RESEARCH ARTICLE

Editorial Process: Submission:02/29/2024 Acceptance:07/12/2024

The Association of *pri-miR34 b/c* Gene Polymorphism and Clinicopathologic Data in Breast Cancer Patients

Thaworn Kongton[1], Sirima Sanguansin[2], Pensri Saelee[3], Acharawan Thongmee[4], Wanida Pongstaporn[4]*

Abstract

Background: MiR-34b/c takes an important role in various aspects of carcinogenesis. Notably, *pri miR34b/c* (rs4938723) T>C polymorphism has been identified as a significant biomarker in various kinds of cancer. The objective of this study was to explore whether *pri-miR34b/c* rs4938723) T>C was associated with breast cancer susceptibility. Moreover, the association of *pri-miR34b/c* (rs4938723) T>C and clinicopathologic data, including survival outcomes, were studied in Thai breast cancer patients. **Methods:** DNA extracted from the blood of 100 Thai female breast cancer patients and 100 Thai healthy women were investigated for *pri-miR34b/c* (rs4938723) T>C polymorphism using polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP). **Results:** There was no statistically significant difference between the frequency of *pri miR34b/c* (rs4938723) T>C genotype between Thai breast cancer patients and normal subjects. This study showed that there is no association between *pri-miR34b/c* (rs4938723) genotypes and breast cancer susceptibility, clinicopathologic parameters, and survival time. However, age greater than 50 and histologic grade III were the prognostic factors affecting survival in breast cancer patients (p=0.017, p=0.010, respectively). **Conclusion:** The *pri-miR34b/c* (rs4938723) genotypes had no association with cancer susceptibility and clinicopathologic parameters in Thai breast cancer patients. Patients with older age and patients with higher histologic grade, but not the *pri miR34b/c* (rs4938723) genotype, affected survival time among breast cancer patients.

Keywords: *pri-miR34b/c*- Polymorphism- Breast cancer

Asian Pac J Cancer Prev, **25 (7)**, 2415-2420

Introduction

Breast cancer is the most common cancer in women, with 2.3 million patients diagnosed and 685,000 deaths reported globally in 2020 [1]. The number of breast cancer patients in Thailand is increasing rapidly. It is estimated that 19,452 patients will be newly diagnosed in 2025. One study in Thailand found that more than half of the patients are pre-menopausal women [2]. The development of breast cancer is influenced by both environmental and genetic factors. The investigation of genetic abnormality may contribute to understanding breast cancer pathogenesis and developing personalized treatment approaches in the future.

MiRNAs (microRNAs) are non-coding RNA of length 18-23 nucleotides, may be either tumor suppressor genes or oncogenes which regulate several processes in cell development. MiRNAs have a role in targeting mRNAs of the target genes and resulting in cleavage or translation repression [3]. Dysregulation of miRNAs is the possible relationship with carcinogenesis of several types of cancers and affects the pathogenesis of cancer such as cell proliferation, cell signaling, cell evading growth suppressor, and resistance to cell death [4-5]. MiRNAs have been first established as novel biomarkers. MiRNAs have been clinically adopted as prognostic biomarkers to assess tumorigenesis progression and treatment response in cancer patients [6].

Single nucleotide polymorphisms (SNPs) of miRNA genes might lead to aberrant maturation of miRNA and affect the specific transcription and target binding activity [7]. It has been reported that SNPs of miRNA may relate to breast cancer susceptibility including metastasis and worse prognosis of breast cancer [8-9]. It is widely acknowledged that p53 can regulate the expression of the miR34 family, which is composed of miR34a, miR34b, and miR34c. MiR34b and miR34c share a common primary transcript known as *pri-miR34b/c*. The promoter region of *pri-miR34b/c* transcripts contains binding sites for p53 [10]. There are several studies about the association of the *pri-miR34b/c* (rs4938723) variants and the various types of cancer susceptibility [11-12].

*[1]Biomedical Science Graduate Program, Department of Medical Science, Faculty of Science, Rangsit University, Pathum Thani 12000, Thailand. [2]Department of oral biology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand. [3]Devision of Research and Academic Support, National Cancer Institute Thailand. [4]Department of Medical Science, Faculty of Science, Rangsit University, Pathum Thani 12000, Thailand. *For Correspondence: wanida.po@rsu.ac.th*

However, these investigations are still inconsistent.

Therefore, this research aimed to study the association between the *pri-miR34b/c* (rs4938723) T>C polymorphism and breast cancer susceptibility, prognostic factors, and survival of Thai breast cancer patients.

Materials and Methods

Specimen

In this retrospective study, a total of 200 blood samples were provided by the National Cancer Institute, Thailand. Among these, 100 blood specimens were derived from female breast cancer patients, while the remaining 100 blood samples were collected from healthy women individuals as the normal control group. All participants in the study belonged to the Thai ethnicity. The age interval of the participants was from 26 to 77 years, with an average of age 57.9 years and a standard deviation of 21.28 years. The clinicopathologic data collected from breast cancer cases in this study included grading, tumor size, staging of tumor, lymph node infiltration, and metastasis. The study also collected immunohistochemistry results such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and triple-negative status (ER-negative, PR-negative, and HER2-negative) from the pathology laboratory of the National Cancer Institute of Thailand.

This research received ethical approval from the Ethics Committee of the National Cancer Institute to ensure the highest ethical standards and protect the rights and welfare of the participants (Code number 031_2020RB_OUT67).

DNA extraction

EDTA blood specimens were derived from both breast cancer cases and healthy controls. The genomic DNA isolation process utilized the High Pure PCR Template Preparation kit (Roche Molecular Diagnostics, Mannheim, Germany). The purity of the DNA sample was measured by spectrophotometry at the absorbance of wavelengths at 260 nm and 280 nm. The 260/280 ratio was within 1.8-2.0. This ratio is generally considered pure DNA with minimal protein contamination. In each PCR reaction, the DNA concentration was 20 ng, and the total reaction volume was 25 microliters.

Genotyping

Genotyping of *pri-miR34b/c* (rs4938723) was conducted using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method with modification [13]. The 212-bp DNA fragments were amplified using 5'-CCTCTGGGAACCTTC TTTGACCTGT-3' as the forward primer and 5'-CCTGGGCCTTCTA GTCAAATAGTGA-3' as the reverse primer. The condition of PCR was as follows: a 5-minute preheating step at 95˚C, 30 cycles of PCR which included denaturation at 95˚C for 30 seconds, annealing at 57˚C for 30 seconds, and extension at 72˚C for 30 seconds. Afterward, there was a final extension step for 10 minutes at 72˚C. Subsequently, the restriction step was composed of PCR product digested with Tsp45I restriction enzyme (BioLabs® Inc., New England Biolabs, Ipswich, MA, USA). The TT genotype, which represents the wild type, resulted in an undigested 212 bp fragment. On the other hand, the CC genotype, corresponding to homozygous polymorphism, produced a pattern with two fragments of 26 and 186 bp. The TC genotype, representing heterozygous polymorphism, resulted in three fragments with patterns of 26, 186, and 212 bp [13]. The genotyping quality control was performed by DNA sequencing in 10% of the cases, and the results were consistent with the PCR-RFLP results (data not shown).

Statistical analysis

Age differences between breast cancer cases and healthy controls were analyzed using Student's t-test. The allele frequencies of *pri-miR34b/c* (rs4938723) genotypes were calculated to determine Hardy-Weinberg equilibrium. Pearson's Chi-square test and odds ratios (OR) with 95% confidence intervals (CI) investigated the association between *pri-miR-34b/c* rs4938723 genotypes and breast cancer susceptibility. The reference group consisted of carriers of the genotype TT. The association between *pri-miR34b/c* (rs4938723) genotype frequencies and clinicopathologic data in breast cancer cases was also examined by Pearson's Chi-square test. The Kaplan-Meir method and the log-rank test assessed the relationship between *pri-miR34b/c* (rs4938723) genotype frequencies and overall survival. The multivariate Cox regression determined the prognostic marker for the survival of breast cancer patients. Statistical analysis was based on two-tailed probabilities. A p-value of <0.05 was considered statistically significant.

Results

The *pri-miR34b/c* (rs4938723) genotypes were investigated through restriction digestion with *Tsp45I* restriction enzyme in 100 Thai breast cancer cases and 100 normal control subjects. The interval age of all cases was 26 -77 years, with a median age of 52.5 years. The average age between breast cancer patients (51.01±10.41 years) and normal subjects (51.39±10.55 years) was not statistically different. (p=0.798).

Association between pri-miR34b/c (rs4938723) genotypes in breast cancer cases and controls

The distribution of *pri-miR34b/c* (rs4938723) genotypes in 100 breast cancer cases and 100 control subjects is shown in Table 1. There was no significant association between the *pri-miR34b/c* (rs4938723) TC genotype with breast cancer susceptibility (p=0.664) (Table 1). The allele frequency of *pri-miR34b/c* (rs4938723) genotypes was calculated for both study populations as shown in Table 1.

Association of pri-miR34b/c (rs4938723) genotypes with clinicopathologic data

The relationship between *pri-miR34b/c* (rs4938723) genotypes and clinicopathologic data included age, grading, tumor size, staging of tumor, lymph node infiltration, tumor metastasis, and ER/PR/HER2 status in breast cancer cases were examined using Pearson

Chi-square analysis. The findings revealed no statistical association between the *pri-miR34b/c* (rs4938723) genotypes and any clinicopathologic parameters (Table 2).

Survival Analysis of Breast Cancer Patients

The overall survival analysis was conducted using Kaplan-Meir survival curves and the Log-rank test. The mean survival time among the TT genotype was 4.57 years, and that in the TC genotype was 3.89 years. The Log-rank test indicated no statistically significant difference in overall survival time between the *pri-miR34b/c* (rs4938723) variant genotype (TC genotype) and the TT genotype in breast cancer patients (p=0.687), as illustrated in Figure 1. Moreover, the multivariate Cox regression method assessed prognostic markers for the survival of breast cancer patients by comparing the survival time between various prognostic factors including age, tumor size, histologic grade, tumor stage, lymph node invasion, triple-negative status, and genotype of *pri-miR34b/c* (rs4938723) T>C. It was revealed that the *pri-miR34b/c* (rs4938723) T>C genotype was not an independent prognostic factor influencing breast cancer survival. However, further analysis through multivariate Cox regression demonstrated that age greater than 50 years (p=0.017) and histologic grade III (p=0.010) were the prognostic factors affecting breast cancer survival (Table 3).

Discussion

MiRNAs are non-coding RNA of length 18-23 nucleotides and may function as either tumor suppressors or oncogenes. The function of miRNAs is to regulate several cellular processes, by targeting mRNAs of the target genes which results in cleavage or translation repression [3]. The miR34 family consists of miR34a, miR34b, and miR34c. The miR34 family has an important role in tumor development by inhibiting cell migration, invasion, and proliferation. There are many researches reveal that the miR34 family is associated with several kinds of cancer such as breast cancer, colorectal cancer, lung cancer, prostate cancer, osteosarcoma, and hematological neoplasm [14]. MiR34b/c is known as a downstream transcriptional target of p53. Several researches reveal the down-regulation of miR34b/c in various types of cancer, primarily attributed to hyper-methylation. The hyper-methylation of the miR34b/c CpG island has been proposed as a biomarker for multiple cancers and is linked to cancer progression and prognosis [15].

SNPs in miRNA genes involve its expression regulation and can significantly impact cancer susceptibility and development. It has been found that the rs4938723 variant in the promoter region of *pri-miR34b/c* may influence the binding of the transcription factor GATA-X, consequently impacting the expression of *pri-miR34b/c* [16]. Several studies found a significant association between the *pri-miR34b/c* (rs4938723) variant and cancer risk. A significantly increased risk of papillary thyroid carcinoma (PTC) was reported in patients with the *pri-miR34b/c* (rs4938723) TC, CC, and TC/CC genotypes compared to those with the TT genotype [10]. In a meta-analysis involving Chinese patients, the collective findings indicated that the *pri-miR34b/c*

Table 1. Distribution of *pri-miR34b/c* Genotypes in 100 Breast Cancer Cases and 100 Controls

Genotype	Case N=100	Control N=100	p-value
pri-miR34b/c (rs4938723)			
TT	38	41	
TC	62	59	
CC	0	0	
TT	38	41	0.664
TC + CC	62	59	
Allele frequency			
T allele	0.695	0.705	
C allele	0.305	0.295	

Significance p-value< 0.05; OR, odds ratio; CI, Confidence interval

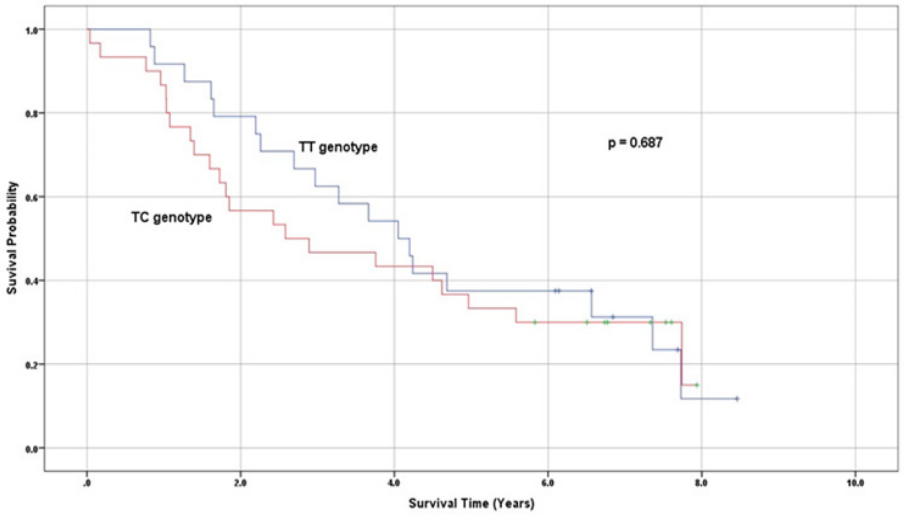

Figure 1. The Survival Curves were Analyzed by the Kaplan–Meier Method and the Log-Rank Test was Used to Compare the Survival Time between the TC Genotype Compared with TT Genotype. (p=0.687)

Table 2. Association of *pri-miR34b/c* Genotypes and Clinicopathologic Data of Breast Cancer Patients

Clinicopathologic data	Number	Genotype		OR (95% CI)	p-value
		TT (%)	TC (%)		
Age (Year)	95				
≤ 50	43	16 (37.2)	27 (62.8)		
> 50	52	20 (38.5)	32 (61.5)	0.948 (0.412, 2.182)	0.9
Histologic grade	84				
I+II	31	8 (25.8)	23 (74.2)		
III	53	25 (47.2)	28 (52.8)	0.390 (0.148, 1.026)	0.053
Tumor size (cm)	91				
≤ 2	43	16 (37.2)	27 (62.8)		
> 2	48	18 (37.5)	30 (62.5)	0.988 (0.422, 2.313)	0.977
Tumor stage	82				
I+IIA+IIB	46	18 (39.1)	28 (60.9)		
IIIA+IIIB+IV	36	14 (38.9)	22 (61.1)	1.010 (0.413, 2.470)	0.982
Lymph node	86				
Positive	50	21 (42.0)	29 (58.0)	0.690 (0.283, 1.685)	0.415
Negative	36	12 (33.3)	24 (66.7)		
Estrogen receptor	91				
Positive	31	8 (25.8)	23 (74.2)	2.199 (0.848, 5.701)	0.101
Negative	60	26 (43.3)	34 (56.7)		
Progesterone receptor	91				
Positive	24	7 (29.2)	17 (70.8)	1.639 (0.599, 4.485)	0.333
Negative	67	27 (40.3)	40 (59.7)		
HER2	91				
Positive	16	3 (18.8)	13 (81.2)	3.053 (0.802, 11.623)	0.09
Negative	75	31 (41.3)	44 (58.7)		
Triple-negative	91				
Yes	35	10 (28.6)	25 (71.4)	0.533 (0.216, 1.318)	0.171
No	56	24 (42.9)	32 (57.1)		
Distant metastasis	89				
Yes	9	2 (22.2)	7 (77.8)	2.100 (0.402, 10.776)	0.365
No	80	30 (37.5)	50 (62.5)		

*Significant p-value < 0.05; OR, Odd Ratio; CI, Confidence Interval; Histologic grade I, well differentiated; grade II, moderately differentiated; grade III, poorly differentiated; Triple-negative is ER-negative, PR-negative, and HER-negative

Table 3. Multivariate COX Regression Methods of Prognostic Marker for Survival of Breast Cancer Patients

Clinical variables	HR	OR	p-value
Age			
>50	0.393	0.183-0.846	0.017*
Tumor size (cm)			
>2	0.484	0.217-1.080	0.076
Histologic grade			
Grade III	0.342	0.151-0.773	0.010*
Tumor stage			
IIIA+IIIB+IV	1.91	0.854-4.269	0.115
Lymph node invasion			
Positive	1.819	0.801-4.133	0.153
Triple-negative tumor (ER/PR/HER2)			
Yes	1.649	0.706-3.852	0.248
pri-miR34b/c (rs4938723)			
TC	1.252	0.561-2.796	0.584

*Significant p-value < 0.05; HR, Hazard ratio; OR, Odds Ratio; CI, Confidence Interval

(rs4938723) polymorphism significantly lowered the risk of digestive cancer (p=0.001) [17]. In addition, the study of meta-analysis of 11 studies including 6169 cases and 6337 controls showed that *pri-miR34b/c* (rs4938723) polymorphism may be associated with the risk of cancers, including nasopharyngeal cancer, osteosarcoma, and renal cell carcinoma [11].

However, two studies in Iranian patients with bladder and breast cancer found that the *pri-miR34b/c* (rs4938723) polymorphism was not associated with cancer susceptibility. [13, 16]. In this study, it was found that *pri-miR-34b/c* rs4938723 polymorphism was not associated with breast cancer susceptibility. This may be due to the difference in ethnicity and genetic diversity in Thai breast cancer patients and other ethnicity. However, this finding is consistent with Sanaei et al. [13].

The study in Iranian breast cancer patients revealed a significant association between the *pri-miR34b/c* (rs4938723) genotypes and clinicopathologic characteristics, such as grade, PR, and HER2 status ($p<0.05$) [13]. In contrast, the present study showed no association between pri miR 34b/c rs4938723 genotypes and clinicopathologic parameters. A study by Tsiakou et al. [9] demonstrated that *pri-miR34b/c* (rs4938723) polymorphism in Greek triple-negative breast cancer patients showed a significant association between survival of the patients with TC or CC alleles ($p< 0.001$) [9]. The inconsistent findings regarding the association between *pri-miR34b/c* (rs4938723) polymorphism and the survival of the patients were detected in our study. However, the present study found that age greater than 50 years (0.017) and histologic grade III (0.010) were the prognostic factors affecting breast cancer survival. Various factors such as ethnicity, genetic diversity, environmental influences, and potential gene-diet interactions, might operate in diverse ways to either elevate or diminish the risk of different cancers in various regions of the world. To comprehensively understand the impact of rs4938723 on breast cancer, further large-scale studies are essential.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

The authors are sincerely thankful to the Biomedical Science Department, Faculty of Science, Rangsit, University, Pathumthani, Thailand, for their supporting grant. This research is the thesis of a graduate student from the Biomedical Science Department, Faculty of Science, Rangsit, University, as a research assistant under the supervision.

Ethical Declaration

The ethical issue was approved by the ethical committee of the National Cancer Institute (Code number 031_2020RB_ OUT67).

Conflict of Interest

There is no conflict of interest.

References

1. Lei S, Zheng R, Zhang S, Wang S, Chen R, Sun K, et al. Global patterns of breast cancer incidence and mortality: A population-based cancer registry data analysis from 2000 to 2020. Cancer Commun (Lond). 2021;41(11):1183-94. https://doi.org/10.1002/cac2.12207.
2. Insamran W, Sangrajrang S. National cancer control program of thailand. Asian Pac J Cancer Prev. 2020;21(3):577-82. https://doi.org/10.31557/apjcp.2020.21.3.577.
3. Bartel DP. Micrornas: Genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97. https://doi.org/10.1016/s0092-8674(04)00045-5.
4. Peng Y, Croce CM. The role of micrornas in human cancer. Signal Transduct Target Ther. 2016;1:15004. https://doi.org/10.1038/sigtrans.2015.4.
5. Hwang HW, Mendell JT. Micrornas in cell proliferation, cell death, and tumorigenesis. Br J Cancer. 2006;94(6):776-80. https://doi.org/10.1038/sj.bjc.6603023.
6. Reddy KB. Microrna (mirna) in cancer. Cancer Cell Int. 2015;15:38. https://doi.org/10.1186/s12935-015-0185-1.
7. Siasi E, Solimani M. Associations of single nucleotide polymorphism in mir-146a gene with susceptibility to breast cancer in the iranian female. Asian Pac J Cancer Prev. 2020;21(6):1585-93. https://doi.org/10.31557/apjcp.2020.21.6.1585.
8. Malhotra P, Read GH, Weidhaas JB. Breast cancer and mir-snps: The importance of mir germ-line genetics. Noncoding RNA. 2019;5(1). https://doi.org/10.3390/ncrna5010027.
9. Tsiakou A, Zagouri F, Zografos E, Samelis G, Gazouli M, Kalapanida D, et al. Prognostic significance of mir-34 rs4938723 t > c polymorphism in triple negative breast cancer patients. Clin Biochem. 2019;68:9-14. https://doi.org/10.1016/j.clinbiochem.2019.03.009.
10. Chen P, Sun R, Pu Y, Bai P, Yuan F, Liang Y, et al. Pri-mir-34b/c and tp-53 polymorphisms are associated with the susceptibility of papillary thyroid carcinoma: A case-control study. Medicine (Baltimore). 2015;94(38):e1536. https://doi.org/10.1097/md.0000000000001536.
11. Wang X, Lu X, Fang Y, Chen H, Deng X, Peng C, et al. Association between mir34b/c polymorphism rs4938723 and cancer risk: A meta-analysis of 11 studies including 6169 cases and 6337 controls. Med Sci Monit. 2014;20:1977-82. https://doi.org/10.12659/msm.892350.
12. Ma XP, Zhang T, Peng B, Yu L, Jiang de K. Association between microrna polymorphisms and cancer risk based on the findings of 66 case-control studies. PLoS One. 2013;8(11):e79584. https://doi.org/10.1371/journal.pone.0079584.
13. Sanaei S, Hashemi M, Rezaei M, Hashemi SM, Bahari G, Ghavami S. Evaluation of the pri-mir-34b/c rs4938723 polymorphism and its association with breast cancer risk. Biomed Rep. 2016;5(1):125-9. https://doi.org/10.3892/br.2016.690.
14. Zhang L, Liao Y, Tang L. Microrna-34 family: A potential tumor suppressor and therapeutic candidate in cancer. J Exp Clin Cancer Res. 2019;38(1):53. https://doi.org/10.1186/s13046-019-1059-5.
15. Ji TX, Zhi C, Guo XG, Zhou Q, Wang GQ, Chen B, et al. Mir-34b/c rs4938723 polymorphism significantly decreases the risk of digestive tract cancer: Meta-analysis. Asian Pac J Cancer Prev. 2015;16(14):6099-104. https://doi.org/10.7314/apjcp.2015.16.14.6099.
16. Xu Y, Liu L, Liu J, Zhang Y, Zhu J, Chen J, et al. A potentially functional polymorphism in the promoter region of mir-34b/c is associated with an increased risk for primary hepatocellular carcinoma. Int J Cancer. 2011;128(2):412-7.

https://doi.org/10.1002/ijc.25342.
17. Hashemi M, Hasanpour V, Danesh H, Bizhani F, Narouie B. Association between pri-mir-34b/c rs4938723 polymorphism and bladder cancer risk. J Biomed Res. 2018;33(1):24-9. https://doi.org/10.7555/jbr.31.20170044.