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Research Article

Genetic Association of *PvuII* & *XbaI* in *ESR1* with the Risk of Breast Cancer in the North Indian Population: A Case-Control Study

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Abstract
Background: Breast Cancer (BC) is the most prevalent cancer among women worldwide and is influenced by a combination of environmental and genetic factors. Among the genetic contributors, the estrogen receptor 1 (*ESR1*) gene and its associated polymorphisms, specifically *XbaI* and *PvuII*, have demonstrated variable risk patterns across different populations.
Aim: This study aims to investigate the relationship between the *XbaI* and *PvuII* genetic variants of the *ESR1* gene and the risk of developing BC in women from Haryana, North India.
Methods: A total of 200 participants (100 cases and 100 controls) were enrolled. Blood samples were collected for DNA extraction and genotyping using PCR-RFLP with *XbaI* and *PvuII* enzymes. Statistical analyses, including t-tests and odds ratios (OR), were performed to assess the associations, with a significance threshold of $p < 0.05$.
Results: The analysis revealed no significant age-related difference between the case and control groups ($p > 0.05$). Across all genetic models, the *XbaI* variant was not associated with BC risk. In contrast, the *PvuII* polymorphism showed significant associations in the dominant (OR = 2.0692; p = 0.0141) and over-dominant (OR = 1.9091; p = 0.0242) models, with a borderline trend in the allelic model (OR = 1.4839; p = 0.0605).
Conclusion: The *PvuII* polymorphism is significantly associated with increased BC risk in this population, while no significant association was found for *XbaI*.

Keywords: BC, *XbaI*, *PvuII*, genetic polymorphisms, risk factors

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Introduction

BC is among the most prevalent cancers globally, with considerable variation in incidence rates across different regions, ranging from lower levels in some countries to significantly higher levels in others (**GBD Compare | IHME Viz Hub**). In India, BC is the most common cancer among women, posing a significant burden on public health (**Madhav et al., 2018**). It is, however, critical to delve deeper into how the prevalence of BC is influenced by a complex interplay of environmental and genetic factors. One such critical genetic factor is *ESR1*, a gene that encodes the estrogen receptor alpha (ERα), playing a pivotal role in the pathogenesis of BC.

The *ESR1* gene, known for its high polymorphism, contains two key variants, rs2234693 (*PvuII*, T>C) and rs9340799 (*XbaI*, A>G), that have garnered significant attention in BCresearch (**Chattopadhyay et al., 2014; Ghali et al., 2018; Al-Eitan et al., 2019**). These genetic variations in intron 1 of the *ESR1* gene (Sudershan et al., 2024) are linked to increased BC susceptibility. While several studies have examined their association with BC risk in various populations (**Surekha et al., 2007; Shen et al., 2006; AL-Tahhan and Refaat, 2010**), no research has been conducted in Haryana, India. This study aims to explore the association between these *ESR1* polymorphisms and BC risk in women from Haryana, addressing a critical issue for the North Indian population.

Method

Ethical permission, Consent to Participant& Sample Collection

The study was conducted in accordance with ethical guidelines and received approval from the Institutional Ethics Committee (IEC) of Maharishi Markandeshwar Institute of Medical Science & Research, Mullana (MMIMSR), Ambala (Reference No: IEC/2349, Dated: 09-12-2022). All participants provided informed consent before being included in the study.

Patient recruitment occurred from January 2023 to June 2024 at the Department of Oncology, MMIMSR, Mullana, Ambala, using a convenience sampling method. Patients diagnosed with pre-operative BC (incident cases) were identified and approached for participation. An experienced oncologist (AKS) conducted detailed assessments. A "sample availability calculator" (**Sudershan et al., 2022**) was used to estimate participant availability.

Control subjects were selected from the hospital's general Outpatient Department (OPD) through hospital-based sampling. Controls were carefully screened to ensure they had no signs of BC, other cancers, or hereditary conditions. The study included 100 patients and 100 controls, maintaining a balanced 1:1 ratio and matching individuals by age to minimize group differences.

Blood samples were collected in 5 ml EDTA vials to preserve sample integrity and stored at -20°C for future genetic analysis. After collection, the samples were transported from MMIMSR, Ambala to the Molecular Genetic Lab at the Institute of Human Genetics, University of Jammu. Strict preventive measures were taken during transportation, including packing the samples in ice containers to preserve their quality and prevent degradation. Upon arrival at the lab, the samples were carefully verified for integrity and proper labeling to ensure accurate and reliable results in subsequent analyses.

DNA Isolation and Genotyping

Blood samples from both cases and controls were collected for DNA extraction using the standard Phenol-chloroform-isoamyl alcohol method. Following extraction, DNA quality was assessed by mixing each sample with 1 µl of 1× DNA loading dye containing bromophenol blue, glycerol, and EDTA, and loading it onto a gel for analysis. DNA concentration was measured using spectrophotometry to ensure accurate quantification for downstream analyses.

Pre-designed primers (**F:** 5'-CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC-3' & **R:** 5'-TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA -3') were employed to amplify the genotypes of the variants under investigation. The PCR reaction mixture (Forward Primer 0.5 µl, Reverse Primer 0.5 µl, Nuclease-Free Water 7 µl, Green Master Mix 7 µl, Genomic DNA 2 µl; Total Volume 17 µl) was optimized to maximize target DNA yield. The amplification was carried out using a series of temperature cycles: an initial denaturation at 94°C for 4 minutes, followed by 35 cycles consisting of denaturation at 94°C for 50 seconds, annealing at 62°C for 50 seconds, and extension at 72°C for 1 minute. A final extension step at 72°C for 7 minutes was performed to ensure complete extension of all DNA fragments. These conditions were carefully optimized to ensure efficient and specific amplification of the target variants for subsequent analysis.

Genotyping was performed using the RFLP method with the enzymes *XbaI* and *PvuII* for the selected variants. The reaction mixture consisted of Double Distilled Water (8 µl), Restriction Buffer (1.5 µl), Restriction Enzyme (0.5 µl), and PCR Product (6 µl). Genotyping was validated by randomly selecting samples for repeated testing. The PCR-RFLP products were analyzed by electrophoresis on a 3% agarose gel and visualized using the ChemiDoc XRS+ Gel Imaging System (**Figure 1A & B**).

Statistical Analysis

Statistical analysis was conducted to assess the relationship between genetic variants and BC risk. Genotype and allele frequencies were determined using descriptive statistics in Microsoft Excel, while demographic characteristics were summarized to characterize the study population. A t-test, performed using GraphPad, compared the means between the case and control groups. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using MedCalc to evaluate the strength of associations across different genetic models. A p-value of less than 0.05 was considered statistically significant, indicating a meaningful difference between the groups.

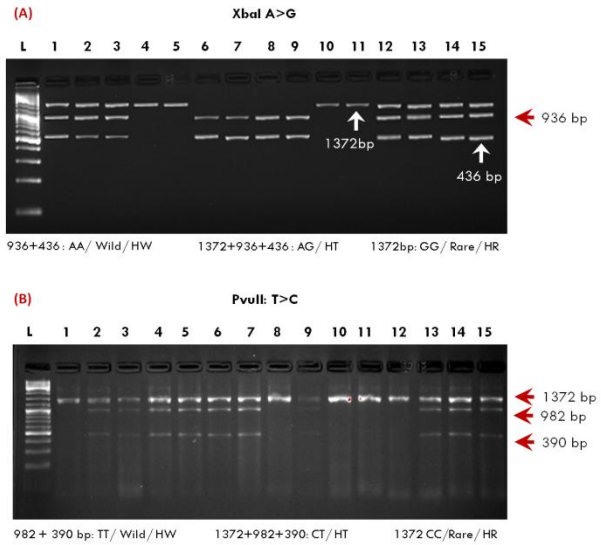

Figure 1A &B: L represents the 100 bp ladder. Wells 1-3 and 12-15 represent heterozygote (AG). Wells 4, 5, 10, and 12 represent homozygous (GG). Wells 6-9 represent homozygous wild-type (AA). **(B):** L represent the 100bp ladder, Well 1, 8, 10-12 showed Homozygous Recessive (CC) genotype, Well 2-7,9, & 13-15 showed heterozygous (CT)

Result

Demographic Profile

A total of 200 participants were included in this analysis, with 100 cases and 100 controls (1:1 ratio). The mean age of the cases was 55.05 ± 11.37 years, while the controls had a mean age of 52.64 ± 4.87 years. A t-test comparison showed no statistically significant age difference between the two groups ($p > 0.05$), suggesting that the age distribution was similar across both groups.

Genetic Association

After evaluating the demographic features, we proceeded with the genetic association analysis, starting with the Hardy-Weinberg Equilibrium (HWE) analysis. For the *XbaI* variant, the control group exhibited a higher frequency of homozygous wild-type genotypes (38% vs. 30%) and a higher wild-type allele frequency (0.63 vs. 0.565) compared to the case group. Both groups were in HWE ($p > 0.05$) (**Table 1**). Regarding the genetic association of the *XbaI* variant, none of the genetic models showed statistically significant associations with disease risk. The odds ratios varied from 1.1277 in the over-dominant model to 1.7944 in the codominant model. However, all p-values exceeded 0.05, indicating no significant association between the *XbaI* variant and disease risk across the tested models. Although the codominant model yielded the highest odds ratio (1.7944), suggesting a potential increased risk, the result was not statistically significant (**Table 2**).

Table 1: Genotypic and Allelic Frequencies of *XbaI* and *PvuII* Variants in Cases and Controls with Hardy-Weinberg Equilibrium Analysis

Variant	Subject Type	Genotypic frequency			HWE
		HW	HT	HR	p-value
XbaI	Case	30 (30.00%)	53 (53.00%)	17 (17.00%)	>0.05
	Control	38 (38.00%)	50 (50.00%)	12 (12.00%)	>0.05
PvuII	Case	30 (30.00%)	60 (60.00%)	10 (10.00%)	0.01
	Control	47 (47.00%)	44 (44.00%)	9 (9.00%)	0.1

For the *PvuII* variant, the control group displayed a higher frequency of homozygous wild-type genotypes (47% vs. 30%) and a higher wild-type allele frequency (0.69 vs. 0.60) compared to the case group. While the case group deviated from Hardy-Weinberg Equilibrium (HWE) ($p = 0.01$), the control group remained in equilibrium ($p > 0.05$). Chi-square analysis revealed a significant deviation from HWE in the case group for *PvuII*, unlike the *XbaI* variant, which showed no significant deviation (**Table 1**). Further analysis of the *PvuII* variant revealed significant associations with disease risk in the dominant model (OR = 2.0692; CI: 1.1578 to 3.6980; $p = 0.0141$) and the over-dominant model (OR = 1.9091; CI: 1.0882 to 3.3493; $p = 0.0242$). These findings suggest that certain genotypes of *PvuII* are significantly associated with a higher risk of disease. Additionally, the allelic model (OR = 1.4839; CI: 0.9827 to 2.2406; $p = 0.0605$) showed a near-significant trend, indicating that the recessive allele may also contribute to disease risk (**Table 2**).

Table 2: Association Analysis of Gene Variants *XbaI* and *PvuII* Under Different Genetic Models

Variant	Genetics Model	OR	CI	p-Value
XbaI	Recessive	1.502	0.67 to 3.33	= 0.31
	Dominant	1.4301	0.79 to 2.57	= 0.23
	OD	1.1277	0.64 to 1.96	= 0.67
	Allelic	1.3109	0.87 to 1.95	= 0.18
	HR vs HW	1.7944	0.74 to 4.3	= 0.19
	HR vs HT	1.3365	0.58 to 3.07	= 0.49
	HT vs HW	1.3427	0.72 to 2.48	= 0.34
PvuII	Recessive	1.12	0.43 to 2.89	= 0.80
	Dominant	2.0692	1.15 to 3.69	= 0.01
	OD	1.9091	1.08 to 3.34	= 0.02
	Allelic	1.4839	0.98 to 2.24	= 0.06
	HR vs HW	1.7407	0.63 to 4.78	= 0.28
	HR vs HT	0.8148	0.30 to 2.17	= 0.68
	HT vs HW	2.1364	1.17 to 3.89	= 0.01

Discussion

BC's high prevalence is influenced by both environmental and genetic factors, with the *ESR1* gene playing a key role in its pathogenesis. The rs2234693 (*PvuII*) and rs9340799 (*XbaI*) polymorphisms, located in intron 1 of the gene, have been linked to increased

BC susceptibility. These variations are commonly studied for their association with BC risk (**Surekha et al., 2007; González-Mancha et al., 2008; Houtsma et al., 2021; Shen et al., 2006; AL-Tahhan and Refaat, 2010**). This analysis aims to evaluate the relationship between these polymorphisms and BC risk in women from Haryana, representing the north Indian population.

In the present study, the *XbaI*variant showed no significant association with BC risk in women from the North Indian population. However, significant associations were observed between the *PvuII*variant and disease risk in both the dominant and over-dominant models, with the allelic model indicating a near-significant trend, suggesting the potential contribution of the recessive allele to disease risk.

Several studies have explored the relationship between the *XbaI* and *PvuII* polymorphisms and BC risk, yielding varying results across different populations. For instance, Lu et al. (2005) found that the *XbaI* rare allele was associated with a reduced risk of BC, particularly in postmenopausal women. In contrast, the *PvuII* polymorphism showed no significant association with BC risk in their study population. Several other studies have also reported diverse findings related to the *PvuII* polymorphism. Surekha et al. (2007) found a significant association between the *PvuII* rare allele with increased BC risk, particularly in premenopausal women. Similarly, González-Mancha et al. (2008) suggested that the *PvuII* rare allele might serve as a germline risk factor for familial BC and was associated with specific tumor phenotypes, though it did not affect disease-free survival. Further research by Shen et al. (2006) observed that the *PvuII* and *XbaI* polymorphisms, possibly in linkage disequilibrium, were associated with a non-significant increase in BC risk in a Chinese cohort. In another study, AL-Tahhan and Refaat (2010) found that the *PvuII* polymorphism significantly increased BC risk in an Egyptian population, while the *XbaI* polymorphism showed no notable association.

While many studies highlight an increased risk of BC (BC) among women in various populations, some intriguing research has shown a reduced risk, prompting further exploration into the genetic factors involved. For instance, Atoum and Alzoughool (2017) found a significant association between the *XbaI* polymorphism and a decreased BC risk, suggesting it might act as a protective factor in the Jordanian population. Interestingly, while they did not find a direct link between the *PvuII* polymorphism and BC risk, a combined analysis of both *PvuII* and *XbaI* revealed a significantly reduced risk of BC, raising the possibility of a synergistic protective effect. Further adding to this complexity, Houtsma et al. (2021) uncovered that the *PvuII* variant was linked to improved overall survival (OS) and disease-free survival (DFS) in postmenopausal, hormone receptor-positive patients treated with exemestane. This suggests that *PvuII* may have not only a risk-modifying effect but also a potential prognostic role in BC outcomes. In another study, Ramalhinho et al. (2013) demonstrated that the *XbaI* polymorphism was associated with a significantly reduced BC risk in Portuguese women, particularly in those carrying the homozygous and heterozygous *X* allele. Although *PvuII* was linked to a non-significantly reduced risk, the combined *PvuII* and *XbaI* polymorphisms revealed a striking reduction in BC risk in individuals. Moreover, their findings suggested a fascinating interaction between these polymorphisms, as they were found to be in linkage disequilibrium, hinting at a complex genetic interplay that could shape BC susceptibility. On the other hand, Madeira et al. (2014) uncovered a different aspect, showing that the *PvuII* polymorphism was significantly associated with an increased risk of BC, especially in cases of estrogen receptor alpha (ERα)-positive BC. Interestingly, the *pp* and *xx* genotypes were exclusively found in patients who responded to tamoxifen therapy, suggesting that the *P* and *X* alleles might not only influence cancer risk but also enhance chemotherapy responsiveness.

These diverse findings ranging from protective genetic factors to those that may increase BC risk underline the complexity of BC genetics. They spark curiosity about the interplay between different genetic variants and their potential to modify both the risk and prognosis of BC across populations. Could certain polymorphisms be shielding women from BC, while others may make them more vulnerable or even influence their response to treatment? The answers may lie in these intricate genetic interactions, waiting to be further explored.

Strengths, Limitations, and Future Perspectives

This study focuses on women from Haryana, North India, providing valuable insights into an underrepresented population. The use of PCR-RFLP ensures accurate genotyping, and the inclusion of cases and controls enhances validity. However, the small sample size (200 participants) limits generalizability, and confounding factors like environment and lifestyle were not explored. Future research should involve larger, diverse cohorts and investigate gene-environment interactions, with longitudinal studies to establish causal links between genetic polymorphisms and breast cancer risk.

Conclusion

This study examined the *XbaI* and *PvuII* genetic variations in relation to BC risk in women from Haryana, North India. While no significant link was found for *XbaI*, the *PvuII* variation was strongly associated with increased BC risk in specific genetic models. Further research with larger cohorts is needed to confirm these findings and explore genetic and environmental contributions to BC risk.

Abbreviation

OMIM - Online Mendelian Inheritance in Man
ERα - Estrogen Receptor Alpha
ESR1 - Estrogen Receptor 1
SNP - Single Nucleotide Polymorphism
PCR - Polymerase Chain Reaction
RFLP - Restriction Fragment Length Polymorphism
HWE - Hardy-Weinberg Equilibrium
OR - Odds Ratio

CI - Confidence Interval
BMI - Body Mass Index
OPD - Outpatient Department
IEC - Institutional Ethics Committee

Declaration

Statement of Ethics & Consent to Participate: The study was conducted per ethical standards, with approval from the Institutional Ethics Committee, MMIMSR, Mullana, Ambala (Ref: IEC/2349, 09-12-2022). Written informed consent was obtained from all participants.

Conflict of Interest Statement: No conflict of interest.

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Data Availability Statement: All data from this study are included in the article. Further inquiries can be directed to the corresponding author.

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Author Contributions: **PK** and **SM** conceptualized the study and provided supervision; **SP**diagnosed the patients, **IB** prepared all the data; **AS** and **PK** conducted all the statistical analysis and interpretation; **IB &AS** drafted the manuscript, **KB**edited pictures and tables; **SP**,**KB** &**PK** edited the manuscript; **PK** and **SM** finalized the manuscript. All authors provided critical feedback on drafts and approved the final manuscript.

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