55	
BMI	Weak	4	5.71	8	17.77	0.002
	Normal	30	42.85	22	48.88	
	Overweight	19	27.14	15	33.33	
	Obese	17	24.28	0	0	
Diet	Meat	6	8.57	8	17.77	0.029
	Vegetarian	30	42.85	9	20	
	Both	34	48.57	28	62.22	

Table IV: Clinical characteristics of BC patients

Genetic factors	Conditions	Frequency	Percentage
Estrogen receptor (ER) status	Positive	39	55.71
	Negative	31	44.29
Progesterone receptor (PR) status	Positive	44	62.86
	Negative	26	37.14
Human epidermal growth receptor (HER2) status	Positive	32	45.71
	Negative	38	54.29
Types of carcinoma in BC patients	Lobular carcinoma	11	15.71
	Ductal carcinoma	35	50
	Mammary carcinoma	23	32.86
	Mucinous carcinoma	1	1.43
BC stages	I	11	15.71
	II	21	30
	III	28	40
	IV	10	14.29
Tumor size	1	13	18.57
	2	14	20
	3	21	30
	4	22	31.42
Lymph node status	0	15	21.43
	1	22	31.42
	2	23	32.86
	3	10	14.29
Metastasis of BC	Positive metastasis	18	25.71
	Negative metastasis	52	74.29

DNA isolation by agarose gel electrophoresis

Isolated DNAs were observed in (1%) agarose gel by UV-transilluminator.

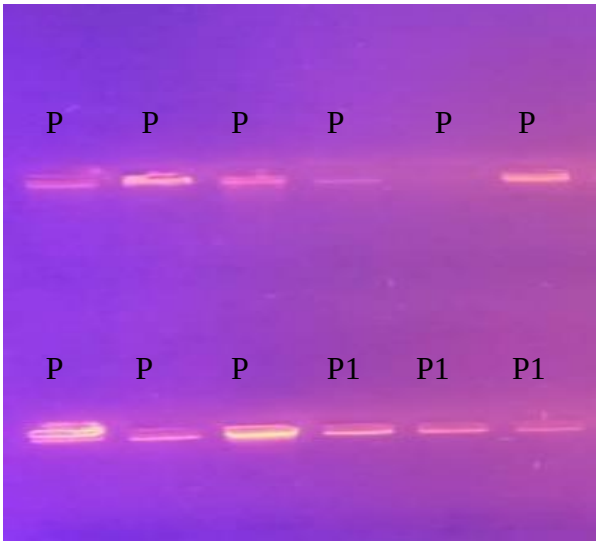

Figure 2. Representative Agarose gel (1%) of Patient's DNA (P1-P32)

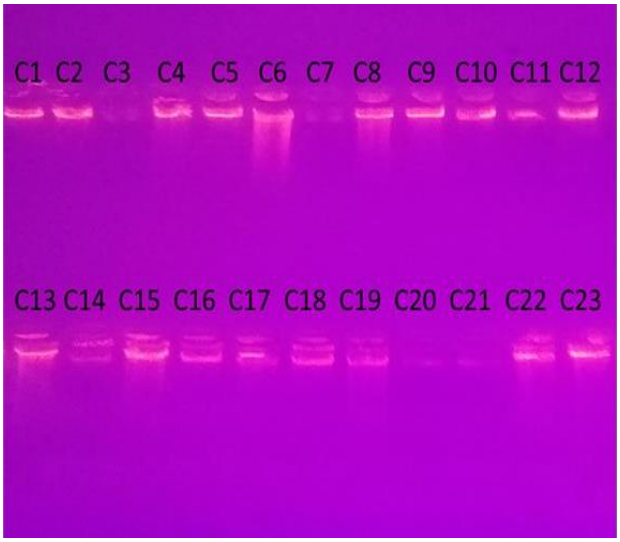

Figure 3. Representative Agarose gel (1%) of control's DNA (C1-C23)

PCR amplification of SNP rs4784227 by gel electrophoresis

SNP rs4784227 was amplified by tetra ARMS PCR and PCR products wereobserved in (1.5%) agarose gel by UV-transilluminator.

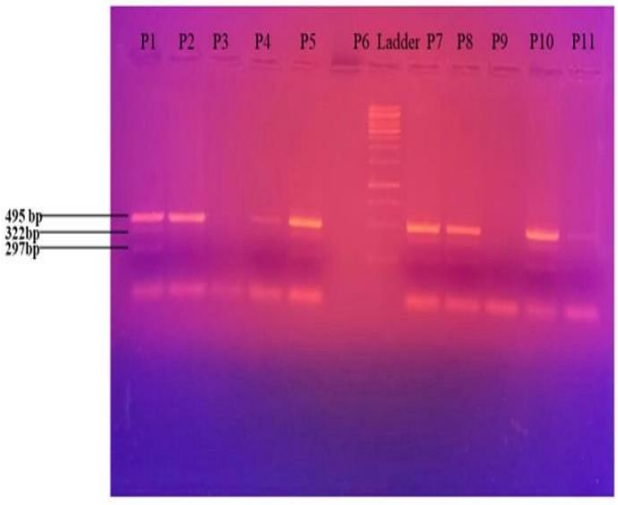

Figure 4. Representative Agarose gel (1.5%) showing amplification controls (C1-C8) in the well 3 the 1KB ladder is present

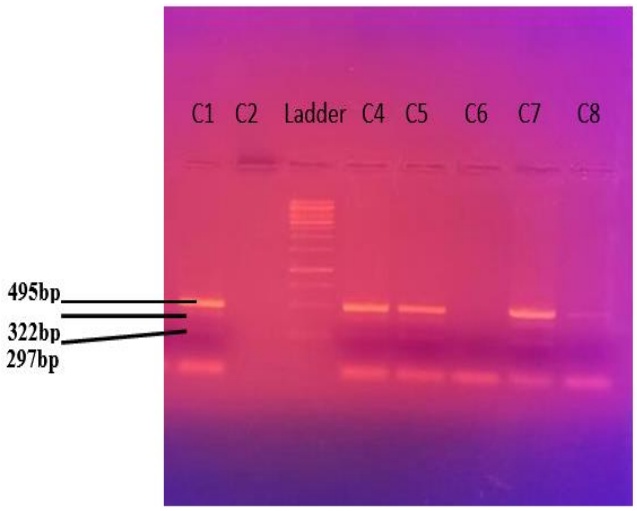

Figure 5. Representative agarose gel ((1.5%) showing SNP rs4784227 of SNPrs4784227 patients (P1-P11) in the well 7 the 1KB ladder is present

Table V: Allelic frequency of SNP rs4784227 of patients and controls

SNP	Allele	Patients		Controls	
		Frequency	Percentage	Frequency	Percentage
Rs4784227	C	0.54	54	0.58	58
	T	0.46	46	0.42	42

Table VI: Genotypic frequency of SNP rs4784227 of patients and controls

SNP	Genotype	Patients		Controls		P-Value
		Frequency	Percentage	Frequency	Percentage	
Rs4784227	CC	6	17.64	7	21.87	0.005
	TT	3	8.82	2	6.25	
	CT	25	73.52	23	71.87	

Sequence of SNP rs4784227 with its flanking region

16 dna: chromosome chromosome: GRCh38:16:52564876:52565676:1

TTGACCCAGGCAGTAGTTTAAAAAAGTTTTAATTTGTTGTTCACATTTAAAAACTGGACA

ATTTCTACATAAAAATCTGAATTACTCATGTCTCTTAAAAAAATAACATCTAGCAATGGT

AGGCCCACATTCCTTCCTGAAAATAATTAGCTGGGAAAGAGTAGGGACTGACCCCTTTAG

ACACGGTATAAATAGCATGGGAGTTGATCAGTAAATATTTGCTGAATGAAAGAATACATG

AATGAAAAGTCAGAGCCCTATAGGTCAGCATGGACGGCGGTAAAGGAACCTGGCTGAGCC

TGAAAGAGAATGTGATCTAAGATTAAATCCAGGATATGCTGGTAAATGTTTAACAGCCAA

CTCTTTGGGGAGGAAAAAAGTCCCAATTTGTAGTGTTTGC**C**GATTATTGTGATGTAAATA

CTCCCATCATGACCAATTTCAAGCTACCAACATGCTGACACTGAACTTGGAGTTGGAAGG

AGATGAACAGGCATAATCAGGTCTCGTGAGATGGCCCAAGCCGGCCCCAGCACTCCACTG

TTATATATGAGGCTAGAATTACTACATAACTGGAATAGCAACTTTCTGGACCATATGCCT

GGAACACAGCAGGTGCTGAATAAATGTTTGTTGATCCAGGAACTGACTGTGTTGAAGCCC

ACAGATGGGAAATCAGTAGAAGGCAGGTAAGAGTAAAAAGAAGGGCAGAGAATTGGGGGT

ACAGACCCCTGAACCATAAGTCAGAGGAATGTTGTACATGTTTTCAGATCCCTCACTGGT

CAAATGAAGGCAAAGGGTTAG

Risk factors

Age

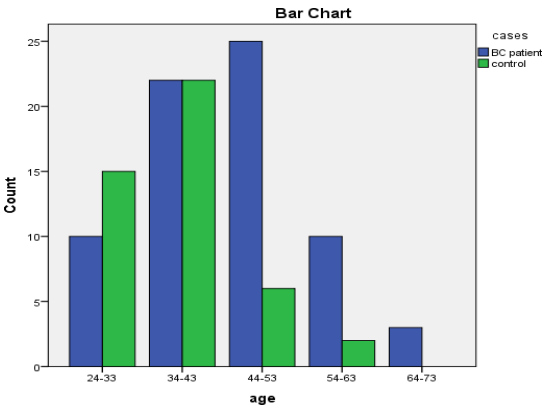

Figure 6. Graph showing association between age and BC Incidence

Family history of BC

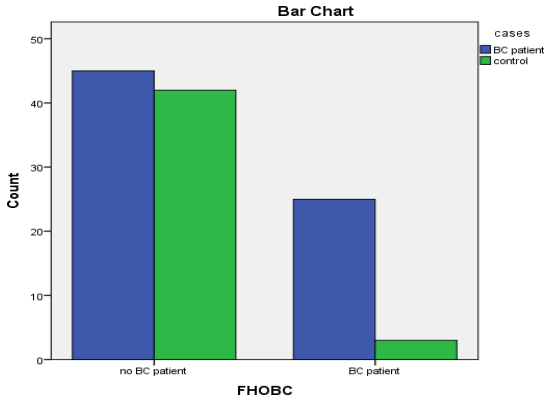

Figure 7. Graph showing association between family history of BC and BC

Family history of cancer

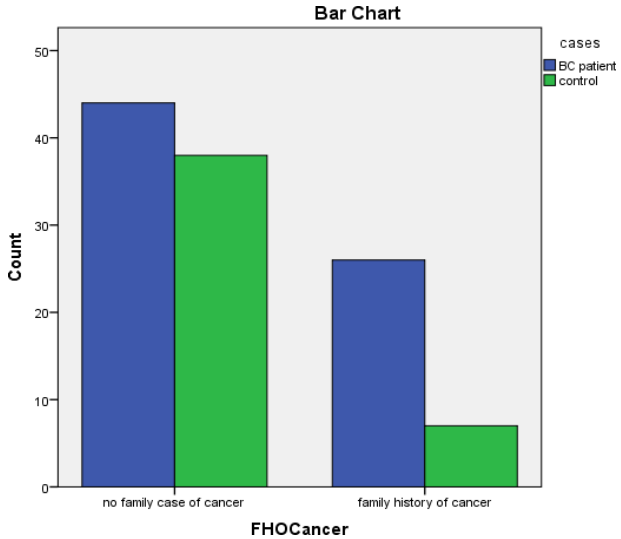

Figure 8. Graph showing no association between family history of other cancers and BC incidence

Menarche age

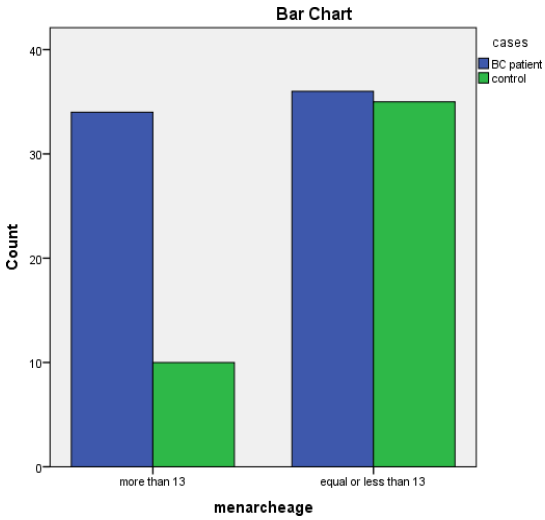

Figure 9. Graph showing association between menarche age and BC

Diet

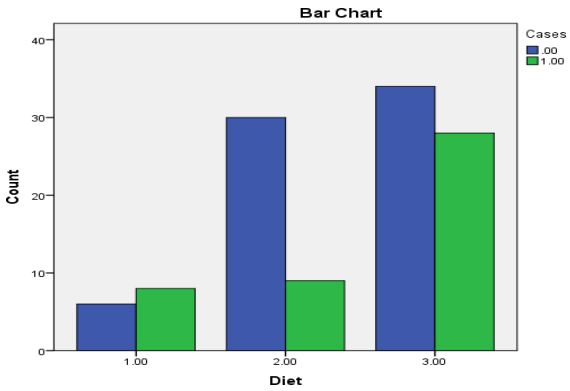

Figure 10. Graph showing association between diet and BC

Menopause age

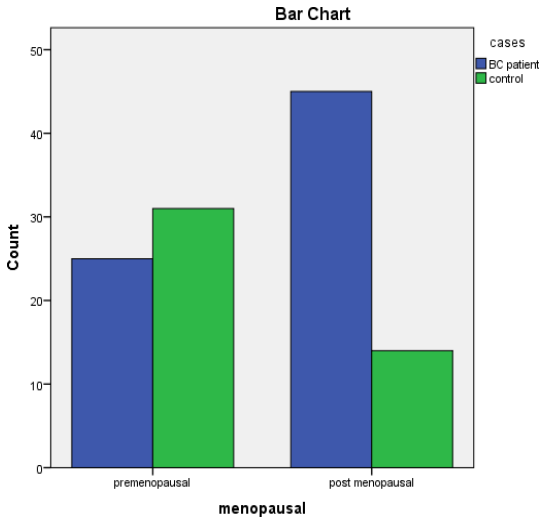

Figure 11. Graph showing association between menopause and BC

Marital status

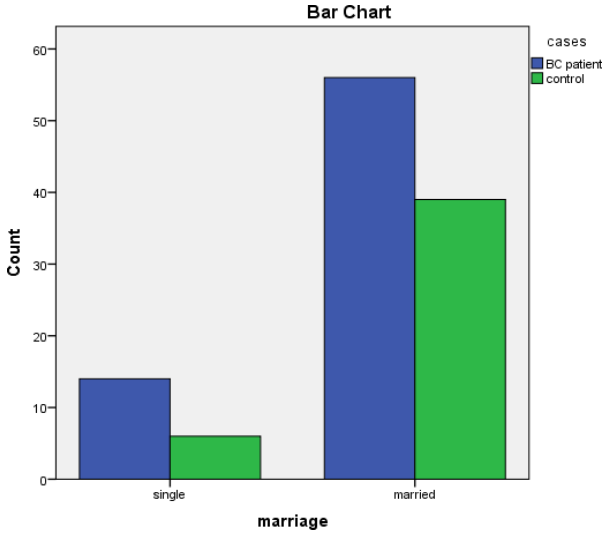

Figure 12. Graph showing no association between marriage and BC

Breast feeding

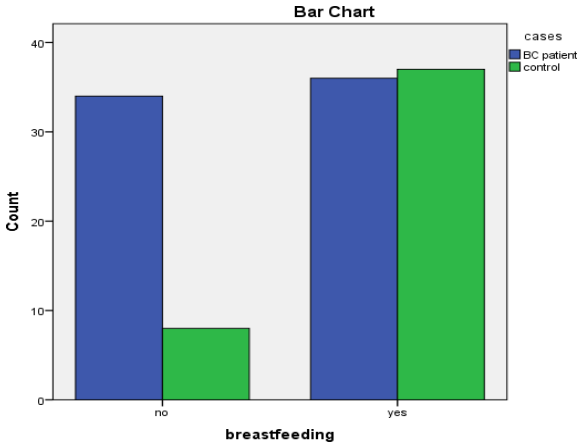

Figure 13. Graph showing association between breast feeding and BC

Any disease

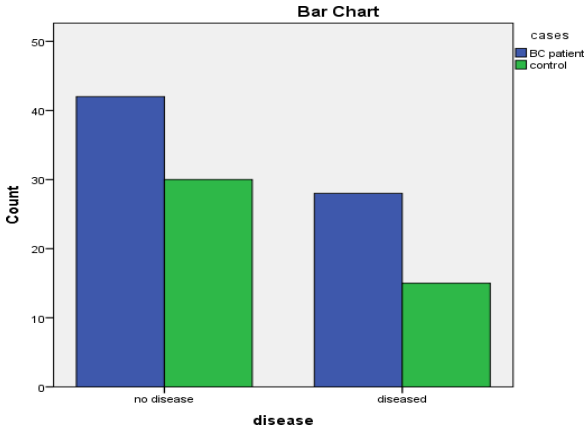

Figure 14. Graph showing no association between disease and BC

Smoking status

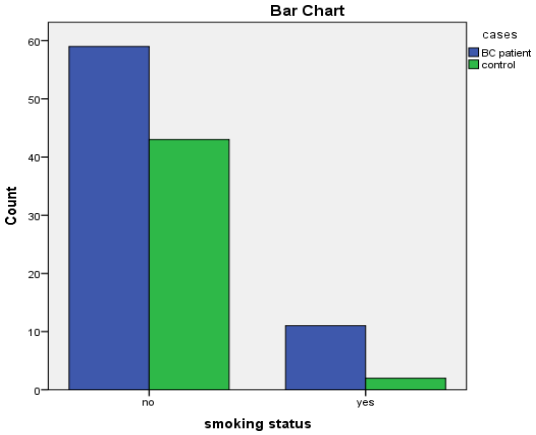

Figure 15. Graph showing no association between smoking status and BC

BMI

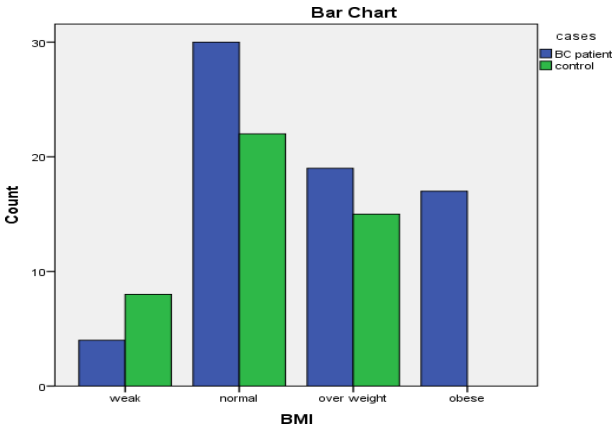

Figure 16. Graph showing association between BMI and BC

ER status

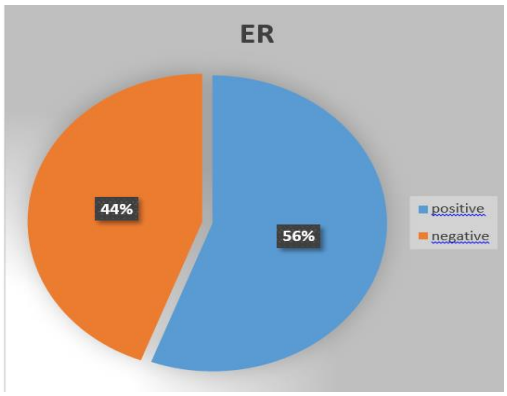

Figure 17. Pie chart showing ER percentages

PR status

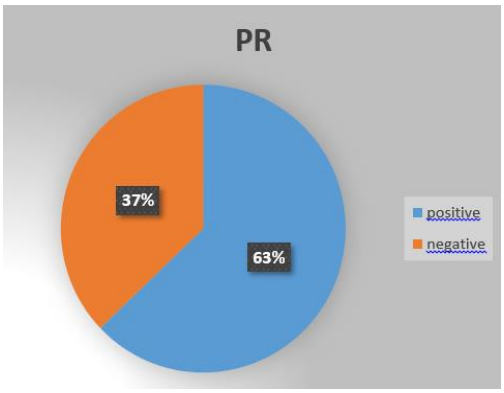

Figure 18. Pie chart showing PR percentages

HER2 status

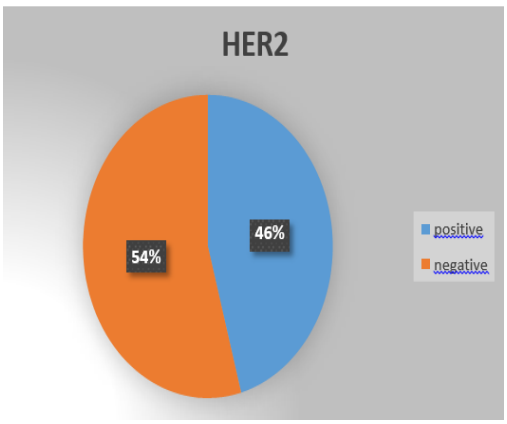

Figure 19. Pie chart showing HER2 percentages

Carcinoma of BC

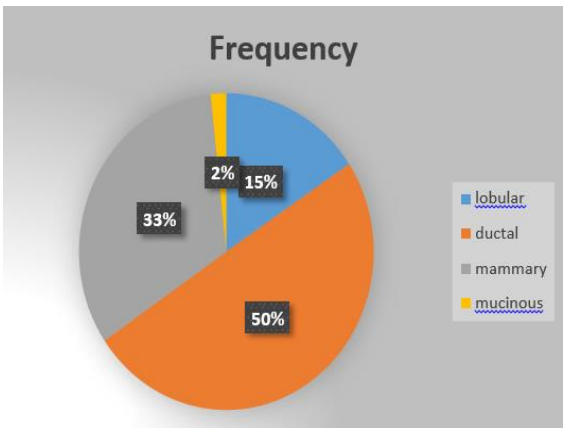

Figure 20. Pie chart showing types of carcinoma

BC stages

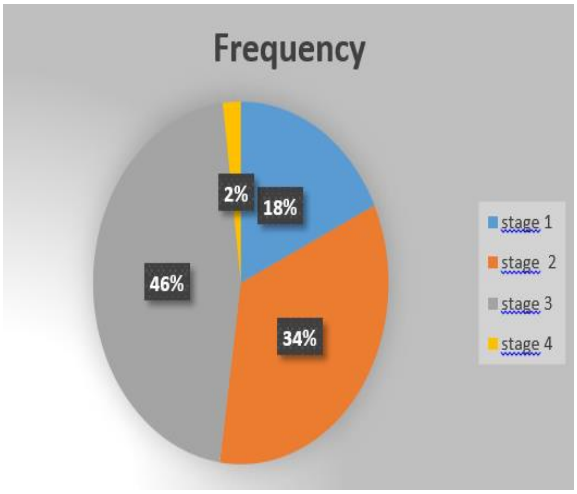

Figure 21. Pie chart showing BC stages

Tumor sizes

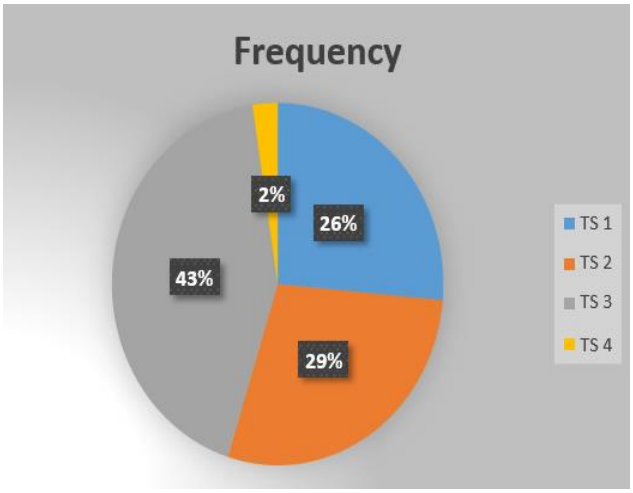

Figure 22. Pie chart showing tumor sizes of BC

Lymph node status

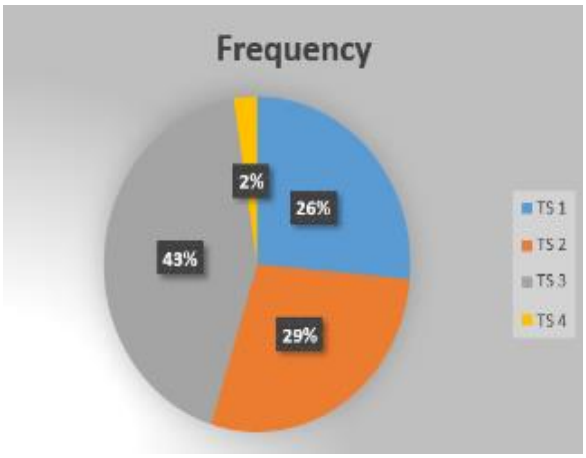

Figure 23. Pie chart showing lymph node status of BC

Metastasis of BC

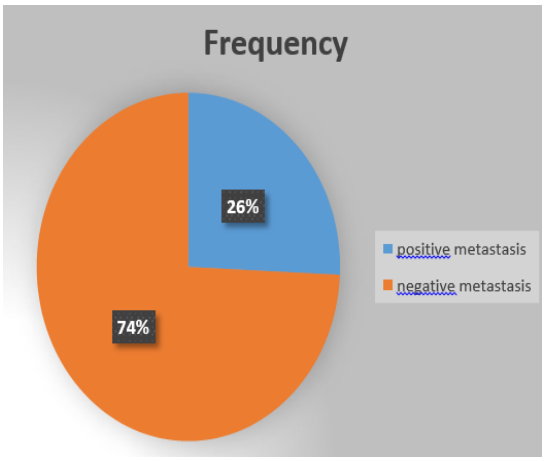

Figure 24. Pie chart showing metastasis of BC

DISCUSSION

Breast cancer (BC) is a complex disease characterized by the uncontrolled growth of malignant cells in breast tissue. It is the most commonly diagnosed cancer among women worldwide, with its incidence rising rapidly, particularly in lower-income countries where mortality rates are higher. The disease shows significant regional variation, with higher age-standardized rates (ASR) in developed regions like North America (99.4 per 100,000) compared to lower rates in less-developed areas such as Africa and Asia. In Pakistan, BC is the most common cancer among women, with a high incidence of younger patients presenting advanced stages. Studies show a strong association between family history, age, and BC risk, particularly in women with early menarche or late menopause, while factors like marital status and smoking showed no significant correlation. Additionally, factors such as breastfeeding, body mass index (BMI), and diet have been linked to BC risk. Breastfeeding for extended periods is associated with a reduced risk, while postmenopausal women with higher BMI levels face increased risk. Diet, particularly high consumption of processed and red meats, has also been implicated in raising BC risk. In contrast, certain genetic factors, such as the SNP rs4784227-CASC16, have shown conflicting associations with BC risk across different populations, highlighting the disease's genetic complexity. Overall, the data underscores the importance of genetic, lifestyle, and demographic factors in understanding and managing BC incidence and outcomes.

Conclusion

In conclusion breast cancer is a complex, heterogeneous disease with rising global incidence, particularly in developing regions like Pakistan. This study highlights the significant role of non-genetic risk factors, including family history, age, early menarche, late menopause, BMI, diet, and breastfeeding, in influencing breast cancer risk. Genetic factors, such as the CASC16-rs4784227 polymorphism were analyzed that shows a strong association was found with breast cancer in the studied Pakistani population. The findings underscore the need for a comprehensive approach that considers both genetic and lifestyle factors in understanding breast cancer risk and improving prevention and treatment strategies.

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BIBLIOGRAPHY

1. Ambrosone, C. B., Freudenheim, J. L., Graham, S., Marshall, J. R., Vena, J. E., Brasure, J. R. and Harrington, A. M., 1996. Cigarette smoking, N-acetyltransferase 2 genetic polymorphisms, and breast cancer risk. *Jama,* **276(18):** 1494-1501.

2. Amarante, M. K. and Watanabe, M. A. E., 2009. The possible involvement of virus in breast cancer. *Journal Of Cancer Research And Clinica Oncology*, **135(3):** 329-337.

3. Apter, D. A. N. and VIHKO, R.., 1983. Early menarche, a risk factor for breast cancer, indicates early onset of ovulatory cycles. *The Journal of Clinical Endocrinology & Metabolism*, **57(1):** 82-86.

4. Athma, P., Rappaport, R. and Swift, M., 1996. Molecular genotyping shows that ataxia-telangiectasia heterozygotes are predisposed to breast cancer. *Cancer Genetics And Cytogenetics,* **92(2):** 130-134.

5. Atlanta, G. A. C. S., 2008. American Cancer Society. *Cancer Facts & Figures*, **20(08):**12-31.

6. Bastiaannet, E., Liefers, G. J., De Craen, A. J. M., Kuppen, P. J. K., Van De Water, W., Portielje, J. E. A. and Westendorp, R. G. J. 2010. Breast cancer in elderly compared to younger patients in the Netherlands: stage at diagnosis, treatment and survival in 127,805 unselected patients. *Breast Cancer Research And Treatment*, **124(3):** 801-807.

7. Bernardo, G. M., Lozada, K. L., Miedler, J. D., Harburg, G., Hewitt, S. C., Mosley, J. D. and Abdul-Karim, F. W., 2010. FOXA1 is an essential determinant of ERα expression and mammary ductal morphogenesis. *Development,* **137(12):** 2045-2054.

8. Bernstein, L., Ross, R. K., Lobo, R. A., Hanisch, R., Krailo, M. D. and Henderson, B. E., 1987. The effects of moderate physical activity on menstrual cycle patterns in adolescence: implications for breast cancer prevention. *British Journal Of Cancer*, **55(6):** 681-685.

9. Bignell, G. R., Barfoot, R., Seal, S., Collins, N., Warren, W. and Stratton, M. R., 1998. Low frequency of somatic mutations in the LKB1/Peutz-Jeghers syndrome gene in sporadic breast cancer. *Cancer Research*, **58(7):** 1384-1386.

10. Boardman, L. A., Thibodeau, S. N., Schaid, D. J., Lindor, N. M., McDonnell, S. K., Burgart, L. J. and Hartmann, L. C. 1998. Increased risk for cancer in patients with the

Peutz-Jeghers syndrome. *Annals Of Internal Medicine*, **128(11):** 896-899.

11. Børresen, A. L., 1992. Role of genetic factors in breast cancer susceptibility. *Acta Oncologica*, **31(2):** 151-155.

12. Børresen, A. L., Andersen, T. I., Garber, J., Barbier-Piraux, N., Thorlacius, S., Eyfjörd, J. and Friend, S. H., 1992. Screening for germ line TP53 mutations in breast cancer patients. *Cancer Research*, **52(11):** 3234-3236.

13. Boardman, L. A., Thibodeau, S. N., Schaid, D. J., Lindor, N. M., McDonnell, S. K., Burgart, L. J. and Hartmann, L. C. 1998. Increased risk for cancer in patients with the Peutz-Jeghers syndrome. *Annals Of Internal Medicine*, **128(11):** 896-899.

14. Brody, J. G., Moysich, K. B., Humblet, O., Attfield, K. R., Beehler, G. P. and Rudel, R. A., 2007. Environmental pollutants and breast cancer: epidemiologic studies. Cancer: *Interdisciplinary International Journal Of The American Cancer Society*, **109(13):** 2667-2711.

15. Bray, F. and Møller, B., 2006. Predicting the future burden of cancer. *Nature Reviews Cancer*, **6(1):** 63-74.

16. Byrne, C., Brinton, L. A., Haile, R. W. and Schairer, C. 1991. Heterogeneity of the effect of family history on breast cancer risk. *Epidemiology*, **201(21):** 276-284.

17. Catsburg, C., Miller, A. B. and Rohan, T. E., 2015. Active cigarette smoking and risk of breast cancer. *International Journal Of Cancer*, **136(9):** 2204-2209.

18. Cowper-Sal, R., Zhang, X., Wright, J. B., Bailey, S. D., Cole, M. D., Eeckhoute, J. and Lupien, M., 2012. Breast cancer risk–associated SNPs modulate the affinity of chromatin for FOXA1 and alter gene expression. *Nature Genetics*, **44(11):** 1191-1198.

19. Couch, F. J., DeShano, M. L., Blackwood, M. A., Calzone, K., Stopfer, J., Campeau, L. and Cobleigh, M. A. 1997. BRCA1 mutations in women attending clinics that evaluate the risk of breast cancer. *New England Journal Of Medicine*, **336(20):** 1409-1415.

20. DeSantis, C., Siegel, R., Bandi, P. and Jemal, A., 2011. Breast cancer statistics, 2011, CA: *A Cancer Journal For Clinicians*, **61(6):** 408-418.

21. Denic, S. and Bener, A., 2001. Consanguinity decreases risk of breast cancer–cervical cancer unaffected. *British Journal Of Cancer*, **85(11):** 1675-1679.

22. DeSantis, C., Ma, J., Bryan, L. and Jemal, A., 2014. Breast cancer statistics, 2013. CA: *A Cancer Journal For Clinicians*, **64(1):** 52-62.
23. Dumitrescu, R. G. and Shields, P. G., 2005. The etiology of alcohol-induced breast cancer. *Alcohol*, **35(3):** 213-225.
24. Dunham, I., Birney, E., Lajoie, B. R., Sanyal, A., Dong, X., Greven, M. and Dekker, J., 2012. *An Integrated Encyclopedia Of DNA Elements In The Human Genome,* **19(8):** 714. Easton, D. F., Bishop, D. T., Ford, D. and Crockford, G. P., 1993. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. *American Journal Of Human Genetics*, **52(4):** 678.
25. Ferlay, J., Bray, B., Pisani, P. and Parkin, D. M., 2004. GLOBOCAN 2002: Cancer incidence, mortality and prevalence worldwide. IARC CancerBase NO. 5. version 2.0, Lyon, France: *Iarcpress***, 34(5):** 55-58.
26. Ghoussaini, M., Pharoah, P. D. and Easton, D. F., 2013. Inherited genetic susceptibility to breast cancer: the beginning of the end or the end of the beginning. *The American Journal Of Pathology,* **183(4):** 1038-1051