RESEARCH ARTICLE

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Association of ESRα-PvuII and ESRα-XbaI Polymorphisms with Breast Cancer Susceptibility in an Iranian Population: A Hospital-Based Case-Control Study

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Abstract

Background: Genetic variations in estrogen receptor genes are implicated in the development of breast cancer (BC). Among these, the ESR1 PvuII and XbaI polymorphisms have been frequently evaluated, but findings remain inconsistent. This study aimed to examine the association of the PvuII and XbaI polymorphisms with breast cancer risk in an Iranian female population. **Materials and Methods:** A case–control study was conducted on 100 breast cancer (BC) patients and 100 healthy, age-matched controls. Genotyping of the ESR1 PvuII (rs2234693 T>C) and XbaI (rs9340799 A>G) polymorphisms was performed using PCR-RFLP, with results validated by direct DNA sequencing. Associations between genotypes, clinicopathological features, and disease risk were analyzed. **Results:** Nominal differences in genotype distributions were observed. After applying multiple-testing correction across the pre-specified models, several associations remained statistically significant; however, marked deviations from Hardy–Weinberg equilibrium (HWE) in controls warrant cautious interpretation. **Conclusion:** Given the hospital-based design, small sample size, and significant deviations from HWE in controls, the observed ESR1 associations should be considered hypothesis-generating rather than confirmatory. Replication in larger, population-based Iranian cohorts with orthogonal genotyping approaches is required before any clinical implications are drawn.

Keywords: Breast cancer- Estrogen receptor- Pvull- Xbal- Single nucleotide polymorphism

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Introduction

Breast cancer (BC) is a complicated and heterogeneous ailment which comprise the most prevalent malignancy and the second cause of the cancer related death in the female population worldwide [1, 2]. Breast malignancies are diagnosed by a hard cancerous mass in the breast which may spread to the axillary lymph nodes and eventually throughout the whole body [3]. While the infiltrating ductal carcinoma (IDC) makes up about 80% of BC cases, invasive lobular carcinoma (ILC) type includes 10-15% of diagnosed patients, and the rest are classified as non-invasive lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS) types which remain localized to the lobules or ducts [4].

The etiology of BC is attributed to the multiple interactive reasons, including epidemiological risk factors, genetic alterations, and hereditary elements [5, 6]. Survival rate of patients has significant correlation with early diagnosis and appropriate timely therapies [7]. With regards to the increased prevalence and mortality rate of BC in recent years, prognosis evaluation or early-stage detection is a critical measure to prevent the occurrence and reduce the mortality of disease [8, 9].

It is well-known that various genetic changes in multiple genes are associated with the incidence of BC [10, 11]. In recent years, genetic profiling of BC tumors has provided precise information toward predictive and prognostic markers in this regard [12]. Single nucleotide polymorphisms (SNPs) are one of the major classes of genetic factors which implicates in susceptibility to BC and characterize inter-individual discrepancy in BC risk and outcome [13, 14]. SNPs can affect a wide number of cell functions, attenuate innate protective mechanisms, and increase the probability of malignancy induced by carcinogenic agents [15, 16]. Therefore, tracing and identification of potential SNPs may play a critical role in the prognosis and applying preventive strategies, early

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diagnosis, and appropriate treatments which subsequently lessen the fatality rate of BC [17, 18].

Hormone dependent breast cancer (HDBC) is the major type of BC in both women and men, which account for two-thirds of new diagnosed BC cases. The hallmark of HDBC which is also referred to as hormone receptor-positive (HR positive) BC is the expression of ovarian steroid hormones (e.g., estrogen and progesterone) receptors, especially estrogen receptor [ER] [19, 20]. These endocrine messenger molecules play a pivotal role in growth and proliferation of luminal epithelial cells of the normal mammary gland. Therefore, genetic variations that lead to elevated levels of these hormones or high expression of their receptors are associated with an increased risk of developing BC [21]. ER gene harbors several variants which may lead to the change in gene expression pattern and correlated with BC susceptibility. Among them, the variant alleles of two SNPs, rs2234693 (Pvull; T>C, chr6:151842200) and rs9340799 (Xbal: A>G, chr6:151842246) are associated with a high risk of BC [22, 23]. Identification of these polymorphisms can be considered as a preventive measures to reduce the risk of BC. Accordingly, we conducted a case-study analysis in an Iranian women population with breast cancer living in the Kashan city to investigate the association of ESRα-Pvull and ESRα-Xbal polymorphisms with BC risk.

Materials and Methods

Study population

This clinical base case-control research was conducted in Shahid Beheshti Hospital of Kashan, Kashan, Iran. The study included 200 female participants, 100 patients who were diagnosed with breast cancer and 100 healthy females as the control group. Informed consent document was signed by all participants after being informed about the research process. All patients were pathologically confirmed sporadic breast cancer, and clinical pathological data including histological grade, tumor type and size, ER/ progesterone receptor (PR)/ human epidermal growth factor receptor 2 (HER-2) status, and lymph node metastasis (LNM) were derived from the Electronic Medical Records of Shahid Beheshti Hospital (Table 1). All participants provided written informed consent prior to enrollment. The study protocol was approved by the Research Ethics Committee of Islamic Azad University, Qom Branch (Approval ID: IR.IAU.QOM.REC.1398.030). All procedures adhered to the Declaration of Helsinki (latest revision).

The control group was age-matched with the BC patients in the experimental group, without any history of malignancy or any other chronic disease.

Control selection and potential bias

Controls were recruited from hospital visitors without prior malignancy or chronic disease. As a hospital-based sample, controls may not perfectly represent the source population, and healthcare-access or referral patterns could introduce selection biases. We address this limitation in the Discussion.

Table 1. Demographic and Pathological Characteristics of the Study Participants

Variables	Description
Age (years) mean±SD	44.51±6.68
BMI (Kg/m^2) mean±SD	25.89±4.99
Menopausal status	
Premenopausal	50 (50.00%)
Postmenopausal	50 (50.00%)
Tumor type	
IDC	79 (79.00%)
ILC	18 (18.00%)
IDC+ILC	3 (3.00%)
Histological grade	
I	55 (55.00%)
II	21 (21.00%)
III	6 (6.00%)
Not identified	18 (18.00%)
Tumor size	
< 2 cm	65 (65.00%)
≥ 2 cm	35 (35.00%)
Estrogen receptor	
Positive	69 (69%)
Negative	31 (31.00%)
Progesterone receptor	
Positive	59 (59.00%)
Negative	41 (41.00%)
Her-2/neu	
Positive	30 (30.00%)
Negative	70 (70.00%)
Lymph node metastasis	
No	46 (46.00%)
Yes	54 (54.00%)

DNA extraction and PCR reaction

The genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using a DNA isolation kit according to the manufacturer's protocol (Sinaclon, Iran). The quality and quantity of extracted DNA were evaluated by gel agarose electrophoresis and NanoDrop spectrophotometer (Tecan M200). The specific primer sequences for amplification of fragments containing PvuII and XbaI polymorphisms are listed in Table 2.

The PCR reaction was conducted in a total volume of 50µl. ER-PvuII polymorphism amplification was performed under the following conditions: 5 min at

Table 2. Primer Sequences for Amplification of Fragments Containing PvuII and XbaI Polymorphisms

SNP		Sequence	Product size
ESR-Pvull	F	AGTCTTGCTATATTGTGCAGGC	369
	R	GAACCATTAGAGACCAATGCTC	
ESR-Xbal	F	GGAGTCTTGCTATATTGTGC	509
	R	GAGGGGAAATTGTTTATTGC	

94°C, followed by 40 cycles of 40 sec at 94°C, 40 sec at 62.5°C, 40 sec at 72°C, and then 5 min at 72°C. PCR reaction for ESR-XbaI polymorphism was also done with the following temperature program: 5 min at 94°C, followed by 40 cycles of 45 sec at 94°C, 45 sec at 58.7°C, 40 sec at 72°C, and then 5 min at 72°C. PCR products were analyzed by 2% agarose gel and the presence of multiallelic polymorphism was investigated by considering specific bands under UV light.

Genotype determination

All specimens were genotyped for rs2234693 T>C and rs9340799 A>G by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) technique. For this purpose, the PCR products were digested with restriction enzymes, with recognition sequence at the 5′...CAG/CTG...3′ and 5′...T/CTAGA...3′ for PvuII and XbaI, respectively. The enzymatic digestion reaction consists of 4µl PCR product, 0.4µl restriction enzyme, 0.6µl of 10x buffer (buffer G for PvuII enzyme and Tango buffer for XbaI enzyme) in a final volume of 10µl. The mixture was incubated at 37°C for 16 hours to complete enzymatic digestion and then placed at 60°C for 10 minutes to inactivate the enzyme. DNA fragments were stained with ethidium bromide, separated by agarose gel electrophoresis, and visualized under UV light.

DNA sequencing

PCR–RFLP genotyping results were confirmed by direct DNA sequencing on PCR products (three PCR amplicons per RFLP pattern). Each PCR product was sequenced in forward direction, and sequencing information was analyzed using Chromas Sequence chromatogram viewer (http://www.technelysium.com.au/chromas_lite.html).

Genotyping quality control

Call rate was 100% for both loci with no missing genotypes (Table 3). Representative amplicons from each PCR-RFLP pattern (three per pattern) were confirmed by Sanger sequencing. No inter-run duplicate genotyping or inter-laboratory validation was performed, which is acknowledged as a limitation.

Statistical analysis

Statistical analysis was conducted by SPSS version 22 software (SPSS Inc., Chicago, IL, USA). Statistical differences between quantitative and qualitative variables among case and control groups were evaluated by independent sample t-test and chi-square test, respectively. Chi-square test was also applied for comparison of different allele frequencies and genotypes. The logistic regression analysis was used to evaluate the association between PvuII and XbaI polymorphisms and the risk of breast cancer. $P<0.05$ was considered significant. Hardy–Weinberg equilibrium (HWE) was assessed in the control group using a 1-df chi-square goodness-of-fit test ($\alpha=0.05$). HWE was also checked in cases as a sensitivity analysis; deviations were noted and considered in interpretation. Multiple-testing was addressed using both Bonferroni (family-wise $\alpha=0.05/8\approx0.006$ across eight prespecified tests in Table 3) and the Benjamini–Hochberg false discovery rate (FDR). All tests were two-sided, and odds ratios (ORs) with 95% confidence intervals (CIs) are reported. In addition to unadjusted p-values, Bonferroni-corrected p-values (p_Bonf) were computed as p_Bonf = min(p × 8, 1.0000) across the eight prespecified tests (Table 3), alongside FDR q-values (Benjamini–Hochberg).

Power and sample size

We conducted post hoc power calculations for a

Table 3. Association of ESR1 PvuII (rs2234693) and XbaI (rs9340799) with Breast Cancer Risk. ORs (95% CIs) from two-sided logistic models. Multiplicity addressed by Bonferroni (p_Bonf) across eight prespecified tests and FDR (Benjamini–Hochberg, q). HWE p (controls): PvuII=4.0×10^{-6}; XbaI=4.7×10^{-7}.

Pvull polymorphism (rs2234693) Genotype /Allele	Case (n=100)	Control (n=100)	OR (95% CI)	p- value	p_Bonf (Bonferroni-adjusted)	q (FDR-BH)
CC	20 (20%)	8 (8%)	-	-	-	-
CT	55 (55%)	72 (72%)	0.31 (0.13-0.76)	0.0092*	0.0736	0.0184
TT	25 (25%)	20 (20%)	0.50 (0.18-1.37)	0.1781	1	0.2035
CT+TT	80 (80%)	92 (92%)	0.35 (0.15-0.83)	0.0177*	0.1416	0.0245
C	95 (47.5%)	88 (44%)	-	-	-	-
T	105 (52.5%)	112 (56%)	0.87 (0.59-1.29)	0.4824	1	0.482

Note: OR, odds ratio; CI, confidence interval; *Significant differences between cases and controls. HWE p (controls): PvuII = 4.0×10^{-6}; XbaI = 4.7×10^{-7}.

Xbal polymorphism (rs9340799) Genotype /Allele	Case (n=100)	Control (n=100)	OR (95% CI)	p- value	p_Bonf (Bonferroni-adjusted)	q (FDR-BH)
GG	41 (41 %)	15(15%)	=	=	-	-
GA	52 (52%)	75 (75%)	0.25 (0.13-0.51)	0.0001*	0.0008	0.0004
AA	7 (7%)	10 (10%)	0.26 (0.08-0.79)	0.0184*	0.1472	0.0245
AA+GA	59 (59%)	85 (85%)	0.25 (0.13-0.50)	0.0001*	0.0008	0.0004
G	134 (67%)	105 (52.5%)	= -	=	=	=
A	66 (33%)	95 (47.5%)	0.54 (0.36-0.82)	0.0032*	0.0256	0.0085

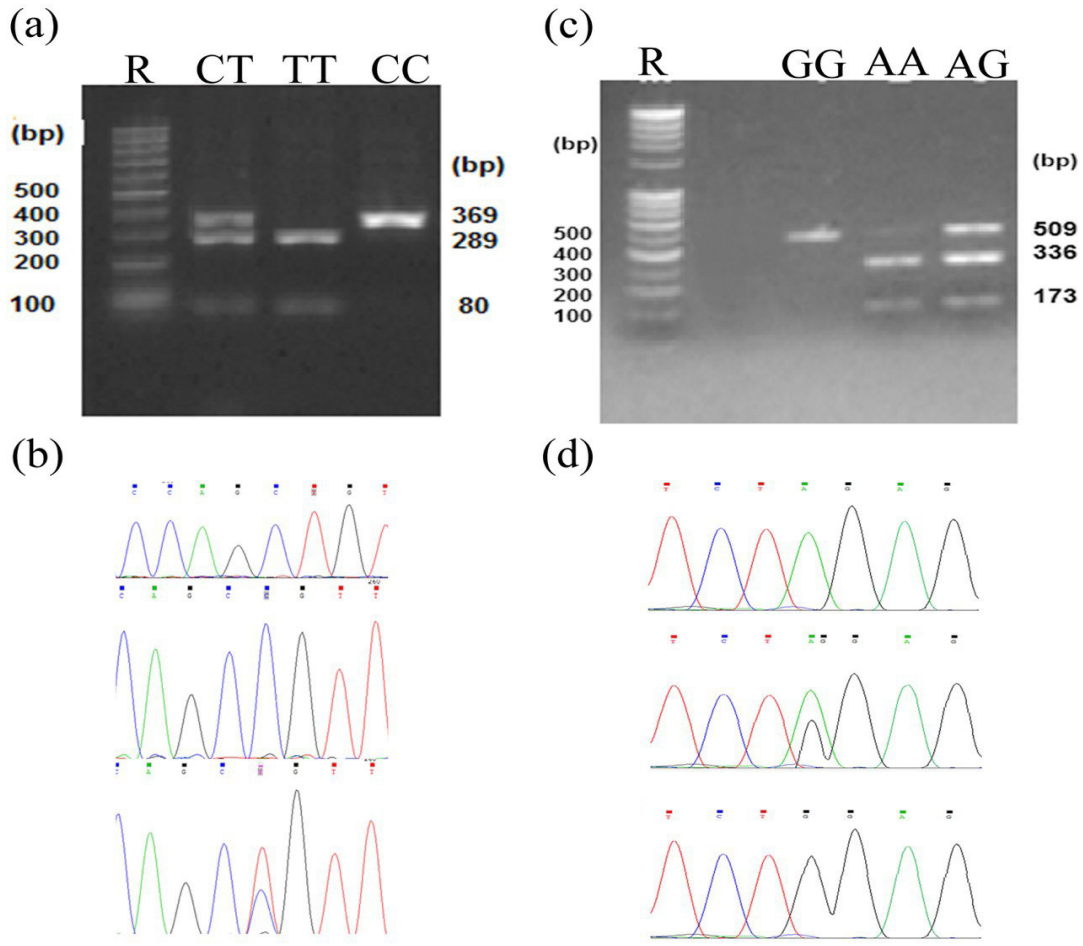

Figure 1. PvuII and XbaI Genotyping and Sanger Validation. (a) PCR–RFLP bands for PvuII arrows mark diagnostic fragments (TT: 369 bp; TC: 369/289/80 bp; CC: 289/80 bp). Lane order (left to right): R, TC, TT, CC. (b) PvuII chromatograms arrows indicate the variant position: T (TT), T/C (TC), C (CC). (c) PCR–RFLP bands for XbaI arrows mark diagnostic fragments (GG: 509 bp; GA: 509/336/173 bp; AA: 336/173 bp). Lane order (left to right): R, GG, AA, GA. (d) XbaI chromatograms arrows indicate the variant position: G (GG), G/A (GA), A (AA). R = 100-bp DNA ladder.

1:1 case–control design (α=0.05, two-sided) using the observed control allele frequencies (PvuII T≈0.56; XbaI A≈0.48; Table 3). With 100 cases and 100 controls, power to detect odds ratios (ORs) ≤1.5 was <50% for both loci, and ~60–75% for ORs around 1.7–2.0 depending on minor allele frequency. Consequently, our study is underpowered for modest genetic effects and results should be interpreted as exploratory.

Results

Demographic and pathological characteristics in breast cancer patients

According to data presented in Table. 1, the risk factors for both premenopausal and postmenopausal BC cases were found to be similar, except for late menopause in postmenopausal patients. Approximately 79% of tumors were IDC type and 18% of them were ILC type. Overexpression of ER and PR was observed in 69% and 59% of patients respectively, and compared to ER-negative, ER-positive tumors were significantly associated with histological grade 1 (P = 0.0003). Only 34% of ER-positive patients (12/35) were diagnosed with tumors larger than 2 cm, and 66% of them (43/65) had tumor sizes smaller than 2 cm. No significant relationship was found between people with overexpression of estrogen and progesterone receptors and lymph node metastasis.

PvuII and XbaI polymorphisms analysis and DNA sequencing

PvuII (rs2234693). All three genotypes (TT, TC, CC) were identified across the 200 participants, yielding the expected PCR–RFLP bands TT: 369 bp; TC: 369/289/80 bp; CC: 289/80 bp (Figure 1a–b). Sanger chromatograms show single clean peaks in homozygotes and a clear, symmetric double peak at the polymorphic position in heterozygotes. XbaI (rs9340799). Likewise, all three genotypes (GG, GA, AA) were observed GG: 509 bp; GA: 509/336/173 bp; AA: 336/173 bp (Figure 1c–d). Sequencing traces confirmed the expected base calls at the variant site, with heterozygous GA displaying the characteristic double peak.

Genotyping performance and internal validation. Call rate was 100% for both loci with no missing genotypes. Representative amplicons from each PCR–RFLP pattern (three per pattern) were verified by Sanger sequencing

with 100% concordance to the RFLP calls; no inter-run duplicates or inter-laboratory validation were performed.

Genotype distributions by case–control status

Genotype and allele counts/percentages for cases and controls are summarized in Table 3. These descriptive results are carried forward to the association analyses in §3.3, where we also report HWE testing in controls and multiplicity-adjusted p-values.

Statistical analysis of PvuII (rs2234693) and XbaI (rs9340799)

Hardy–Weinberg equilibrium

Control genotypes deviated markedly from HWE for both loci (PvuII: $\chi^2(1)=21.26$, $p=4.0\times10^{-6}$; XbaI: $\chi^2(1)=25.38$, $p=4.7\times10^{-7}$), driven by heterozygote excess. Case genotypes did not deviate significantly (PvuII: $\chi^2(1)=1.06$, p=0.304; XbaI: $\chi^2(1)=3.10$, p=0.079).

Unadjusted and adjusted associations

As shown in Table 3, PvuII CT vs CC (OR=0.31, 95% CI 0.13–0.76, p=0.0092, p_Bonf=0.0736, q=0.0184) and (CT+TT) vs CC (OR=0.35, 95% CI 0.15–0.83, p=0.0177, p_Bonf=0.1416, q=0.0245) were significant under FDR but not Bonferroni. For XbaI, GA vs GG (OR=0.25, 95% CI 0.13–0.51, p=0.0001, p_Bonf=0.0008, q≈0.0004), (GA+AA) vs GG (OR=0.25, 95% CI 0.13–0.50, p=0.0001, p_Bonf=0.0008, q≈0.0004), and the A vs G allele contrast (OR=0.54, 95% CI 0.36–0.82, p=0.0032, p_Bonf=0.0256, q≈0.0085) remained statistically significant after multiplicity correction, whereas AA vs GG met FDR (q≈0.0245) but not Bonferroni (p_Bonf=0.1472). Given the pronounced HWE deviations in controls, all associations should be interpreted cautiously.

Exploratory subgroup analyses

Exploratory cross-tabulations of genotypes with tumor size and lymph node status are presented in Supplementary Table 1. Given the limited sample and further subdivision, these analyses are underpowered and were not adjusted for multiplicity; therefore, no inferential claims are made.

Discussion

It is well-known that ovarian steroid hormones, including estrogens, are correlated with BC development in premenopausal and postmenopausal females [20]. Whether from endogenous or exogenous sources, estrogens are identified as carcinogens due to their stimulatory effect on the proliferation of normal or mutated breast cells [24]. Human estrogen receptor (ER) comes in two main form including ERα and ERβ. The coding gene of ERα which involves in breast cancer-promoting effects of estrogens is located at 6q25.1 and contains 8 exons [11].

Since ER acts as a hormone-regulated nuclear transcription factor, ER gene variations play a pivotal role in BC susceptibility and highlight inter-individual variation role toward the BC risk and outcome [25]. PvuII (rs2234693) and XbaI (rs9340799), the most studied ERα gene polymorphisms, are located in the first intron with a nucleotide distance separation of about 50 base pair [22, 26]. Intronic variations and synonymous polymorphisms may alter mRNA transcript splicing or change the function of the final protein products which results in notable shift in the function of genes [27]. These SNPs have been previously correlated with various disorders and cancer types and the effects of their allelic variation have been investigated in different populations in relation to risk of BC [16]. So the aim of this study was to investigate any potential evident relationship between mentioned SNPs occurrence and BC risk, within the Iranian female's population.

Our findings should be interpreted in the context of larger syntheses. A comprehensive meta-analysis of 80 studies reported little to no overall effect of ESR1 PvuII on cancer susceptibility, and evidence for XbaI remains mixed across populations. Accordingly, our nominal protective associations observed under FDR but not uniformly under Bonferroni may reflect small-study fluctuations and/or design-specific factors. Replication in well-powered, population-based Iranian cohorts is therefore essential [22, 23].

In our study population, IDC type represent approximately 79% of the tumors which is consistent with retrospective studies [28]. According to the published report by Gulam Nabi Sofi [29]. the percentage of ER/PR-positive tumors in our population is lower than similar cases in the West and Europe. No significant relationship was found between people with overexpression of estrogen and progesterone receptors and lymph node metastasis. Our findings were consistent with the studies of Gulam Nabi Sofi. Although Her2/neu receptor was positive in 30% of cases, this receptor alone cannot be considered as a breast cancer marker in patients.

PCR-RFLP genotyping analysis of PvuII SNP demonstrated that there is a significant relationship between CT genotype and diminished risk of breast cancer. While T allele possessed a higher frequency in the control group, no association was evident between T allele and BC risk. The frequency of heterozygous allele (CT) compared to homozygous alleles (CC and TT) has a significant relationship with reducing the risk of cancer.

On the other hand, genotype analysis of XbaI polymorphism revealed that GA and AA genotypes frequency is significantly linked to a decreased risk of breast cancer. A significant association was also observed between allele A frequency and reduced possibility of breast cancer. Shen et al. evaluated CYP1A1 MspI, ERα PvuII, and ERα XbaI polymorphisms in the Chinese females' population [30]. They reported that in comparison to the homozygous genotype of CYP1A1 MspI polymorphism (m1/m1; m2/m2), its heterozygous genotype (m1/m2) is associated with the increased risk of breast cancer. Their data also showed that PvuII and XbaI polymorphisms were less associated with breast cancer but they occurred more in women with a family history. The analysis of these polymorphisms suggests that estrogen metabolizing genes and estrogen receptors may play a substantial role in breast cancer incidence. In parallel with our study, another group investigated

the Pvull and Xbal polymorphisms correlation with BC risk in the Brazilian postmenopausal women population Association of PvuII and XbaI polymorphisms on estrogen receptor alpha [14]. Their results revealed that while the Pvull SNP was slightly correlated with the prevalence of breast cancer, heterozygous individuals for Xbal SNP had a higher risk of developing BC. Limitations: Control genotypes deviated from HWE for both ESR1 variants, largely due to heterozygote excess. In hospital-based case–control designs, such deviations may reflect sampling/selection factors, subtle population structure, or technical issues in PCR-RFLP (e.g., partial digestion) that inflate heterozygote calls. Although representative amplicons were Sanger-validated, genotyping artefact cannot be ruled out. Accordingly, the observed associations should be regarded as exploratory pending replication with orthogonal genotyping in population-based samples. Generalizability. This single-center study (Shahid Beheshti Hospital, Kashan) limits generalizability to broader Iranian or Middle Eastern populations and different care settings. Multi-center, population-based sampling would improve representativeness. Confounding. Beyond age-matching, harmonized data on key confounders (e.g., family history, reproductive factors, BMI/physical activity, socioeconomic status) were not available for both cases and controls. As a result, we could not fit fully adjusted logistic models; residual confounding cannot be excluded. Population-specific effects. Although ethnic heterogeneity in ESR1 associations has been reported, demonstrating population-specific protection requires substantially larger samples, ancestry-informative markers to control subtle stratification, and replication in independent Iranian cohorts. No principal component adjustment was possible here; population structure may therefore contribute to the observed patterns.

In conclusion, in this hospital-based Iranian sample, several ESR1 associations met FDR significance, whereas only XbaI GA/GA+AA and the A allele met Bonferroni thresholds. However, pronounced HWE deviations in controls, limited power, and potential selection bias preclude causal inference. These results are hypothesis-generating and should be validated using orthogonal genotyping in larger, population-based cohorts with adjustment for key confounders.

Author Contribution Statement

All authors contributed equally in this study.

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Ethics approval

Research Ethics Committee of Islamic Azad University, Qom Branch; Approval ID: IR.IAU.QOM.REC.1398.030.

Conflict of interest

The authors declare that they have no conflict of interest.

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