Association of SNP Rs34678647 with breast cancer risk in the Vietnamese population: An initial study

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ABSTRACT

Breast cancer remains a leading cause of mortality among women worldwide. In Vietnam, the rising incidence underscores the urgent need for early diagnostic markers. Genetic factors, particularly Single Nucleotide Polymorphisms (SNPs), play a critical role in breast cancer susceptibility. The SNP rs34678647, located downstream of the miR-221/222 cluster targeting Estrogen Receptor alpha (ERα), is hypothesized to influence breast cancer risk by modulating cancer-related pathways. This study investigates the association between rs34678647 and breast cancer risk in the Vietnamese population. A total of 234 DNA samples, comprising 131 breast cancer cases and 103 healthy controls, were genotyped using the Polymerase Chain Reaction-High Resolution Melting (PCR-HRM) technique, which is selected for its high sensitivity and specificity in SNP detection. Genotype frequencies were determined, and statistical analyses were conducted to evaluate associations. The PCR-HRM method successfully genotyped rs34678647 with high accuracy. The T allele was observed in 23% of cases and 19% of controls; however, no significant association with breast cancer risk was identified (OR = 1.19, 95% CI: 0.77-1.85, p = 0.43). Genotype distributions conformed to the Hardy-Weinberg equilibrium in both groups, supporting the representativeness of the sample. In conclusion, while the T allele of rs34678647 showed a nonsignificant trend toward increased breast cancer risk among Vietnamese women, the results were not statistically conclusive. These findings highlight the need for larger-scale studies to further explore the potential role of rs34678647 as a genetic risk factor and its applicability as an early diagnostic biomarker.

1. Introduction

Breast cancer is the leading cause of cancer-related mortality in women worldwide, with about 666,000 deaths reported in 2022, according to the World Health Organization. In Vietnam alone, breast cancer accounted for 10,008 deaths out of 24,563 newly diagnosed cases, underscoring the urgent need for improved diagnostic and treatment strategies (Bray et al., 2024). As the incidence of breast cancer continues to rise both globally and nationally, identifying early diagnostic markers and understanding risk factors are essential to improving outcomes for affected women.

Genetic predispositions, such as Single-Nucleotide Polymorphisms (SNPs), have emerged as key factors in understanding breast cancer susceptibility (Ripperger et al., 2009; Zhang et al., 2022). Identifying specific SNPs linked to breast cancer can provide valuable insights into individual risk and help in the stratification of patients for targeted therapies. By exploring the genetic underpinnings of breast cancer, researchers aim to improve early detection and treatment, particularly in populations that are currently understudied.

The miR-221/222 cluster has garnered significant attention among cancer-related microRNAs due to its ability to regulate key oncogenes and tumor suppressors involved in breast cancer progression. Specifically, this cluster modulates critical signaling pathways, including PTEN, HER2, cyclin-dependent kinases, and FOXO3, thereby influencing cell proliferation, apoptosis, and tumor growth (Li et al., 2021; Wu et al., 2022). Of particular relevance, miR-221/222 has been implicated in the development of Estrogen Receptor alpha (ERα)-negative breast tumors, suggesting that genetic variations affecting its expression or function may influence breast cancer susceptibility (Li et al., 2023).

Single-Nucleotide Polymorphism (SNP) rs34678647, located downstream of the miR-221/222 cluster, is hypothesized to affect the post-transcriptional regulation of Estrogen Receptor alpha (ERα) and related cancer pathways (Di Leva et al., 2010; Wilk & Braun, 2018). Given its potential functional relevance, investigating this SNP may offer insights into the molecular mechanisms underlying breast cancer, particularly in ERα-negative cases. Moreover, data from the 1000 Genomes Project indicate that the T allele of rs34678647 is relatively common in several Asian populations, including Koreans (25.97%), Japanese (26.19%), and the Kinh population of Ho Chi Minh City, Vietnam (27%) (The 1000 Genomes Project Consortium, 2015), making it a suitable target for association studies in Vietnamese cohorts.

Despite its plausible biological significance and frequency in Asian populations, rs34678647 remains underexplored in the context of breast cancer risk, particularly among Vietnamese women. Based on the potential regulatory role of rs34678647 in miR-221/222 activity and its association with ERα pathways, we hypothesize that this SNP may be associated with breast cancer susceptibility in Vietnamese women. Therefore, this study aims to evaluate the association between rs34678647 and breast cancer susceptibility in the Vietnamese population, addressing a critical gap in the genetic understanding of breast cancer predisposition.

2. Materials and methods

2.1. Study population

Two hundred thirty-eight whole blood samples were collected from Vietnamese women aged 30 - 60 years (mean age ~ 40), all of whom belonged of the Kinh ethnic group. Written informed consent was obtained, and the study was approved by the Medical Ethics Council of Oncology Hospital, Ho Chi Minh City; Approval No. 177/ĐĐ-CDT (Oncology Hospital Ho Chi Minh City, 2022).

The case group (n = 135) included women clinically and histologically diagnosed with malignant breast tumors. Inclusion criteria: aged 30 - 60, confirmed breast cancer (Female patients with suspicious breast lesions detected on imaging (mammography and/or ultrasound); Histopathological confirmation of malignancy (biopsy positive for breast cancer), Kinh ethnicity, and consent to participate. Exclusion: other malignancies or current/prior enrollment in cancer treatment trials.

The control group (n = 103) consisted of healthy women undergoing routine health screening at the same hospital. Inclusion criteria: no history or clinical evidence of breast cancer, age and ethnicity matched with cases, and informed consent. Exclusion: any history of breast or other malignancies.

2.2. DNA extraction

DNA was extracted from the collected blood samples using a modified salting-out method, as described by Nguyen et al. (2012). The purity and concentration of the DNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), with an absorbance ratio (A260/A280) of 1.8 to 2.0, indicating high-quality DNA.

2.3. PCR-HRM methodology

The SNP genotyping process using the Polymerase Chain Reaction - High Resolution Melting (PCR-HRM) method began with in silico design, where flanking sequence data for SNP rs34678647 were obtained from the NCBI database. Primers were designed using Primer3plus, UCSC Genome Browser, Oligo Analyzer, NCBI BLAST, and uMelt HETs. Primers were optimized for GC content (50 - 60%) and had a melting temperature (Tm) difference between the forward and reverse primers of less than 2°C. The primer pair successfully amplified a 67-bp fragment. PCR-HRM determined the optimal annealing temperature to of between 57°C and 67°C using an Eppendorf thermal cycler and HotStarTaq DNA polymerase (Qiagen). The PCR reaction mixture consisted of 1X PCR buffer, 0.2µM dNTP, 0.2µM of each primer (forward and reverse), 0.05 units of HotstarTaq, 10ng of DNA, and H2O. Thermal cycling conditions consisted of an initial denaturation at 95°C for 05 minutes, followed by 40 cycles of 95°C for 30 seconds, an optimal annealing temperature (Ta) for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. High-resolution melting analysis was conducted on genotype samples. Three distinct melting curves corresponding to the GG, GT, and TT genotypes were identified. SNP genotyping assays were performed employing the LightCycler 480 High-Resolution Melting Master (Roche Diagnostics, Germany) and a LightCycler 96 Instrument equipped with a 96-well thermal block (Roche Diagnostics). A control sample was selected based on its unique melting curve patterns and validated by Sanger sequencing. PCR products were verified by electrophoresis on a 1.5% agarose gel at 90V for 30 minutes to confirm the target product size and identify any by-products.

The PCR-HRM method was validated by assessing its sensitivity, specificity, and stability (Saadi-Ben Aoun et al., 2024). Sensitivity was measured by calculating the number of failed samples on the first HRM run without repetition. Specificity was determined by the method's ability to distinguish between genotypes based on melting temperatures (Tm). An ANOVA test was conducted on the samples, and a p-value of less than 0.05 indicated high specificity. Stability was evaluated based on variations in Tm among samples of the same genotype, with control samples showing negligible deviation around the average Tm. A T-test was performed using R (R i386 3.4.0 and RStudio), and a p-value above 0.05 confirmed high stability. The epiR package in R i386 3.4.4 is used to estimate the statistical power for epidemiological studies.

2.4. Statistical analysis

Allele frequencies were calculated using the formulas:

$$f(G) = f(GG) + \frac{1}{2}f(GT)) \text{ and } f(T) = f(TT) + \frac{1}{2}f(GT) \quad (1)$$

Equation (1). The allele frequencies are determined from genotype frequencies (Hartl & Clark, 2007).

Hardy-Weinberg Equilibrium (HWE) was evaluated for both the case and control groups. Binary logistic regression was utilized to estimate Odds Ratios (ORs) and 95% Confidence Intervals (CIs) across various genetic models (allelic, additive, dominant, and recessive). These models were chosen to capture the potential range of effects of rs34678647 on breast cancer risk based on its hypothesized mode of inheritance.

3. Results and discussion

3.1. Result

3.1.1. PCR-HRM optimization

The optimization of the PCR-HRM method was performed to ensure accurate detection of SNP rs34678647. Primers were designed explicitly for this SNP, and their performance was evaluated during the optimization process. Details regarding the primer sequences and their melting temperatures (Tm) are provided in Table 1.

Table 1

Primer Sequences and Melting Temperatures for PCR-HRM Genotyping of SNP rs34678647

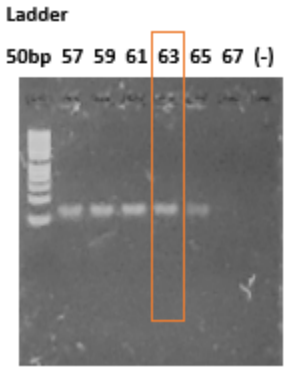
Primer	Sequence (5’ - 3’)	Tm (°C)
HRM-F	TCCAACAGATACTGACTGAGTGACA	61.1
HRM-R	TCTCCAGCACCTAAGAAAATATGTGG	63.7

Note. Primers designed and analyzed by the author

A gradient PCR was performed to identify the ideal annealing temperature for the PCR-HRM assay. The annealing temperature ranged from 57°C to 67°C, with the most precise band observed at 63°C. This temperature was chosen as the optimal setting for future PCR reactions (Figure 1).

Figure 1

Optimization of Annealing Temperature for HRM Primer Set Used in Genotyping SNP rs34678647



Note. Gel electrophoresis image generated by the author

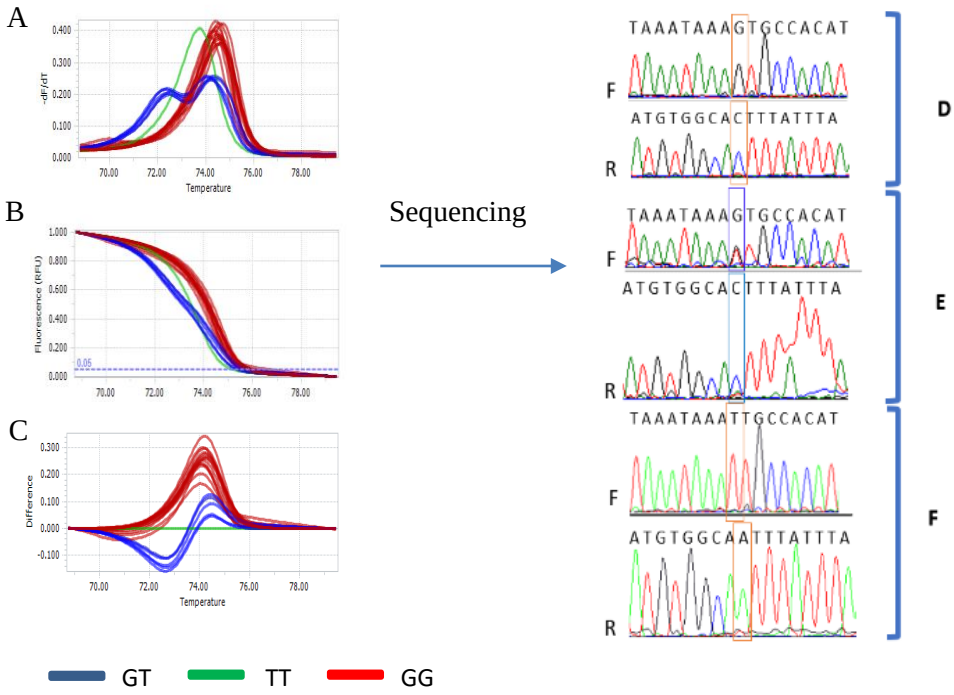
3.1.2. PCR-HRM validation

The accuracy and reliability of the PCR-HRM method were validated using Sanger sequencing. Positive control samples representing each genotype (GG, GT, and TT) were

sequenced to confirm the genotypes identified by the PCR-HRM method. The Sanger sequencing results aligned with the genotypes predicted by PCR-HRM, as shown in Figure 2. Distinct melting curves were observed for each genotype, with clear differentiation between the GG, GT, and TT genotypes (Figures 2A, 2B, and 2C). These results were further validated by Sanger sequencing (Figures 2D, 2E, and 2F).

Figure 2

HRM Analysis of SNP rs34678647 genotypes. (A) Melting Peak, (B) Melting Curve, (C) Difference Plot, (D) GG genotype, (E) GT genotype, and (F) TT genotype

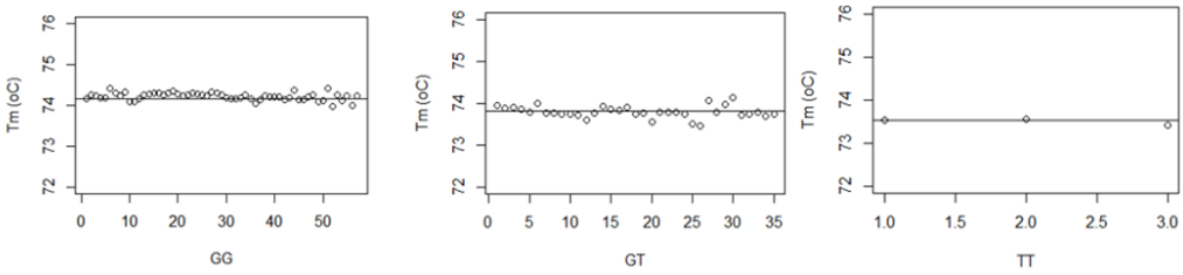


Note. HRM and Sanger sequencing data were obtained and analyzed by the author

The stability of the PCR-HRM method was evaluated by analyzing the variation in Tm values among samples with the same genotype. The results demonstrated minimal fluctuation in Tm values, indicating the high stability of the method. Figure 3 shows that the Tm values for control samples with the same genotype varied insignificantly around the average Tm for each genotype, confirming the robustness and consistency of the PCR-HRM method.

Figure 3

Stability of HRM Method: Variation in Melting Temperatures (Tm) Across Samples with the Same Genotype

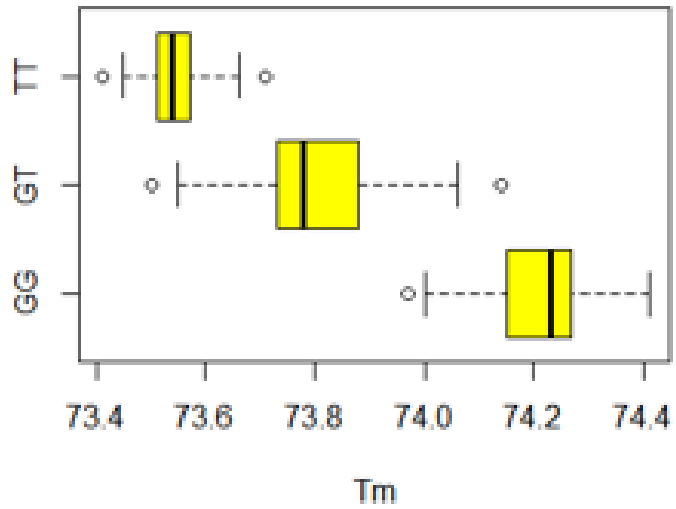


Note. Data analyzed by the author

The sensitivity of the PCR-HRM method was 98.3%, with 04 out of 238 samples failing to amplify during the first attempt. Specificity was assessed by analyzing the differences in melting temperatures (Tm) between genotypes. The ANOVA test revealed statistically significant differences in Tm values for the three genotypes ($p < 0.001$), indicating high specificity in distinguishing between GG, GT, and TT genotypes. The box plot in Figure 4 further illustrates the distinct separation of melting curve temperatures for each genotype, with no overlap between them, highlighting the method's high specificity.

Figure 4

Box Plot of Melting Curve Temperatures (Tm) for Distinguishing SNP rs34678647 Genotypes



Note. Data analyzed by the author

3.1.3. Statistical analysis

The allele and genotype frequencies for rs34678647 were analyzed in both the case and control groups (Table 2). In the case group, the T allele was observed at a frequency of 23%, while the G allele was present at a frequency of 77%. In the control group, the T allele frequency was slightly lower at 19%, while the G allele frequency was 81%. The genotype distribution in the case group was 61% GG, 33% GT, and 6% TT, compared to 66% GG, 29% GT, and 5% TT in the control group. The genotype frequencies in both the case and control groups adhered to HWE, with a p-value greater than 0.05, indicating that the control population is representative of the general population. Adherence to HWE suggests that the allele frequencies were not influenced by selection, mutation, or genetic drift, ensuring the validity of the genetic association results.

Table 2

Allele and Genotype Frequencies of SNP rs34678647 in Breast Cancer Cases and Controls

	Genotype			Allele		P_{HWE}
	TT	**GT**	**GG**	**T**	**G**	
Case (131)	8 (6%)	43 (33%)	80 (61%)	59 (23%)	203 (77%)	0.46
Control (103)	5 (5%)	30 (29%)	68 (66%)	40 (19%)	166 (81%)	0.53

Note. The author analyzed allele and genotype frequencies in breast cancer cases; control data were obtained from the 1000 Genomes Project

No significant association was detected between SNP rs34678647 and breast cancer risk across all genetic models. In the allelic model, the OR was 1.19 (p = 0.43); additive comparisons showed ORs of 1.36 (TT vs. GG) and 1.22 (TG vs. GG, p = 0.67); the dominant model (TT + TG vs. GG) had an OR of 1.24 (p = 0.43), and the recessive model (TT vs. TG + GG) had an OR of 1.27 (p = 0.67). Although these findings were not statistically significant, the ORs across models suggested a possible trend toward increased breast cancer risk associated with the T allele. To further explore this potential association, a power analysis was conducted under the allelic model. With 131 cases and 103 controls, the study showed low power (5%). Sample size estimation indicated that 7,860 cases and 6,180 controls would be needed to achieve 80% power - an unfeasible number given our current resources. These findings highlight the need for future studies with larger sample sizes to verify the potential association and clarify the suggested risk trend of rs34678647 in breast cancer.

Table 3

Association of SNP rs34678647 with Breast Cancer Risk Analyzed Across Different Genetic Models

Analysis model		OR	95% CI	p-value
Allele	T vs G	1.19	0.77 – 1.85	0.43
Addictive	TT vs GG	1.36	0.42 – 4.35	0.67
	TG vs GG	1.22	0.69 – 2.15	
Dominance	[TT + TG] vs GG	1.24	0.72 – 2.12	0.43
Recessive	TT vs [TG + GG]	1.27	0.40 – 4.02	0.67

Note. Data on the association between SNP rs34678647 and breast cancer risk across different genetic models were analyzed by the author

3.2. Discussion

In this study, we successfully optimized and validated the PCR-HRM method for genotyping the rs34678647 variant, demonstrating high sensitivity, specificity, and stability. The technique enabled accurate discrimination between GG, GT, and TT genotypes, ensuring the accuracy of our study's genetic data. The PCR-HRM method used in this study was successfully optimized and validated, showing high sensitivity (98.3%) and specificity, making it an effective tool for genotyping SNP rs34678647. The method demonstrated stability, with minimal variation in melting temperatures across replicates, and allowed for clear differentiation between GG, GT, and TT genotypes. The successful application of PCR-HRM in this study highlights its utility for genotyping in resource-limited settings.

The association between SNP rs34678647, located downstream of the miR-221/222 cluster, and breast cancer risk in Vietnamese women was explored. Although an Odds Ratio (OR) of 1.19 was observed for the T allele and the association was not statistically significant (p = 0.43), the OR value suggested a potential association with increased breast cancer risk. Therefore, the biological role of the T allele is significant and warrants further investigation in a larger population. MiR-221/222 plays a crucial role in regulating oncogenes and tumor suppressor genes, such as PTEN and p27, which are key regulators of cell growth, apoptosis, and tumor progression (Garofalo et al., 2012). By downregulating PTEN, miR-221/222 promotes tumor growth and resistance to cell death, crucial in breast cancer development (Li et al., 2021; Yin et al., 2020). SNP rs34678647, located downstream of this miRNA cluster,

may influence miRNA processing or target recognition, potentially altering gene expression related to tumor suppression and cell proliferation (Ravegnini et al., 2019; Yang et al., 2021). Thus, even a small effect of the T allele could contribute to breast cancer risk of modulating these pathways, emphasizing the need for additional functional studies. The results of this study are consistent with those of An et al. (2019) and Yang et al. (2021), who also reported no significant association between rs34678647 and either hypertension or cervical cancer. These consistent results across different diseases suggest that rs34678647 may have a limited role in influencing susceptibility to cancer and other conditions. However, differences in genetic architecture, environmental exposures, and sample sizes across studies may account for the variability in results (Liu et al., 2012). Our study, although focused on breast cancer, reinforces the idea that the potential role of rs34678647 in disease development might be modest. One of the primary limitations of this study is the relatively small sample size, consisting of 131 breast cancer cases and 103 controls. To achieve the desired 80% power for detecting a moderate effect size, a significantly larger sample size would be required. Therefore, the nonsignificant findings reported here may be a consequence of limited statistical power rather than the absence of an actual biological effect. Additionally, the study focused on a single SNP, whereas breast cancer susceptibility is likely influenced by multiple genetic and environmental factors that were not captured in this analysis.

Future studies should focus on expanding the sample size to enhance statistical power and improve the reliability of the results. Larger cohort studies would help validate the observed trend and better assess the contribution of rs34678647 to breast cancer risk. In addition to expanding the sample size, future studies should explore the functional effects of rs34678647 on miRNA processing, target binding, and gene expression. Investigating how this SNP interacts with other genetic variants and environmental factors, such as hormone exposure or lifestyle factors, could provide a more thorough understanding of its role in breast cancer. Additionally, it may be valuable to examine the association of rs34678647 with specific breast cancer subtypes, particularly ER-negative tumors, where miRNA regulation plays a more pronounced role.

4. Conclusions & recommendations

The optimized PCR-HRM protocol demonstrated high sensitivity, stability, and specificity, confirming its suitability for genotyping rs34678647 in the Vietnamese population. While the T allele of rs34678647 showed a higher frequency among breast cancer cases compared to controls, this difference was not statistically significant. Therefore, no conclusive evidence of an association between this SNP and breast cancer risk was observed in this study. These findings underscore the need for larger-scale studies to validate the potential role of rs34678647 as a genetic marker. The PCR-HRM method, as demonstrated in this study, provides an efficient and reliable approach for SNP genotyping and should be considered in future investigations aimed at identifying genetic risk factors for breast cancer.

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NO CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest.

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