POLYMORPHISM OF *MiR34 b/c* IN THAI BREAST CANCER PATIENTS

BY

THAWORN KONGTON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
IN BIOMEDICAL SCIENCES
FACULTY OF SCIENCE

GRADUATE SCHOOL, RANGSIT UNIVERSITY
ACADEMIC YEAR 2024

Thesis entitled

POLYMORPHISM OF *MiR34 b/c* IN THAI BREAST CANCER PATIENTS

by

THAWORN KONGTON

was submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biomedical Sciences

Rangsit University
Academic Year 2024

Assoc. Prof. Sirima Sanguansin, Ph.D.
Examination Committee Chairperson

Assoc. Prof. Kanyanan Kritsiriwuthinan, Ph.D.
Member

Asst. Prof. Oraphan Wanakhachornkrai, Ph.D.
Member

Assoc. Prof. Wanida Pongstaporn, Ph.D.
Member and Advisor

Asst. Prof. Pol.Lt. Acharawan Thongmee, Ph.D.
Member and Co-Advisor

Approved by Graduate School

(Prof. Suejit Pechprasarn, Ph.D.)
Dean of Graduate School
May 30, 2025

ACKNOWLEDGEMENTS

The success of this thesis is supported by many qualified individuals. I would like to express my sincere gratitude to my major advisor Assoc. Prof. Dr. Wanida Pongstaporn from Rangsit University. I am deeply appreciative of her kind assistance, extensive support, and profound knowledge, as well as her thoughtful suggestions, document review and editing to address all defects. I am immensely grateful to Dr. Pensri Saelee from the Division of Research at the National Cancer Institute, Bangkok, Thailand, for providing the DNA samples of breast cancer patients and consultation on statistical analysis issue.

I express my sincere gratitude to Asst. Prof. Pol. Lt. Dr. Acharawan Thongmee and Asst. Prof. Dr. Oraphan Wanakhachornkrai from Rangsit University for their dedications for education and advice on preparing this thesis. Their guidances on laboratory techniques significantly contributed to the research work.

I would like to thank chairperson of thesis committee, Assoc. Prof. Dr. Sirima Sanguansin from Oral Biology division, Faculty of Dentistry, Mahidol University and Assoc. Prof. Dr. Kanyanan Kritsiriwuthinan from Faculty of Medical Technology, Rangsit University for valuable advice and suggestions for this research.

In addition, I would like to thank Pathobiology unit, Faculty of Science, Rangsit University for providing the facilities that supported this research.

Finally, I would like to give special thanks to the parents, family, and friends for their encouragement, assistance and their support in all aspects.

Thaworn Kongton
Researcher

6204311	:	Thawon Kongton
Thesis Title	:	Polymorphism of *MiR34 b/c* in Thai Breast Cancer Patients
Program	:	Master of Science in Biomedical Sciences
Thesis Advisor	:	Assoc. Prof. Wanida Pongstaporn, Ph.D.
Thesis Co-Advisor	:	Asst. Prof. Pol.Lt. Acharawan Thongmee, Ph.D.

Abstract

MiR-34b/c plays a critical role in various aspects of carcinogenesis. Notably, the *pri-miR34b/c* (rs4938723) T>C polymorphism has been identified as significant biomarker in several types of cancers. The objective of this study was to investigate whether the *pri-miR34b/c* (rs4938723) T>C polymorphism is associated with breast cancer susceptibility. In addition, the study examined the relationship between this polymorphism and clinicopathologic features, including survival outcomes, in Thai breast cancer patients. DNA samples were extracted from the blood of 100 Thai breast cancer patients and 100 healthy Thai women. Genotyping of the *pri-miR34b/c* (rs4938723) T>C polymorphism was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR - RFLP) analysis.

The results showed no statistically significant difference in the distribution of *pri-miR34b/c* (rs4938723) T>C genotypes between breast cancer patients and healthy controls. This finding suggests no association between this polymorphism and breast cancer susceptibility or clinicopathologic characteristics. However, certain clinical factors specifically age greater than 50 and histologic grade III were identified as significant prognostic indicators for survival, with p-values of 0.017 and 0.010, respectively.

(Total 49 pages)

Keywords: Thai Breast Cancer Patients, *pri-miR34b/c*, Polymorphism

Student's Signature …..... Thesis Advisor's Signature .....

Thesis Co-Advisor's Signature.....

TABLE OF CONTENTS

CONTENTS (CONT.)

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Abbreviation	Meaning
A	Adenine
Bp	Base pair
C	Cytosine
CBC	Complete blood count
^{o}C	Degree Celsius
ddNTPs	Dideoxy nucleotide triphosphates
DNA	Deoxyribonucleic acid
DCIS	Ductal Carcinoma In Situ
FAB	The French American and British
G	Guanine
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
LCIS	Lobular Carcinoma In Situ
ng	nanogram
PCR	Polymerase chain reaction
Rbc	Red blood cells
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
s	Seconds
SNPs	Single nucleotide polymorphism
T	Thymine
Taq	Thermus Aquaticus
µl	microliter
Wbc	White blood cells

CHAPTER I

INTRODUCTION

1.1 Background of the problem

Breast cancer is one of the most widespread cancers, with 2.3 million patients diagnosed and 685,000 deaths reported globally in 2020. It is the most commonly diagnosed cancer and leading cause of cancer-related death among women worldwide (Lie et al., 2021). The number of breast cancer patients in Thailand is increasing rapidly, with an estimated 19,452 new diagnoses for 2025. One study in Thailand found that more than half of patients are pre-menopausal women (Imsamran & Sangrajrang, 2020). The development of breast cancer is influenced by both environmental factors and genetic susceptibility.

Breast cancer is a cancer of epithelial cells lining the mammary ducts or mammary glands. The cancer cells are dividing abnormally and uncontrollably. The risk of developing breast cancer varies greatly with age, ethnicity, gender, menopausal status, mammary gland mass, and hormone replacement therapy. These factors related to differences in risk, clinical manifestations, and prognosis of breast cancer patients. In general, cancer is the result of molecular changes occurring in cancer cells. This can be caused by abnormalities of genes such as dysfunction of tumor suppressor genes and over activity of oncogenes. The degree of gene abnormality becomes greater as the cancer progresses.

MiRNAs (MicroRNAs) are non-coding RNA of length 18-23 nucleotides. MiRNAs may function as either tumor suppressor genes or oncogenes that play an important role in regulation of several cellular processes by targeting mRNAs of the target genes and resulting in cleavage or translation repression (Bartel, 2004).

The expression of target genes is suppressed at the post-transcriptional level when the miRNAs bind to the 3'untranslated regions of the target miRNAs. Dysregulation of miRNAs is the possible relationship with carcinogenesis of several types of cancers, and affect the hallmarks of cancer such as sustaining proliferative signaling, evading growth suppressor and resisting cell death (Peng & Croce, 2016). MiRNAs have been first established as biomarker. It was found that there is downregulation of MiR15a/16-1 in patients with B-cell chronic lymphocytic leukemia (B-CLL) (Lovata et al., 2015). MiRNAs has been clinically adopted as prognostic biomarkers to assess tumorigenesis progression and treatment response in cancer patients (Reddy, 2015).

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation found in human. SNPs of miRNA gene might lead to aberrant maturation of miRNA and affect the specific transcription and target binding activity (Siasi & Solimani, 2020). Moreover, it has been reported that SNPs may relate to the risk of developing breast cancer (Sanaei, Hashemi, & Rezaei, 2016), metastasis and worse prognosis of breast cancer (Tsiakou et al., 2019).

It is widely acknowledged that p53 plays a crucial role in regulating the expression of miR-34 family, encompassing miR-34a, miR-34b, and miR-34c. MiR-34a is encoded by its own transcript, while miR-34b and miR-34c share a common primary transcript known as *pri-miR34b/c*. The promoter region of pri-miR-34b/c transcripts contains binding sites for p53 (Chen et al., 2015). Numerous studies investigated the rs4938723 variants of miR34 b/c on the risk of various cancers. However, the findings from these investigations are still inconsistent (Sanaei et al., 2016).

This research aimed to investigate the relationship between the genetic variation of *pri-miR34b/c* (rs4938723) and the risk of breast cancer. Additionally, the study examined the association between the genetic variations of *pri-miR34b/c* (rs4938723) and clinicopathological factors, including survival rates in Thai breast cancer patients. A retrospective study was conducted for convenience in collecting of specimens and patient clinical histories. DNA samples from 100 breast cancer patients and 100 control subjects were collected and analyzed.

1.2 Objectives of the research

1.2.1 To investigate the association between the genetic variation of *pri-miR34b/c* (rs4938723) T>C polymorphism and susceptibility to breast cancer risk

1.2.2 To determine the association between the genetic variation of *pri-miR34b/c* (rs4938723) T>C polymorphism and clinicopathological data of breast cancer patients which associated with prognosis and treatment outcomes

1.2.3 To determine the relationship between the genetic variation of *pri-miR34b/c* (rs4938723) T>C and survival rates

1.2.4 To determine the prognostic marker for survival of breast cancer patients including age, tumor size, histological grade, tumor stage, lymph node invasion, triple negative status and genotype of *pri-miR34b/c* (rs4938723) T>C. A significant association was considered when the P-value was <0.05

1.3 Research hypothesis

The pri-miR34b/*c* (rs4938723) T>C polymorphism may associate with breast cancer susceptibility and/or some clinical outcomes of Thai breast cancer patients.

1.4 Scope of the research

The association of *pri-miR34b/c* (rs4938723) T>C polymorphism with breast cancer susceptibility in Thai patients and their clinical outcomes were studied. This research is the case-control study. There are 200 DNA samples from two groups, control and breast cancer. DNA samples of control group were extracted from the normal human buffy coat blood of 100 adults who have no history of breast cancer. DNA samples of breast cancer patients were extracted from buffy coat specimen provided by Division of Research, National Cancer Institute, Bangkok, Thailand.

The *pri-miR34b/c* (rs4938723) T>C genotypes of the breast cancer and control samples were investigated using polymerase chain reaction-restriction fragment length

polymorphism (PCR-RFLP) method. The association between *pri-miR34b/c* (rs4938723) T>C genotypes and breast cancer susceptibility including clinicopathological data were analyzed by statistical method.

Kaplan-Meir method and log-rank test were used to investigate the relationship between *pri-miR34b/c* (rs4938723) genotype frequencies and overall survival. The multivariate Cox regression was used to determine the prognostic marker for survival of breast cancer patients including age, tumor size, histological grade, tumor stage, lymph node invasion, triple negative status and genotype of *pri-miR34b/c* (rs4938723) T>C.

1.5 Benefits of the research

The significantly association, *pri-miR34b/c* (rs4938723) can be used as biomarker for breast cancer susceptibility. In addition, the significant association between *pri-miR34b/c* (rs4938723) and clinicopathological characteristics indicates that the polymorphism can be utilized as a prognostic biomarker in Thai breast cancer patients.

CHAPTER 2

LITERATURE REVIEWS

2.1 Breast cancer and anatomy

Breast cancer is the most common malignancy and the second major cause of mortality and morbidity in women worldwide (Khamis, Sahab, & Sang, 2012). There has been a multi-step process to develop breast cancer. Metastasis is the main cause of deaths of the patients due to difficulty of treating and curing, can be found in the nearby and distant organ such as lung, liver, bone, and brain. The patients who have been diagnosed of breast cancer early have a better prognosis and a survival rate than the late diagnosed ones (Sun et al., 2009).

Breast cancer is classified into carcinoma and sarcoma. Carcinoma is the cancer of epithelial which lining lobules as well as terminal ducts. These epithelial cells normally produce the milk. The histology of carcinomas existing in the breast usually called adenocarcinoma (American Cancer Society, 2023). Sarcoma is rarely found and arises from the stromal cells of the breast such as myofibroblasts, blood vessel cells and fibroblast. Normally sarcomas accounts for less than 1% of primary breast cancer (Johns Hopkins University, 2021).

The anatomy of breast comprises of lobules and ducts that produce and secrete milk. The cells that line both the lobules and ducts are epithelial cells. The cancer of these epithelial cells is called carcinoma, which is the most common type of breast cancer.

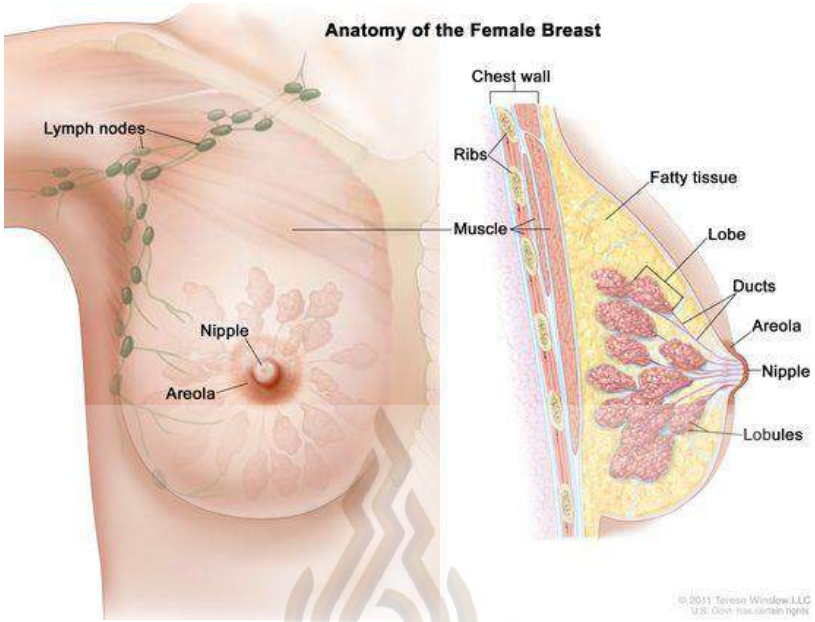

Figure 2.1 Anatomy of female breast

Source: National Cancer Institute, 2021

2.2 Types of Breast cancers (American Cancer Society, 2021)

Breast cancer can be classified into different types in order to better understand this disease and its implications for diagnosis and treatment.

1) Carcinoma in Situ refers to cancer that is confined to the milk ducts or mammary glands and has not yet invaded the surrounding breast tissue. Carcinoma In Situ can be further divided into two subtypes;

1.1) Ductal Carcinoma in Situ (DCIS) occurs in the milk ducts and is highly effective, often involving surgery and/or radiotherapy to remove the cancerous cells.

1.2) Lobular Carcinoma in Situ (LCIS) that occurs in the mammary glands, specifically in the lobules. This type has the risk of becoming invasive in the future.

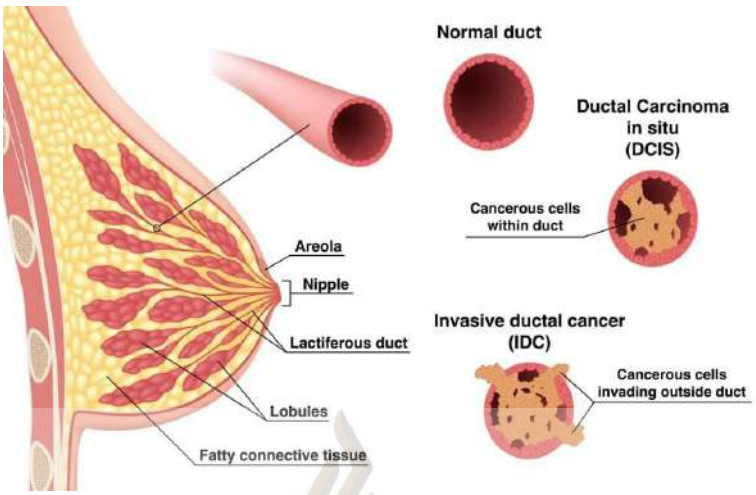

Figure 2.2 Illustration demonstrating the difference between ductal carcinoma in situ and invasive breast carcinoma.
Source: TeachMeSurgery, 2023

2) Invasive Breast cancer refers to cancer has spread from the milk ducts or mammary glands to the breast tissue or the lymph nodes in the armpit. This type of cancer can be further divided into two subtypes;

2.1) Invasive Ductal Carcinoma (IDC) is the most common type that account for 80% of all breast cancers.

2.2) Invasive Lobular Carcinoma (ILC) accounts for 10% of all breast cancers.

3) Uncommon Breast Cancer

3.1) Inflammatory Breast Cancer is found approximately 1 - 5%. The signs and symptoms are skin swollen and redness looks like an orange peel because cancer cells spreading to the skin and obstruct of the lymphatic duct. Cancer cells invade to the other tissue and spread quickly resulted in worse prognosis than above two cancers.

3.2) Paget's disease of the nipple is rarely found about 1-3%, the cancer starts from the ducts of the nipple then spreads to the nipple surface and the areola (Dark circle skin around the areola).

3.3) Phylloides tumor is rare breast tumor, starts in the connective tissue (stromal) of the breast. The tumor is most common with women with their 40s, mostly are benign only small percentage are malignant.

3.4) Angiosarcoma is found less than 1%.

2.3 Epidemiology of Breast cancer

Breast cancer is mostly found between the ages of 50 - 60 years old, in diverse ethnicity. In Thailand, it is very common to people aged 45 - 50 years old, when the patients have their first diagnosis, 80% have been found the early stage of breast cancer (stages 1 - 3) and 10% have metastatic cancer (stage 4). Recently, more screening tests conducted the more detection rate of breast cancer in Thailand.

In comparison between Western countries and Eastern countries, it is found that in Western countries such as the United States, Canada and European countries have more incidence of breast cancer than the Eastern countries such as China, Korea, Japan, and India. The most common age of breast cancer diagnosis is between 40 and 50 years in the Asian countries, whereas between 60 and 70 years in Western countries. Moreover, the incidence of breast cancer in Asian countries is increasing and is associated with increased mortality. In Western countries, the incidence is rising but the mortality rate is decreasing. In addition, it was detected the first stage of breast cancer as many as 60 - 70 percent while mainly stage 2 was detected in the East (Leong et al., 2010).

2.4 Risk factors and causes of Breast cancer

The exact cause of breast cancer is not yet known up to the present but refers to the past study; breast cancer is related to the increased female hormone levels, genetics, and environment. The risk factors refer to

1) Females are more likely to develop breast cancer than males.

2) Women over 40 years old.

3) Women who have age at first menstruation less than 12 years old, or women who menopause after 55 years old.

4) Women who has the first child over the age of 35 or woman without children.

5) Women with a history of using hormone replacement therapy during menopause or long-term use contraceptives.

6) Women with a history of receiving high doses of chest radiotherapy during childhood especially those ages less than 30 years at exposure.

7) Women with a history of atypical ductal / lobular hyperplasia or benign tumors which have potential to develop into breast cancer in the future.

8) Women having family genetic predisposition such as hereditary breast and ovarian syndromes, Li Fraumeni syndrome, familial Cowden syndrome or even if gene mutations were not detected, if they have first degree relatives with breast cancer, the probability of these women to develop breast cancer is much higher than the general women. Normally about 5 – 10% of breast cancer cases are associated with heredity which has direct result from gene mutations passed form a parent. BRCA1 or BRCA2 gene mutation is the most common cause of hereditary breast cancer which normally these genes act as tumor suppressor genes to repair damaged DNA and protect the abnormal cell growth that led to cancer. (American Cancer Society, 2021). In addition, the women who are obese or weight exceed the standard especially in postmenopausal women, less physically active, regularly eat high-calorie or high-fat diets, smoking and drinking alcohol also reportedly increases the risk of breast cancer. Also, the early-stage breast cancer patients who are overweight and lack of exercise, after receiving treatment, there will be a chance of breast cancer recurrence after continued follow-up (National Comprehensive Cancer Network, 2020).

2.5 Signs and symptoms of Breast cancer

Breast cancer can manifest in various signs and symptoms such as

1) Lump in the Breast: This may feel hard or rubbery and can sometimes be painful, swollen, or tender.

2) Changes in Breast Appearance: This includes deformities of the nipple, dimpled skin, or other unusual characteristics.

3) Skin Texture Changes: The skin may resemble the texture of an orange peel, indicating possible underlying issues.

4) Increase in Breast Size: Noticeable enlargement of one breast compared to the other can be a sign.

5) Swollen Lymph Nodes: Tumors may develop in the armpit or neck area if the cancer spreads.

6) Anorexia: A loss of appetite can occur as the disease progresses.

7) Weight Loss: Unexplained weight loss is a common symptom that may accompany the disease.

2.6 Pathogenesis of Breast cancer

Breast cancers usually start from the hyperproliferation of the ducts and then develop into benign tumors. Breast cancer occurs from the environment factor interacts with the genetically susceptible host. The carcinogenic factors normally constantly stimulate the cells to become cancer cells and divide uncontrollably or even metastasize to the other tissues or organs.

The tumor microenvironments or the stroma play crucial roles in breast cancer initiation and progression. The tumor microenvironment is composed of extracellular and cellular tissue network that surrounds and interacts with tumor cells. The cells residing in the microenvironment includes fibroblasts, myofibroblasts, endothelial cells, adipocytes, and various immune cells. These cells are surrounded by an extracellular matrix (ECM) in a three-dimensional structure composed of several substances including collagens, laminin, and fibronectin. In the normal inflammatory process when cells are injured or infected, the tissues undergo an inflammatory process and repair themselves by eliminating the causes of inflammation and eventually the tissues are recovered to the normal state. However, in the chronic inflammation process, it is unable to eliminate the cause of inflammation and finally lead to cancer initiation (Sun et al., 2017; Khamis et al., 2012).

2.7 Metastasis of Breast cancer

Metastasis is the systemic process of the tumor cells break away from the original tumor and enter the blood stream or lymphatic system and finally develop to the secondary tumor and settle at the different organs and tissues (American Cancer Society, 2021).

The metastasis mechanism can be explained as the carcinoma in situ changed to the invasive carcinoma. When the breast cancer becomes invasive, the myoepithelial cell layer is degraded with the underlying basement membrane and cancer cells invade the surrounding microenvironment. In advanced breast cancer, the myoepithelial cell layer and basement membrane are completely lost and result in invasion of epithelial cells, proliferation of stromal cells, and angiogenesis (Khamis et al., 2012).

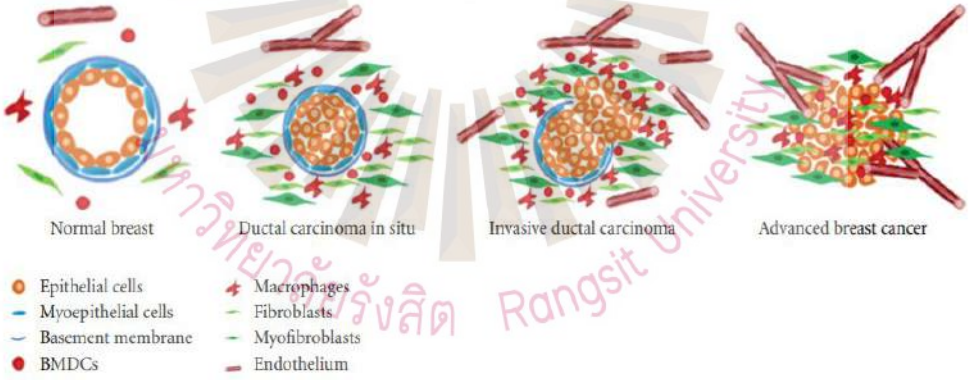

Figure 2.3 Schematic presentation of breast cancer progression accompanied with stromal cells

Source: Khamis et al., 2012

2.8 Diagnosis of Breast cancer

The diagnosis of breast cancer is crucial because the severity of the disease can be determined from the histologic grade and the histologic subtype of breast cancer. These factors are closely related to the prognosis and the choice of treatment

for the patient. The risk classification of Breast cancer can be conducted in two methods;

2.8.1 Risk classification based on histologic grade

Microscopically examination of breast tumor biopsies is conducted after specimen sectioning and staining not only for examining whether the tissue specimen is cancer but also to determine severity level or response to cancer treatment according to tumor grades which varies on a diverse type of cancer and based on the amount of abnormality which can be divided into 3 grades

Grade 1: Well differentiated (low grade), the tumor cells and the organization of the tumor tissue appear close to normal.

Grade 2: Moderately differentiated (intermediate grade)

Grade 3: Poorly differentiated (high grade), tumors tend to grow rapidly and spread faster than tumors with a lower grade.

2.8.2 Risk classification based on receptors status

Normally, every cell has receptors which express on cell membrane cytoplasm and nucleus. Chemical messengers such as hormones bind to receptors, and causes changes in the cell. Breast cancer cells have been studied by several researchers to determine which receptors can be the target of the cancer treatment.

It was found that some of the cancer cells have or not have the expressions of the receptors. The immunohistochemistry (IHC) classification of breast cancer provides both therapeutic and prognostic information according to molecular subtypes that can be categorized into

1) Estrogen receptor (ER). The cells have estrogen receptor called ER positive or ER negative in case no expression.

2) Progesterone receptor (PR). The cells have progesterone receptor called PR positive or PR negative in case no expression.

3) Human Epidermal Growth Factor Receptor or HER2 receptor. If there is a HER2 gene that can translate into HER2 receptor protein called HER2 positive and HER2 negative in case no HER2 receptors protein synthesis.

Cells that have none of these three receptor types are called triple-negative, although they frequently express other hormonal receptors such as androgen receptor and prolactin receptor. ER-positive cancer cells depend on estrogen for their growth, so

they can be treated with estrogen receptor antagonists to block estrogen receptors such as tamoxifen and generally have a better prognosis. Cancer cells that have excessive expression of HER2 gene called HER2-positive cancers that produce too much of the growth-promoting protein exists on the outside of all breast cells. Targeted therapy drugs are used to shut down the HER2 protein cause slowing the growth and killing these cancer cells. HER2-positive breast cancers are worse prognosis than HER2-negative breast cancers but HER2-positive cancer cells respond to drugs such as the monoclonal antibody trastuzumab in combination with conventional chemotherapy and this has improved the prognosis significantly.

In breast cancer treatment, the genetic information of breast cancer cells is increasingly used to support decisions about which treatments are the most suitable. Breast cancer groups include

Group 1 (luminal A): This group includes tumors that are ER positive and PR positive, but negative for HER2. Luminal A breast cancers are likely to benefit from hormone therapy and may also benefit from chemotherapy.

Group 2 (luminal B): This type includes tumors that are ER positive, PR negative, and HER2 positive. Luminal B breast cancers are likely to benefit from chemotherapy and may benefit from hormone therapy and treatment targeted to HER2.

Group 3 (HER2 positive): This type includes tumors that are ER negative and PR negative, but HER2 positive. HER2 breast cancers are likely to benefit from chemotherapy and treatment targeted to HER2.

Group 4 (basal-like): This type is called triple-negative breast cancer, includes tumors that are ER negative, PR negative, and HER2 negative. Basal-like breast cancers are likely to benefit from chemotherapy (Mayo Clinic, 2023).

In comparison, using luminal A as a reference, the triple negative cancer had the worst overall survival and the worst disease-free survival among all of subtypes (Onitilo, Engel, Greenlee, & Mukesh, 2009).

2.9 Severity classification of Breast cancer

The American Joint Committee on Cancer (AJCC) has published the 8th edition of the tumor-nodemetastasis (TNM) system for cancer staging in 2017. Breast cancer has been staged using the AJCC TNM staging system mostly based on anatomic factors: the extent of the primary tumor (T), the extent of spread to the regional lymph nodes (N), and the presence of metastasis (M). The more understanding of biological markers at present such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and their close relationship with prognosis, selection of therapy, and response to therapy, have challenged the conventional TNM staging for breast cancer. The recent edition has changed the principle from relies heavily on anatomical staging into the prognostic biomarkers such as

1) Histologic grade
2) Hormone receptor
3) HER2 expression
4) Oncotype DX score

The Oncotype DX score is the score to determine the opportunity of breast cancer recurrence. The scores are obtained from the test called 'Oncotype DX Breast Recurrence Score Test' that analyzes the activity of a group of genes that can affect the early-stage of breast cancer and the response for treatment. This test is used for helping assess risk of early-stage, ER-positive breast cancer coming back in distant part and to help figure out benefit from chemotherapy. The results of the Oncotype DX Breast Recurrence Score Test combined with other features of the cancer can give information about whether or not to have chemotherapy to treat early-stage, hormone-receptor-positive, HER2-negative breast cancer (Koh & Kim, 2018; Breast cancer organization, 2023).

Breast cancer can generally be classified into several stages, ranging from Stage 0 to Stage IV, based on the size of the tumor and the extent to which the cancer has spread to lymph nodes. The classification are Stage 0, Stage IA, Stage IB, Stage IIA, Stage IIB, Stage IIIA, Stage IIIB, Stage IIIC and Stage IV which the cancer

metastasizes beyond the breast and nearby lymph nodes to other organs such as the lungs, distant lymph nodes, skin, bones, liver, or brain (Breast Cancer Organization, 2023; American Society of Clinical Oncology, 2021).

2.10 Breast cancer screening

Breast cancer screening is the early detection of breast cancer for diagnosis and treament in people who has no underlying disease or precancerous lesion that cause the lower mortality rate from breast cancer. The breast cancer screening in women age over 50 years is recommended (National Comprehensive Cancer Network, 2020; Smith, Brooks, Cokkinides, & Saslow, 2013). There are several breast cancer screening tests such as

1) Breast Self- Examination (BSE): A breast self-exam involves the woman herself looking and feeling for any changes in her breasts. While not a substitute for mammography or clinical exams, it can help women become familiar with their breasts, enabling them to detect any changes more easily.

2) Clinical Breast Examination (CBE): A clinical breast exam is performed by a healthcare professional that uses their hands to feel for lumps or other changes in the entire breast, underarm, and collarbone area. Most of the studies used this method ascociates with the mammograms in screening of breast cancer tests. According to the study of the benefits of this combination, the sensitivity of breast cancer detection was improved but no significant reduction of breast cancer mortality rate was observed (Miller, Teresa, Baines, & Wall, 2000).

3) Mammography: A mammogram is an X-ray of the breast and is the most common screening test for breast cancer. According to the meta-analysis study data in 2009, it was found that the mortality rate from breast cancer could be reduced 15 percent in women aged 39-49 years and 14 percent in women aged 50-59 years and 32 percent in women age 60 - 69 years. In conclusion, it can reduce mortality from breast cancer in women aged 50 - 69 years. In women aged 40 - 49 years, althoug it can reduce the mortality rate from breast cancer but no statistical significance while no definite conclusions can be drawn in the women aged 70 and over (Nelson et al., 2009).

4) Ultrasound. Breast ultrasound uses sound waves to make images of the breast. It is not typically used for routine screening but can be used to further evaluate an area of concern found on a mammogram.

5) MRI (Magnetic resonance imaging). MRI method uses magnets and radio waves to take pictures of the breast. It is sometimes used in addition to mammograms for high-risk women. This method is not recommended for breast cancer screening in the general population because of its high false positives rate.

2.11 Breast cancer treatment

The choice of treatment depends on the type of breast cancer, its stage, and whether the cancer is sensitive to certain hormones or proteins, as well as the patient health and personal preferences. The treatment choice can be either local treatment or systemic treatments. Local treatment aims to treat the tumor without affecting the rest of the body and consists of

1) Surgery: There are two main types of surgery; Breast-conserving surgery is a surgery which only the part of the breast containing the cancer is removed and mastectomy is a surgery which the entire breast is removed.

2) Radiation: Radiation therapy utilizes high-energy rays or particles to target and destroy cancer cells. It is a common treatment method employed after breast-conserving surgery (BCS) or mastectomy to prevent recurrence of breast cancer. It is also used for metastatic breast cancer in order to control the growth of these metastases and alleviate symptoms.

3) Systemic treatments: Systemic treatments are treating of breast cancer by drugs that can be delivered to the cancer cells almost anywhere in the patient body. The drugs can be administered by oral or intravenous routes depending on the type of breast cancer. The drug treatment for breast cancer might be used several methods including

4) Chemotherapy: Chemotherapy involves the use of drugs to kill rapidly dividing cells, including cancer cells. It's often used before surgery (neoadjuvant chemotherapy) to shrink tumors, or after surgery (adjuvant chemotherapy) to eliminate any remaining cancer cells.

5) Hormone therapy: Hormone therapy, also known as endocrine therapy, is used for breast cancers that are hormone receptor-positive (estrogen or progesterone receptor-positive). It works by blocking the body natural hormones from supporting the growth of cancer cells and lowering the levels of hormones in the body that feed cancer growth. Common drugs include tamoxifen, aromatase inhibitors such as anastrozole, letrozole, exemestane.

6) Target therapy: Target therapy is a type of cancer treatment that targets proteins that control growth, division and spreading of cancer cells. Target therapy uses drug delivered to the distance part of body to treat the cancer by targeting some protein such as HER2 which is the growth-factor protein expressed on the cancer cell surfaces. Examples include HER2-targeted therapies such as trastuzumab, pertuzumab, ado-trastuzumab emtansine for HER2-positive breast cancers. CDK4/6 inhibitors such as palbociclib, ribociclib, abemaciclib for HR-positive, HER2-negative breast cancers. PARP inhibitors such as olaparib and talazoparib for patients with BRCA mutations and advanced breast cancer.

7) Immunotherapy: Immunotherapy uses medicines to leveraging the power of a person immune system to identify and eliminate cancer cells more efficiently. This therapy particularly used for triple-negative breast cancer that is PD-L1 positive metastatic breast cancers. Checkpoint inhibitors like pembrolizumab and atezolizumab are examples of immunotherapies used in breast cancer treatment (American Cancer Society, 2023).

2.12 MicroRNA (miRNA)

MiRNAs 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 (Bartel, 2004). The mechanism of miRNAs is the regulation of gene expression to either by repressing mRNA translation and inducing mRNA to degradation, by binding complementary with mRNA targeting at the 3' untranslated regions (3'UTRs) of the mRNAs on post-transcriptional level (Engels & Hutvagner, 2006). Dysregulation of miRNAs is the

possible relationship with carcinogenesis of several types of cancers, and affect the pathogenesis of cancer such as cell proliferation, cell signaling, and cell evading growth suppressor, resistant of cell death (Peng & Croce, 2016). MiRNAs has been clinically adopted as prognostic biomarkers to assess tumorigenesis progression and treatment response in cancer patients (Reddy, 2015).

2.13 MicroRNA biogenesis

The biogenesis of miRNAs is a multi-step process that begins with the transcription of a primary miRNA (pri-miRNA) precursor by RNA polymerase II. Th pri-miRNA is then processed in the nucleus by endonuclease enzymes such as DROSHA and DGCR8, resulting in a precursor hairpin miRNA (pre-miRNA) sequence consisting of approximately 80–100 nucleotides. Exportin-5 plays a crucial role in transporting pre-miRNAs from the nucleus to the cytoplasm, where they undergo further processing. In the cytoplasm, a ribonuclease named Dicer cleaves the pre-miRNA into a double-stranded mature miRNA.

The mature miRNA duplex then binds to Argonaute (Ago) proteins to form the RNA-induced silencing complex (RISC). This complex regulates the translation by recognizing complementary sequences in the 3′ or 5′ untranslated region (UTR) of its target mRNAs, where it causes RISC to silence target through mRNA cleavage, deadenylation or translational repression, where the passenger strand is degraded (Reddy, 2015).

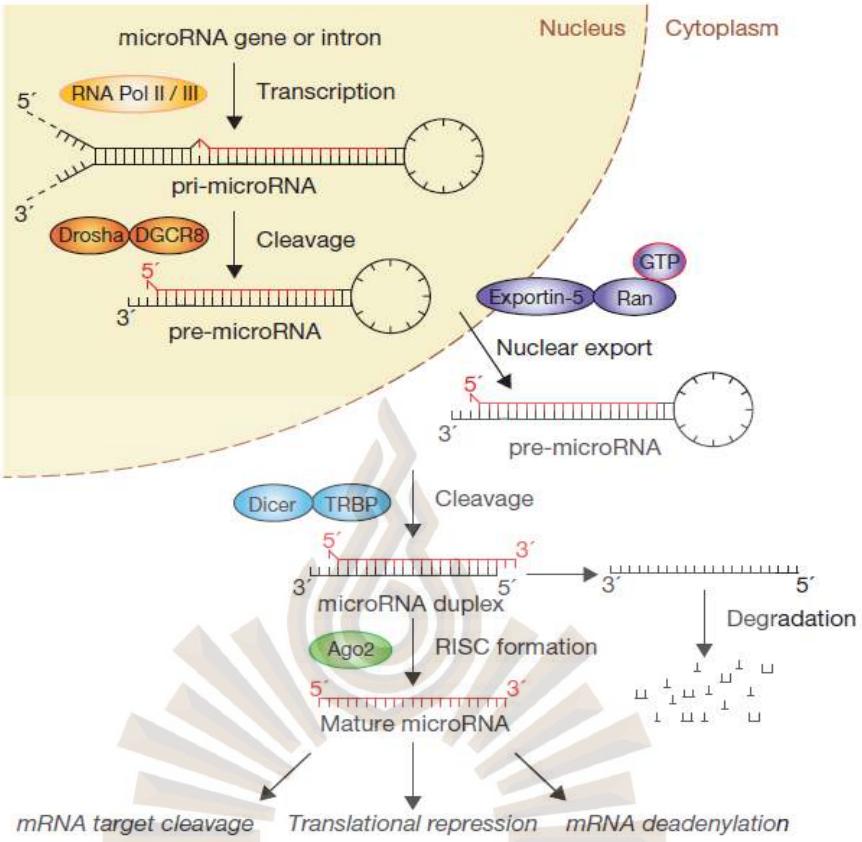

Figure 2.4 miRNA biogenesis

Source: Winter et al., 2009

2.14 MiR34 b/c

MiR-34 family plays an important role in regulating the important biological processes such as cell development, metabolism and differentiation. The miR-34 family contains three members, miR-34a, miR-34b and miR-34c, is encoded by two genes located on chromosomes 1 and 11. MiR34 b/c shares a common primary seed sequence located at one transcription unit on chromosome 11q23.1 while miR-34a is encoded in the second exon of a transcript located on chromosome 1p36.22. The position 2-9 adjacent at the 5' end is called the “seed region” of mature miRNAs of all three members guarantee for recognizing mRNA 3’-UTR. The seed sequence of miR-34a and miR-34c is almost identical, showing that they hold similar mRNA target but miR-34b is a little different (Zhang, Liao, & Tang, 2019). MiR-34a expression levels

are the most in human cells except for lung tissue in humans and brain tissue in mice that miR34 b/c is usually expressed instead (Imani, Wu, & Fu, 2018).

It is widely acknowledged that p53 can regulate the expression of miR-34 family, which composes of miR-34a, miR-34b, and miR-34c. MiR-34b and miR-34c share a common primary transcript known as pri-miR34b/c. The promoter region of pri-miR34b/c transcripts contains binding sites for p53 (Chen et al., 2015). There are several studies about the association of the rs4938723 variants of miR-34b/c and the various types of cancer susceptibility. However, these investigations are still inconsistent (Sanaei et al., 2016).

2.15 Single Nucleotide Polymorphisms (SNPs) of miR34 b/c

Single nucleotide polymorphisms (SNPs) of miRNA gene might lead aberrant maturation of miRNA and affect the specific transcription and target binding activity (Siasi & Solimani, 2020). It has been reported that SNPs of miRNA may relate to breast cancer susceptibility (Sanaei et al., 2016) including metastasis and worse prognosis of breast cancer (Tsiakou, Sagour, & Zografos, 2019). Furthermore, SNPs or epigenetics of the miRNA-34 b/c are also related to various types of cancer such as breast cancer, colorectal cancer, oral squamous cell carcinoma, and malignant melanoma. The polymorphism of miR34 b/c can be used as a prognostic biomarker in breast cancer patients and hepatic cellular carcinoma (HCC) patients (Son et al., 2013; Imani et al., 2018). A putatively functional SNPs rs4938723 T>C is located within the CpG island of the promoter of *pri-miR34b/c and* is a 423-base pair upstream from the transcription start site. The variation of rs4938723 C to T may affect a predicted GATA-X transcription factor binding and subsequently affect the expression and carcinogenesis.

Table 2.1 The association of the miR-34s members and various cancers.

Diseases	miR-34s member	Biological effect	Reference
Breast cancer	a / c	Inhibiting cellular self-renewal, repressing cell proliferation and inducing G2/M cell cycle arrest	Yang et al., 2013; Achari et al., 2014: Zhang et al., 2019
Lung cancer	b / c	Suppress the metastasis ability	Daugaard et al., 2017
Prostate cancer	a / b / c	Regulate Wnt signal pathway negatively to inhibit EMT-associated migration and invasion - inhibition in cell migration, invasion and proliferation, whereas showed no influence on apoptosis	Liang et al., 2015; Fang et al., 2017
Hepatocellular carcinoma	a / b	The mechanism was not clarified but found downregulation of miR-34a and miR-34b in tumor tissues	Xie et al., 2014
Colorectal cancer	a / b / c	Dysregulation of miRNA-34s is found in cancer cells but the results are inconsistent.	Zhang et al., 2014; Hiyoshi et al., 2015; Zhang et al., 2017
Osteosarcoma	a / b	Acts as tumor suppressor, promote apoptosis and cell cycle arrest at G0/G1	Gang et al., 2017; Xi et al., 2018
Hematological neoplasm	a / b / c	Downregulation and cause inhibited autophagy and induced apoptosis	Liu et al., 2017; Van et al., 2018

Source: Adapted from Zhang et al., 2019

CHAPTER 3

MATERIALS AND METHODS

3.1 Study population

This study included 100 patients diagnosed with breast cancer and 100 healthy controls with no cancer history. A total of 200 blood samples were provided by National Cancer Institute, Thailand for the retrospective study. All participants in the study belonged to the Thai ethnicity. Immunohistochemistry results, including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and triple negative status (ER-negative, PR-negative and HER2-negative), were collected from pathology laboratory of National Cancer Institute of Thailand. The specimens used for this research were DNA extracted from buffy coat blood of 100 normal control subjects and 100 breast cancer patients, obtained from the specimen bank from Dr. Pensri Saelee, Division of Research, National Cancer Institute, Bangkok, Thailand. This research received ethical approval from the Ethics Committee of the National Cancer Institute to ensure the highest ethical standards and protects the rights and welfare of the participants (Code number 031_2020RB_OUT67).

3.2 Genotyping of the *pri-miR34b/c* (T>C) polymorphism

Genotyping of *pri-miR34b/c* (rs4938723) was conducted using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method (Sanaei et al., 2016). Evaluation of the *pri-miR34b/c* (rs4938723) polymorphism and its association with breast cancer risk use forward and reverse primers as shown in Table 3.1.

Table 3.1 The concentration and sequence of *pri-miR34b/c* primers**.**

Primers	Sequencing	Concentration
miR-34 b/c F	5'-CCTCTGGGAACCTTCTTT GACCTGT-3'	20 pmol/µL
miR-34 b/c R	5'-CCTGGGCCTTCTAGTCAAATA GTGA-3'	20 pmol/µL

Mater mix preparation for amplified DNA in SNP *pri-miR34b/c* analysis included 10x *i*-Taq ™ plus PCR buffer, 10 mM of dNTP (2.5 mM each), 20 µM for miR34 b/cF and miR34 b/cR, 1U of *i*-Taq ™ plus DNA Polymerase. The details of master mix preparation were shown in Table 3.2.

Table 3.2 Preparation of the Master mix (Intronbio ; *i*-Taq ™ plus PCR System)

Reagent		Final concentration	Volume (µl)/1 Reaction
Autoclaved distilled water			13.8
5x reaction buffer include dNTP mixture and $MgCl_2$		200 µM	5.0
Primers	Forward primer (sense)	20 pmol/µL	0.5
	Reverse primer (antisense)	20 pmol/µL	0.5
i-Taq DNA polymerase		1 unit	0.2
DNA template			5.0
Total			25.0

One PCR tube composed of Master mix 20 uL and DNA template 5 µl. The condition of PCR was as Table 3.3.

Table 3.3 Program settings for DNA amplification

Step	Temperature	Time	Number of cycles
Preheating	95˚C	5 minutes	
Denaturation	95˚C	30 seconds	30x
Annealing	57˚C	30 seconds	
Extension	72˚C	30 seconds	
Final extension	72˚C	5 minutes	

In the subsequently restriction step, the PCR product was digested with Tsp45I restriction enzyme (BioLabs® Inc., New England Biolabs, Ipswich, MA, USA). The TT genotype, representing the wild type, resulted in an undigested 212-bp fragment. In contrast, 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 (Sanaei et al., 2016). The genotyping quality control was performed by DNA sequencing in 10% of the cases, and the results were consistent with the PCR-RFLP results.

3.3 Quality control procedures

To mitigate the risk of PCR contamination, reagents for the PCR were carefully aliquoted. Each assay included a negative control of no DNA template sample to monitor any potential PCR contamination. More than 10% of the samples were randomly selected and subjected to repeat RFLP analysis for result confirmation.

Furthermore, to verify the accuracy of the RFLP results, 10% of the samples from both breast cancer and normal controls were selected for cross-verification through sequencing analysis.

3.4 Statistical analysis

The qualitative data, which is counted as frequency, was analyzed using Chi-square test. The continuous data was analyzed using Student's t-test. Statistical analysis was based on two-tailed probabilities. P-value of <0.05 was considered statistically significant. 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) were used to investigate the association between *pri-miR34b/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 clinical including pathological data in breast cancer cases were also examined by Pearson's Chi-square test.

Furthermore, Kaplan-Meir method and log-rank test were used to investigate the relationship between *pri-miR34b/c* (rs4938723) genotype frequencies and overall survival. The multivariate Cox regression was used to determine the prognostic marker for survival of breast cancer patients including age, tumor size, histological grade, tumor stage, lymph node invasion, triple negative status and genotype of *pri-miR34b/c* (rs4938723) T>C. A significant association was considered when the P-value was less than 0.05.

CHAPTER 4

RESULTS

The demographic data of 100 breast cancer cases and 100 normal controls subjects are shown in Table 4.1. The interval age of all cases was 26 -77 years, with a median age of 52.5 years. The average of age between breast cancer patients (51.01 ± 10.41) and normal subjects (51.39 ± 10.55) is no statistically different (P = 0.798).

4.1 Genotyping of the *pri-miR34b/c* (T>C) polymorphism

Genotyping of the *pri-miR34b/c* (T>C) polymorphism was analyzed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP).

4.1.1 PCR products of *pri-miR34b/c* (rs4938723) in breast cancer patients and normal control subjects.

The PCR products of *pri-miR34b/c* were 212 bp in 2% agarose gel-electrophoresis. The examples of PCR products from breast cancer patients and normal control subjects were shown in figures 4.1 and 4.2, respectively.

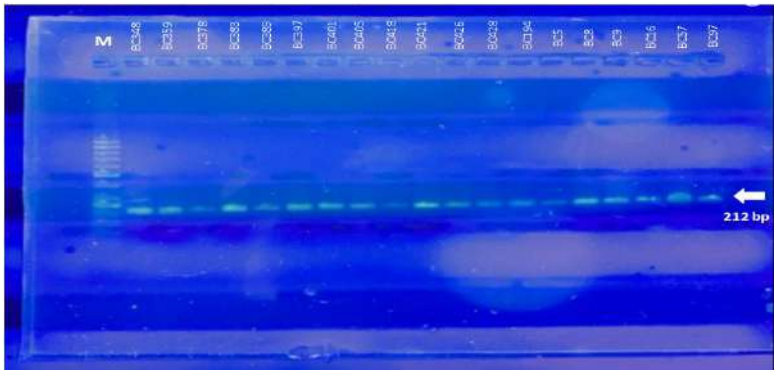

Figure 4.1 Examples of *pri-miR34b/c* PCR products in breast cancer patients.
M=50 bp marker

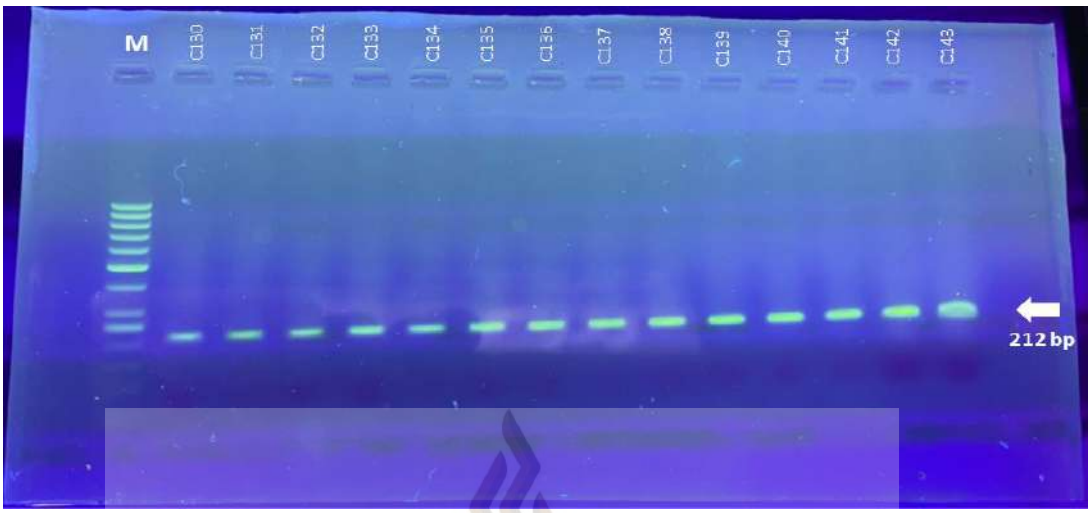

Figure 4.2 Examples of *pri-miR34b/c* PCR products in normal controls.
M=50 bp marker

4.1.2 Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) for *pri-miR34b/c* (T>C) polymorphism

The PCR products of breast cancer patients and normal controls were digested with *Tsp45I* restriction enzyme and produces a 186- and 26-base pair (bp) pattern, while the T allele cannot be digested and shows 212-base pair (bp) pattern. Therefore, the TT (wild type) product shows single DNA band of 212 base pair, TC (heterozygote polymorphism) shows three DNA bands of a 212-, 186- and 26-base pairs, and CC (homozygote polymorphism) shows two DNA bands of 186- and 26-base pairs. However, the 26-base pair band is hardly observed. The examples of PCR-RFLP genotyping of *pri-miR34b/c* (rs4938723) in breast cancer patients and normal control subjects were shown in figures 4.3 and 4.4, respectively.

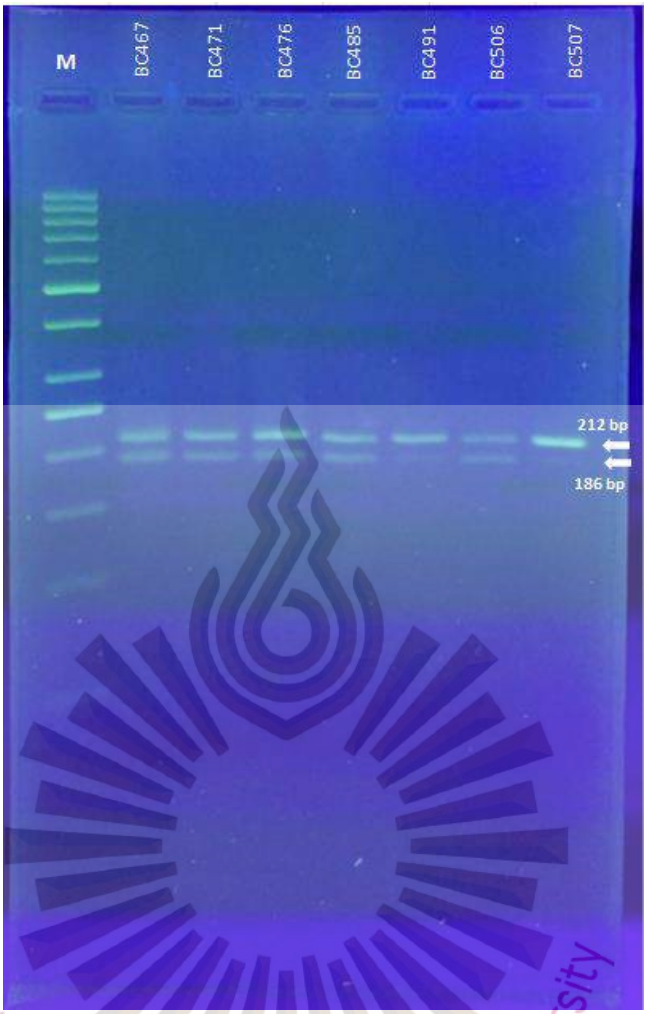

Figure 4.3 PCR-RFLP genotyping of *pri-miR34b/c* (rs4938723) in breast cancer patients.

Lane 1 50 bp Marker
Lane 2 BC 467 : the genotype is TC
Lane 3 BC 471 : the genotype is TC
Lane 4 BC 476 : the genotype is TC
Lane 5 BC 485 : the genotype is TC
Lane 6 BC 491 : the genotype is TC
Lane 7 BC 506 : the genotype is TC
Lane 8 BC 507 : the genotype is TT

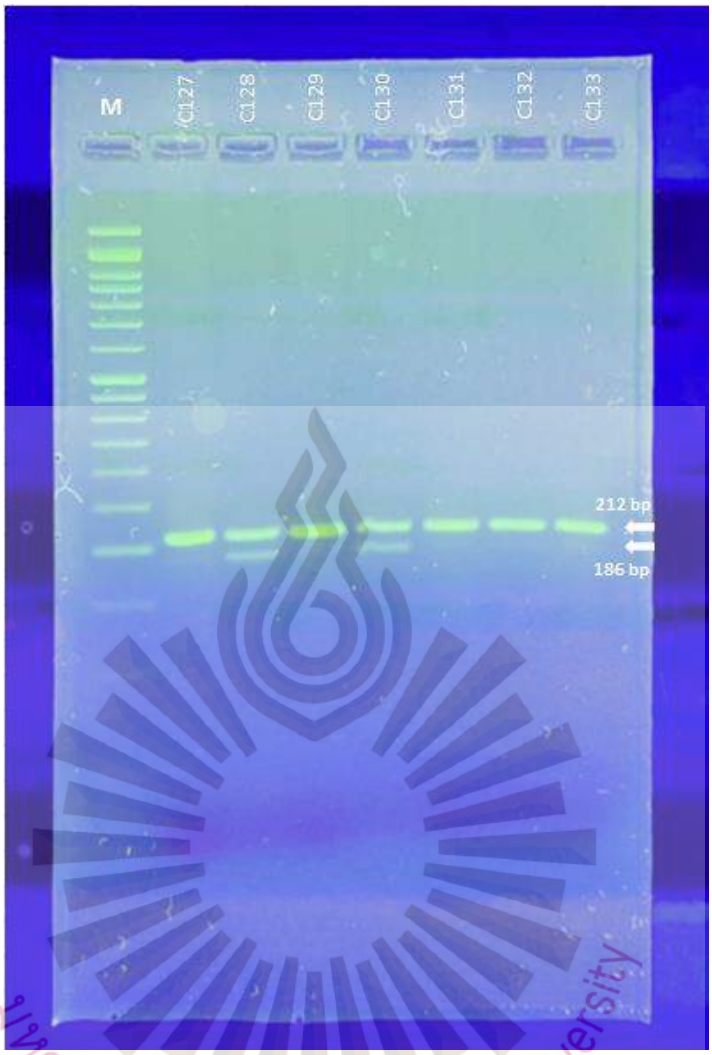

Figure 4.4 PCR-RFLP genotyping of *pri-miR34b/c* (rs4938723) in normal controls.

Lane 1 50 bp Marker
Lane 2 C 127 : the genotype is TT
Lane 3 C 128 : the genotype is TC
Lane 4 C 129 : the genotype is TT
Lane 5 C 130 : the genotype is TC
Lane 6 C 131 : the genotype is TT
Lane 7 C 132 : the genotype is TT
Lane 8 C 133 : the genotype is TT

Cross-verification was carried out using sequencing analysis on 20 samples each from Breast Cancer patients and Normal controls. The samples BC142 and C177 were initially classified as genotype TC through RFLP analysis. Further examinations using the sequencing method revealed that the sequencing chromatogram indicates the presence of both Cytosine and Thymine bases at the 26 bp position from the forward

primer origin, as marked by the bold black arrow in Figures 4.5 and 4.6, respectively. This clearly confirms that the results obtained from the RFLP analysis method have reliability and consistency.

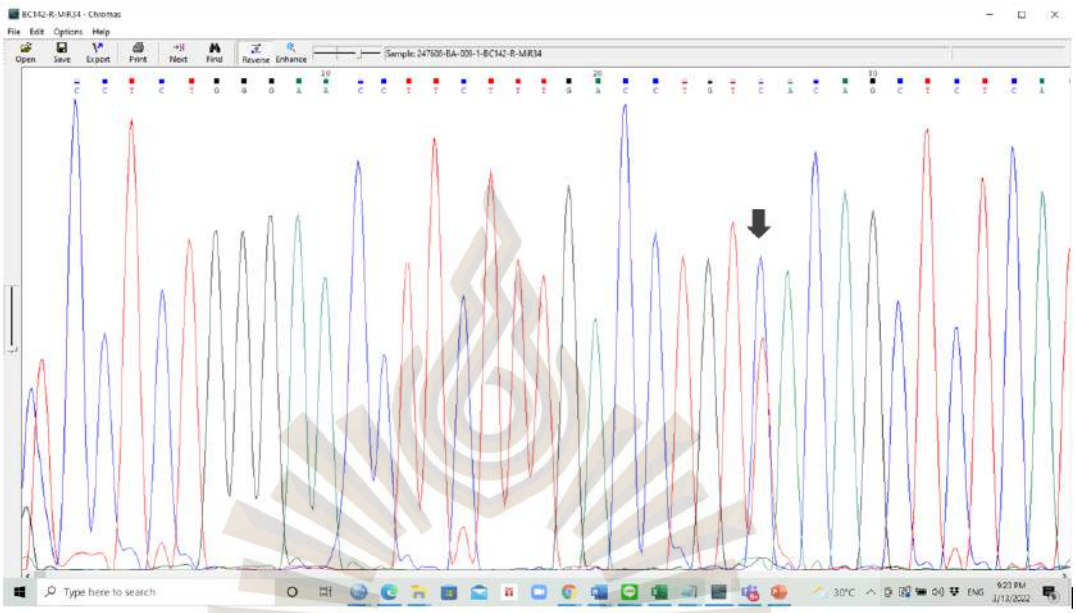

Figure 4.5 Sequencing chromatogram genotyping of *pri-miR34b/c* (rs4938723) in breast cancer patient sample BC142

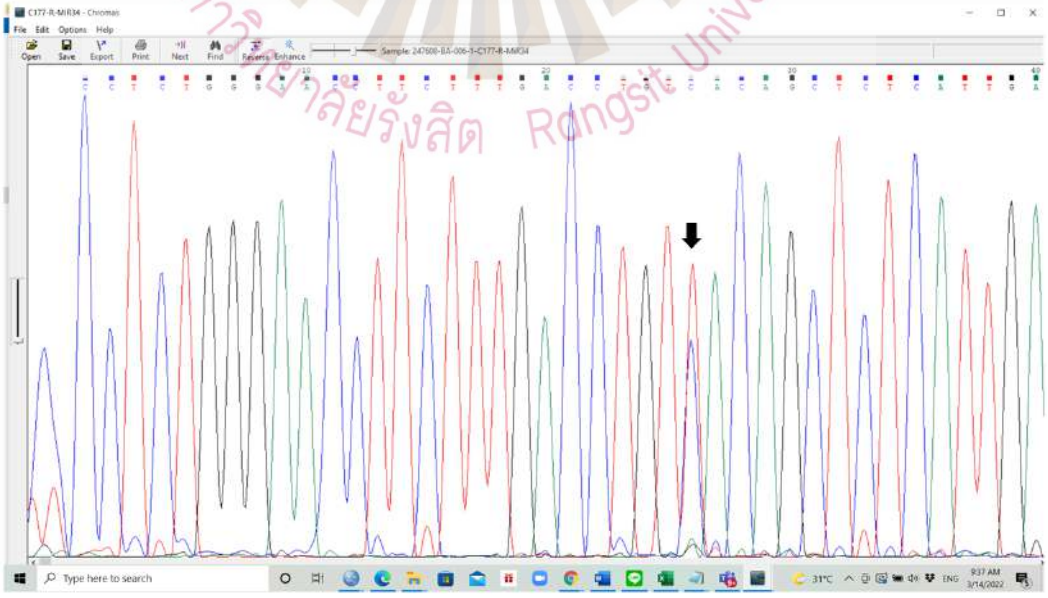

Figure 4.6 Sequencing chromatogram genotyping of *pri-miR34b/c* (rs4938723) in normal controls sample C177

4.2 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 is no significant association between *pri-miR34b/c* (rs4938723) TC genotype with breast cancer susceptibility (P=0.772) (Table 1). The allele frequency of *pri-miR34b/c* (rs4938723) genotypes was calculated for both study populations as shown in Table 4.1

Table 4.1 Distribution of *pri-miR34b/c* (rs4938723) genotypes in 100 Breast cancer cases and 100 Controls

Genotype	Case N=100	Control N=100	P-value
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

4.3 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 as shown in Table 4.2

Table 4.2 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)	0.900
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)	0.090
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.

4.4 Survival Analysis of Breast Cancer Patients

The overall survival analysis was conducted using Kaplan-Meir survival curves and the Log-rank test. The results indicated no statistically significant difference in overall survival time between the *pri-miR34b/c* (rs4938723) variant genotype (TC and CC genotype) and the TT genotype in breast cancer patients (P = 0.832), as illustrated in Figure 4.7. Moreover, the multivariate Cox regression method assessing prognostic markers for the survival of breast cancer patients by comparing the survival time between various prognostic factors including age, tumor size, histological grade, tumor stage, lymph node invasion, triple negative status and genotype of *pri-miR34b/c* (rs4938723) T>C) in breast cancer patients. 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) are the prognostic biomarker affecting breast cancer survival according to Table 4.3

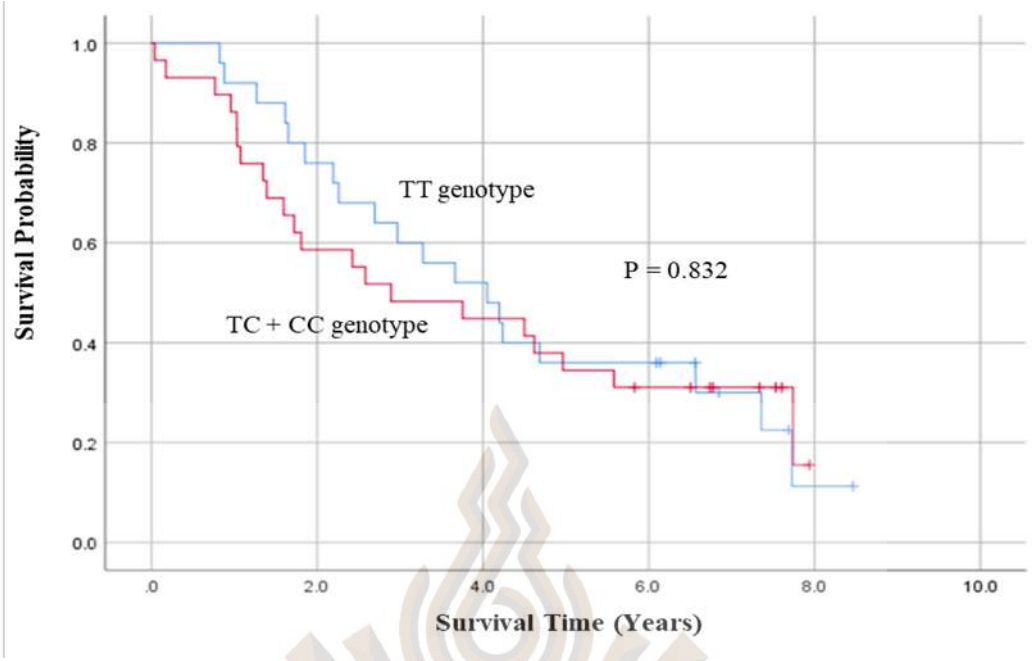

Figure 4.7 The Survival Curves were analyzed by Kaplan–Meier Method and log Rank Test was used to compare the Survival Time between TC+CC Genotype compared with TT Genotype. (P=0.832)

Table 4.3 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	1.910	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 vs TT	1.252	0.561-2.796	0.584

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

CHAPTER 5

DISCUSSION AND CONCLUSION

MiRNAs are non-coding RNA of length 18-23 nucleotides, may function as either tumor suppressor 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 (Bartel, 2004). The miR-34 family consists of miR-34a, miR-34b, and miR-34c. MiR-34 family has an important role in tumor development by inhibiting cell migration, invasion, and proliferation. There are many researches reveal that miR-34 family associates with several kinds of cancer such as breast cancer, colorectal cancer, lung cancer, prostate cancer, osteosarcoma, and hematological neoplasm (Zhang et al., 2019). MiR34 b/c is known as a downstream transcriptional target of p53. Several researches reveal the down-regulation of miR-34b/c in various types of cancer, primarily attributed to hyper-methylation. The hyper-methylation of the miR34 b/c CpG island has been proposed as a biomarker for multiple cancers and is linked to cancer progression and prognosis (Ji et al., 2015).

The presence of SNPs in miRNA genes involves its expression regulation and can significantly impact cancer susceptibility and development. It was 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-miR34 b/c. It was reported that *pri-miR34b/c* (rs4938723) in Greek triple-negative breast cancer patients showed significantly associated with survival of the patients with TC or CC alleles ($P < 0.001$) (Tsiakau et al., 2019). The study on Iranian breast cancer patients revealed a significant association between the *pri-miR34b/c* (rs4938723) genotypes and clinicopathologic characteristics, such as grade, progesterone receptor, and human epidermal growth factor receptor 2 status ($P < 0.05$) (Sanaei et al., 2016). For other types of cancer, a significantly increased risk of papillary thyroid carcinoma (PTC) was

reported in patients with the miR34 b/c rs4938723 CT, CC, and CT/CC genotypes compared to those with the TT genotype (Chen et al., 2015). In a meta-analysis involving Chinese patients, the collective findings indicated that the *pri-miR34b/c* (rs4938723) polymorphism significantly lowered the risk of digestive cancer (P = 0.001) (Ji et al., 2015). Inconsistent results on *pri-miR34b/c* (rs4938723) polymorphism and bladder cancer risk have been reported in Iranian patients (Hashemi, Hasanpour, Danesh, Bahari, & Taheri, 2019).

In this research, the *pri-miR34b/c* (rs4938723) variant was not associated with susceptibility to breast cancer (P=0.772). In addition, there was no statistically association between *pri-miR34b/c* (rs4938723) genotypes and clinicopathologic parameters. This study finding was consistent with the study in Iranian breast cancer patients by Sanaei et al. (2016) that revealed no significant association between pri-miR34 b/c rs4938723 variant and breast cancer susceptibility. However, the Iranian study revealed a significant association between the *pri-miR34b/c* (rs4938723) genotypes and clinicopathologic characteristics, such as grade, PR, and HER2 status ($p<0.05$). This lack of association may be attributed to differences in ethnicity and genetic diversity between Thai breast cancer patients and those of other ethnic backgrounds. A study conducted by Tsiakou et al. 2019 revealed that the *pri-miR34b/c* (rs4938723) polymorphism exhibited a significant association with patient survival in Greek triple-negative breast cancer patients possessing TC or CC alleles ($P<0.001$). The findings in this study regarding association between the *pri-miR34b/c* (rs4938723) polymorphism and patient survival were inconsistent. However, it was observed that age greater than 50 years ($p = 0.017$) and histologic grade III ($p = 0.010$) were significant prognostic factors influencing breast cancer survival. The variability in results could be attributed to factors such as ethnicity, genetic diversity, environmental influences, and potential gene-diet interactions, which might affect cancer risk differently across various global regions. To gain a more comprehensive understanding of the impact of the *pri-miR34b/c* (rs4938723) polymorphism on breast cancer, further large-scale studies are necessary.

In conclusion, the *pri-miR34b/c* (rs4938723)T>C polymorphism did not show a significant association with susceptibility to breast cancer, clinicopathological

characteristics, or survival outcomes among Thai patients. However, certain factors such as age greater than 50 years and histologic grade III were identified as potential prognostic factors that could influence breast cancer survival.

REFERENCES

American Cancer Society. (2023a). *Breast cancer risk factors you cannot change*. Retrieved from https://www.cancer.org/cancer/breast-cancer/risk-and-prevention/breast-cancer-risk-factors-you-cannot-change.htm

American Cancer Society. (2023b). *Surgery for breast cancer*. Retrieved from https://www.cancer.org/cancer/breast-cancer/treatment/surgery-for-breast-cancer.html

American Society of Clinical Oncology. (2023). *What is metastasis?* Retrieved from https://www.cancer.net/navigating-cancer-care/cancer-basics/what-metastasis

Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell, 116*(2), 281–297. doi:10.1016/S0092-8674(04)00045-5

Breastcancer.org. (2023a). *Breast cancer staging*. Retrieved from https://www.breastcancer.org/symptoms/diagnosis/staging

Breastcancer.org. (2023b). *Oncotype DX test*. Retrieved from https://www.breastcancer.org/symptoms/testing/types/oncotype_dx

Chen, P., Sun, R., Pu, Y., Yang, Y., Ma, Y., & Wu, X. (2015). Pri-Mir-34b/c and TP53 polymorphisms are associated with the susceptibility of papillary thyroid carcinoma: A case–control study. *Medicine, 94*(38), e1509. doi:10.1097/MD.0000000000001509

Hashemi, M., Hasanpour, V., Danesh, H., Bahari, G., & Taheri, M. (2019). Association between pri-miR-34b/c rs4938723 polymorphism and bladder cancer risk. *The Journal of Biomedical Research, 33*(1), 24–29. doi:10.7555/JBR.33.20180081

Imani, S., Wu, R. C., & Fu, J. (2018). MiRNA-34 family in breast cancer: From research to therapeutic potential. *Journal of Cancer, 9*, 3765–3775. doi:10.7150/jca.26115

Imsamran, W., & Sangrajrang, W. (2020). National cancer control program of Thailand. *Asian Pacific Journal of Cancer Prevention, 21*(3), 577–582. doi:10.31557/APJCP.2020.21.3.577

REFERENCES (CONT.)

Ji, T. X., Zhi, C., Guo, X. G., Wang, J., & Li, Y. (2015). MiR-34b/c rs4938723 polymorphism significantly decreases the risk of digestive tract cancer: Meta-analysis. *Asian Pacific Journal of Cancer Prevention, 16*(14), 6099–6104. doi:10.7314/APJCP.2015.16.14.6099

Johns Hopkins University. (2023). *Types of breast cancer*. Retrieved from https://pathology.jhu.edu/breast/types-of-breast-cancer

Khamis, Z. I., Sahab, Z. J., & Sang, Q. X. A. (2012). Active roles of tumor stroma in breast cancer metastasis. *International Journal of Breast Cancer, 2012*, Article ID 574025.doi:10.1155/2012/574025

Kho, S. J., & Kim, M. J. (2018). Introduction of a new staging system of breast cancer for radiologists: An emphasis on the prognostic stage. *Korean Journal of Radiology, 20*(1), 69–82.doi:10.3348/kjr.2018.20.1.69

Lei, S., Zheng, R., Zhang, S., Wang, S., Chen, R., Sun, K., . . . Wei, W. (2021). Global patterns of breast cancer incidence and mortality: A population-based cancer registry data analysis from 2000 to 2020. *Cancer Communications, 41*, 1183–1194.doi:10.1002/cac2.12207

Leong, S. P. L., Shen, Z. Z., Liu, T. J., et al. (2010). Is breast cancer the same disease in Asian and Western countries? *World Journal of Surgery, 34*, 2308–2324. doi:10.1007/s00268-010-0696-x

Lovata, F., Fassan, M., Gasparini, P., et al. (2015). miR-15b/16-2 deletion promotes B-cell malignancies. *Proceedings of the National Academy of Sciences of the United States of America, 112*(37), 11636–11641. doi:10.1073/pnas.1511977112

Mayo Clinic. (2023). *Breast cancer*. Retrieved from https://www.mayoclinic.org/diseases-conditions/breast-cancer/in-depth/breast-cancer/art-20045654

Miller, A. B., To, T., Baines, C. J., & Wall, C. (2000). Canadian National Breast Screening Study-2: 13-year results of a randomized trial in women aged 50–59 years. *Journal of the National Cancer Institute, 92*(18), 1490–1499. doi:10.1093/jnci/92.18.1490

REFERENCES (CONT.)

National Cancer Institute. (2021). *Breast*. Retrieved from https://www.cancer.gov/publications/dictionaries/cancer-terms/def/breast

National Comprehensive Cancer Network. (2023). *Recently published guidelines*. Retrieved from https://www.nccn.org/guidelines/recently-published-guidelines

Nelson, H. D., Tyne, K., Naik, A., Bougatsos, C., Chan, B. K., & Humphrey, L. (2009). Screening for breast cancer: An update for the U.S. Preventive Services Task Force. *Annals of Internal Medicine, 151*(10), 727–737. doi:10.7326/0003-4819-151-10-200911170-00009

Onitilo, A. A., Engel, J. M., Greenlee, R. T., & Mukesh, B. N. (2009). Breast cancer subtypes based on ER/PR and Her2 expression: Comparison of clinicopathologic features and survival. *Clinical Medicine & Research, 7*(1/2), 4–13. doi:10.3121/cmr.2009.825

Peng, Y., & Croce, C. M. (2016). The role of microRNAs in human cancer. *Signal Transduction and Targeted Therapy, 1*, Article 15004. doi:10.1038/sigtrans.2016.4

Reddy, K. B. (2015). MicroRNA (miRNA) in cancer. *Cancer Cell International, 15*, Article 38. doi:10.1186/s12935-015-0208-6

Sanaei, S., Hashemi, M., & Rezaei, M. (2016). Evaluation of the pri-miR-34b/c rs4938723 polymorphism and its association with breast cancer risk. *Biomedical Reports, 5*(2), 125–129. doi:10.3892/br.2016.725

Siasi, E., & Solimani, M. (2020). Associations of single nucleotide polymorphism in miR-146a gene with susceptibility to breast cancer in the Iranian female. *Asian Pacific Journal of Cancer Prevention, 21*(6), 1585–1593. doi:10.31557/APJCP.2020.21.6.1585

Smith, R. A., Brooks, D., Cokkinides, V., Saslow, D., & Brawley, O. W. (2013). Cancer screening in the United States, 2013: A review of current American Cancer Society guidelines, current issues in cancer screening, and new guidance on cervical cancer screening and lung cancer screening. *CA: A Cancer Journal for Clinicians, 63*(2), 87–105. doi:10.3322/caac.21166

REFERENCES (CONT.)

Son, M. S., Jang, M. J., Jeon, Y. J., Kim, W. H., Kwon, C. I., Ko, K. H., . . . Kim, N. K. (2013). Promoter polymorphisms of pri-miR-34b/c are associated with hepatocellular carcinoma. *Gene, 524*(1), 156–160. doi:10.1016/j.gene.2013.03.007

Sun, G., Yan, J., Noltner, K., Feng, J., Li, H., Sarkis, D. A., & Sommer, S. S. (2009). SNPs in human miRNA genes affect biogenesis and function. *RNA, 15*(9), 1640–1651. doi:10.1261/rna.1602609

Sun, Y. S., Zhao, Z., Yang, Z. N., Xu, F., Lu, H. J., Zhu, Z. Y., . . . Jiang, J. (2017). Risk factors and preventions of breast cancer. *International Journal of Biological Sciences, 13*(11), 1387–1397. doi:10.7150/ijbs.21635

TeachMeSurgery. (2023). *Breast carcinoma in situ*. Retrieved from https://teachmesurgery.com/breast/malignant-disease/breast-carcinoma-in-situ/

Tsiakou, A., Sagour, F., & Zografos, E. (2019). Prognostic significance of miR-34 rs4938723 T>C polymorphism in triple negative breast cancer patients. *Clinical Biochemistry, 68*, 9–14. doi:10.1016/j.clinbiochem.2019.04.005

Winter, J., Jung, S., Keller, S., Gregory, R. I., & Diederichs, S. (2009). Many roads to maturity: MicroRNA biogenesis pathways and their regulation. *Nature Cell Biology, 11*(3), 228–234. doi:10.1038/ncb0309-228

Zhang, L., Liao, Y., & Tang, L. (2019). MicroRNA-34 family: A potential tumor suppressor and therapeutic candidate in cancer. *Journal of Experimental & Clinical Cancer Research, 38*, 53. doi:10.1186/s13046-019-1066-z

APPENDICES

APPENDIX A
REAGENT PREPARATION

1. Preparation of a stock solution of 10x Tris –borate-EDTA (TBE) buffer

1.1 Tris base	108 g
1.2 Boric acid	55 g
Dissolve in 800 ml distilled water (used stirring mix)	
1.3 Add 0.5 M Na_2EDTA (pH 8.0)	40 ml
Adjust volume to 1,000 ml	

APPENDIX B
SUMMARY DATA

Table B.1 Summary data of Breast patient samples

Code	Age	Histology	Grade	Tumor size (cm.)	Stage	ER	PR	HER2
BC2	42	IDC	II	3	IIIB	neg	neg	neg
BC3	61	IDC	II	3.5	IIIA	neg	neg	neg
BC5	51	IDC	II	4.5	IIIB	neg	neg	2+/equi
BC7	53	IDC	II	1.5	IIA	neg	neg	neg
BC8	64	IDC	-	2.5	IIIA	3+	2+	1+/neg
BC9	51	IDC	II	2.5	IIA	3+	2+	neg
BC11	54	IDC	III	2.5	IIB	neg	neg	neg
BC13	60	IDC	III	2.8	IIIA	neg	neg	neg
BC16	43	IDC	III	1	IIA	3+	3+	neg
BC29	56	IDC	I	2.3	IIB	neg	neg	neg
BC30	51	IDC	III	9	IIIB	neg	neg	3+
BC57	48	IDC	III	10	IIIA	neg	neg	2+/equi
BC80	48	IDC	III	3.5	IIIA	neg	neg	neg
BC91	45	IDC	III	1.5	IIB	1+/neg	neg	1+/neg
BC97	56	MC	II	3.7	IIA	3+	neg	neg
BC116	54	IDC	II	-	II	neg	neg	neg
BC117	55	ILC		2	IIIA	neg	neg	1+/neg
BC129	70	IDC	II	2.5	IIA	3+	3+	1+/neg
BC131	56	IDC	III	3.5	IIIA	3+	2+	neg
BC134	53	IDC	II	5.1	IIIA	2+	3+	1+/neg
BC136	53	IDC	I	0.8	I	2+	2+	2+/equi
BC139	51	IDC	III	5	IIB	neg	neg	neg
BC142	40	IDC	II	2.7	IIA	3+	3+	3+
BC146	32	IDC	II	1.5	IIA	neg	neg	2+/equi
BC147	31	IDC	II	1	I	3+	3+	neg
BC149	41	IDC	III	1.6	III	2+	3+	3+
BC150	64	IDC	III		-	neg	neg	2+/equi
BC151	53	IDC	II	1.3	I	3+	2+	neg
BC152	59	IDC	II	2	IV	neg	neg	neg
BC153	57	IDC	III	1.6	IV	3+	3+	neg
BC154	46	IDC	II	2.5	IIA	2+	1-10/pos	2+/equi
BC157	56	IDC	II	3.53	IIA	neg	neg	neg
BC158	50	IDC	III	3	IIA	neg	neg	neg
BC159	43	IDC	III	3.7	IIIA	3+	2+	neg
BC161	55	IDC	III	1.9	II	3+	neg	2+/equi
BC163	39	IDC	III	6	IIIB	neg	neg	neg
BC166	57	IDC	III	3.5	IIIB	3+	2+/neg	2+/equi
BC168	55	IDC	III	2.5	III	neg	neg	neg

Table B.1 Summary data of Breast patient samples (Cont.)

Code	Age	Histology	Grade	Tumor size (cm.)	Stage	ER	PR	HER2
BC171	60	IDC	II	1.3	IIA	2+	3+	1+/neg
BC172	58	IDC	-		IIIA	3+	neg	neg
BC174	71	IDC	II	3.1	IIA	3+	3+	neg
BC176	34	IDC	III	2.2	IIIA	neg	neg	neg
BC186	75	IDC	II	5.2	IIIA	neg	neg	neg
BC187	43	IDC	II	2.1	IIIA	2+	2+	2+/equi
BC188	31	IDC	III	4.8	IIA	neg	neg	neg
BC191	54	ILC	III	2.5	IIIA	neg	neg	neg
BC194	56	IDC	III	3.5	IIIA	neg	neg	neg
BC195	69	IDC	II/III	3	IIIA	3+	3+	neg
BC202	62	IDC	II	2.3	IIA	neg	neg	neg
BC203	57	IDC	II	1.5	II	2+	neg	2+/equi
BC204	52	IDC	III	2	II	2+	3+	neg
BC208	40	IDC	III	3	IIB	neg	neg	neg
BC211	43	IDC	III	6.5	IV	neg	neg	3+
BC213	42	IDC	III	2	III	neg	neg	neg
BC215	43	IDC	II	1.8	IIA	3+	3+	neg
BC224	56	IDC	III	1.8	I	neg	neg	neg
BC231	56	IDC	II	3.8	IIIB	neg	neg	1+/neg
BC232	38	IDC	III	2.5	IIB	3+	3+	2+/equi
BC236	61	IDC	-	-	-	1+/pos	neg	3+
BC244	52	IDC	III	-	IIIB	neg	neg	neg
BC245	52	IDC	III	2.8	I	neg	neg	neg
BC250	44	IDC	II/III	-	I	3+	neg	neg
BC256	38	IDC	III	3	III	neg	neg	neg
BC262	50	IDC	II	4	IIA	neg	neg	neg
BC265	60	IDC	III	2.5	IV	neg	neg	neg
BC268	46	IDC	III	-	III	3+	3+	3+
BC274	60	IDC	III	2.3	IIA	1+/pos	neg	1+/neg
BC280	53	IDC	-	3	IV	neg	neg	neg
BC282	36	IDC	III	1.3	IIIB	neg	neg	neg
BC287	42	IDC	II	2	I	neg	neg	neg
BC292	62	IDC	III	4	IIB	neg	neg	3+
BC308	41	IDC	III	3	IIIA	3+	neg	neg
BC322	37	IDC	III	-	-	2+	3+	neg
BC326	55	IDC	III	1.5	III	neg	neg	neg
BC348	36	IDC	III	1.5	IIA	neg	neg	neg
BC355	66	IDC	-	-	-	neg	neg	1+/neg

Table B.1 Summary data of Breast patient samples (Cont.)

Code	Age	Histology	Grade	Tumor size (cm.)	Stage	ER	PR	HER2
BC359	44	IDC	II	3	IIA	neg	neg	neg
BC378	59	IDC	III	1.7	IIA	neg	neg	neg
BC379	70	IDC	-	-	-	neg	neg	neg
BC383	58	IDC	II	-	-	neg	neg	1+/neg
BC397	50	IDC	II	-	II	neg	neg	neg
BC401	35	IDC	III	-	-	neg	neg	neg
BC405	46	Secretory	-	-	IIB	neg	neg	neg
BC418	55	NC	II	2	-	neg	neg	neg
BC421	44	IDC	III	4.4	IIB	neg	neg	1+/neg
BC426	77	IDC	III	3	IIA	neg	neg	neg
BC428	55	IDC	II	-	-	neg	neg	neg
BC440	65	IDC	III	4.5	IIB	neg	neg	1+/neg
BC448	56	IDC	III	-	-	neg	neg	neg
BC456	49	IDC	III	-	IIB	neg	neg	neg
BC467	55	IC	-	-	-	neg	neg	neg
BC471	44	IDC	III	-	III	neg	neg	1+/neg
BC476	30	IDC	III	5	IIB	neg	neg	1+/neg
BC485	26	IDC	II	2	IIIA	neg	neg	neg
BC491	44	IDC	III	1.3	IIA	neg	neg	neg
BC506	62	IDC	II	-	-	neg	neg	neg
BC507	49	IDC	III	1.5	II	neg	neg	neg
BC553	60	IDC	III			neg	neg	neg
BC554	57	IDC	III			neg	neg	neg
BC563	37	IDC	III	3.5		neg	neg	neg

BIOGRAPHY

Name	Thaworn Kongton
Education background	Chulalongkorn University Bachelor of Engineering, 2002 Chulalongkorn University Master of Business Administration, 2009 Sukhothai Thammathirat Open University Bachelor of Science in Occupational health and Safety, 2017 Rangsit University Master of Science in Biomedical Sciences, 2024
Place of work	Sahaviriya Steel Industries Public Company Limited
Work position	Assistant Vice President – Business Development