Study of WT1 Gene Expression in Human Breast Cancer

Tapanawan Nasomyon

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Author	Miss Tapanawan Nasomyon
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Major Advisor:

…….....

(Asst. Prof. Dr. Potchanapond Graidist)

Co advisor:

…….....

(Assoc. Dr.Surasak Sangkhathat)

…….....

(Dr. Srila Samphao)

Examining Committee :

……..Chairperson

(Dr. Danai Tiwawech)

……..

(Asst. Prof. Dr. Bhutorn Canyuk)

……..

(Asst. Prof. Dr. Potchanapond Graidist)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Sciences

……..…….....

(Assoc.Prof.Dr.Teerapol Srichana)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……Signature

(Asst. Prof. Dr. Potchanapond Graidist)

Major Advisor

……Signature

(Miss Tapanawan Nasomyon)

Candidate

I hereby certify that this work has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

……Signature

(Miss Tapanawan Nasomyon)

Candidate

ชื่อวิทยานิพนธ์ การศึกษาการแสดงออกของยีนดับบลิวทีวัน (WT1) ในมะเร็งเต้านม
ผู้เขียน นางสาวฐาปนาวรรณ นาสมยนต์
สาขาวิชา ชีวเวชศาสตร์
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บทคัดย่อ

ยีนดับบลิวทีวัน (WT1) ปกติ (wild type) มีบทบาทเป็นยีนก่อมะเร็งในมะเร็งหลายชนิด ยีน WT1 แสดงออกสูงในมะเร็งเต้านม ซึ่งมีความสัมพันธ์กับการพยากรณ์โรคไม่ดีและ มะเร็งเต้านมชนิดร้ายแรง การเกิดกระบวนการตัดที่ตำแหน่ง exon 5 และ exon 9 ของ mRNA ทำให้เกิดการสร้างโปรตีนจำนวน 4 ไอโซอร์มหลัก ได้แก่ ไอโซฟอร์ม WT1(+/+), WT1(+/-), WT1(-/+) และ WT1(-/-) ซึ่งแต่ละไอ โซฟอร์มมีบทบาทแตกต่างกันขึ้นอยู่กับชนิดของเซลล์และโปรตีนที่อยู่ข้างเคียง การศึกษานี้ได้ศึกษาถึง การแสดงออกของ ไอโซฟอร์มของ WT1 ในชิ้นเนื้อมะเร็งเต้านม โดยใช้วิธี RT-PCR และศึกษา ความสัมพันธ์ข องไอโซฟอร์มของ WT1 กับการแสดงออกของโปรตีน ER-α และ HER2 โดยใช้วิธี Western blot analysis รวมทั้งศึกษาถึงบทบาทของไอโซฟอร์มด้วยวิธีการเพิ่มการแสดงออก แบบถาวรในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MCF-7 จากนั้นเซลล์เหล่านี้จะถูกลดการแสดงออกของยีน WT1 โดยใช้ siRNA ที่จำเพาะต่อยีน WT1 ($siRNA_{WT1}$) จากการทดลองพบว่า WT1(17AA-) มีการแสดงอ อกสูงในชิ้นเนื้อเต้านมปกติ ทั้งในระดับ mRNA และโปรตีน ขณะที่พบการแสดงออกของ ทั้ง WT1(17AA+) และ WT1(17AA-) ในระดับ mRNA และโปรตีน โดยมีการแสดงออกที่สูงอย่างมีนัยสำคัญทางสถิติในชิ้นเนื้อมะเร็งเต้านม นอกจากนี้ยังพบว่าการแสดงออกของ ทั้ง WT1(17AA+) และ WT1(17AA-) มีความสัมพันธ์ในทิศทางเดียวกันกับการแสดงออก ที่สูงขึ้นของ โปรตีน ER-α และ HER2 และพบว่า การแสดงออก ที่สูงของ WT1(+/+) และ WT1(+/-) ซึ่งมีความสัมพันธ์กับการแสดงออก ที่สูงขึ้นของโปรตีน ER-α และ HER2 ด้วยยิ่งไปกว่านั้น การ แสดงออก ที่ลดลง ของ WT1(+/+) และ WT1(+/-) ด้วย $siRNA_{WT1}$ นำไปสู่การลดลงของโปรตีน ER-α และ HER2 และจำนวนเซลล์ ดังนั้นจึงสามารถสรุป ได้ว่า WT1(17AA+) อาจมีบทบาทเป็นยีนก่อมะเร็งโดย สัมพันธ์กับการเพิ่มขึ้นของโปรตีน ER-α และ HER2 ในมะเร็งเต้านม อย่างไรก็ตามการควรศึกษาบทบาทของ WT1 เพิ่มเติมเพื่อให้ทราบถึงกลไกที่ชัดเจนในมะเร็งเต้านม

Thesis Title Study of WT1 gene expression in human breast cancer.

Author Miss Tapanawan Nasomyon

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ABSTRACT

The wild-type of WT1 gene plays an oncogenic role in several cancers. WT1 highly expresses in breast cancer that is correlated with poor prognosis and malignant of breast cancer. Alternative splicing at exon 5 and exon 9 generates four major protein isoforms including (-/-), (+/-), (-/+) and (+/+). Each WT1 isoforms plays different roles, depending on cellular and protein context. This study investigated the expression of WT1 isofroms in breast cancer tissues using Reverse transcription-polymerase chain reaction (RT-PCR) and determined the correlation of WT1 with ER-α and HER2 protein using Western blot analysis. Moreover, the correlation of WT1 was investigated in MCF-7 cells, stably overexpressing WT1 isoforms. All overexpressing cells were decreased WT1 expression using $siRNA_{WT1}$. The results showed that WT1(17AA-) mRNA and protein were significantly found in adjacent normal breast tissues. Whereas, mixture of WT1(17AA+) and WT1(17AA-) of mRNA and protein significantly expressed in breast carcinoma tissues. The expression of mixed WT1(17AA+) and (17AA-) isoforms positively correlated with ER-α and HER2 expression. Overexpression of WT1(+/+) and WT1(+/-) associated with high expression of ER-α and HER2. Moreover, the reduction of WT1(+/+) and WT1(+/-) isoforms by $siRNA_{WT1}$ led to a decrease of both ER-α and HER2 proteins and number of cells. These results suggest that WT1(17AA+) may play oncogenic role in breast carcinoma tissues by upregulation of ER-α and HER2. However, function of WT1 isoforms should be further explored to address the mechanism in breast cancer.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	=	Amino acid
Ab	=	Antibody
AI	=	Aromatase inhibitors
AJCC	=	American Joint Committee on Cancer Staging
AKT	=	Protein kinase B
AML	=	Acute Myeloid Leukemia
APS	=	Ammonium persulfate
AP-1	=	Adaptor protein 1/ Adaptin 1
ATCC	=	American Type Culture Collection
Bak	=	Bcl-2 homologous antagonist killer
Bax	=	Bcl-2-associated X protein
Bcl-2	=	B cell lymphoma 2
bp	=	Base pairs
BP	=	Binding protein
BSA	=	Bovine serum albumin
$CaCl_2$	=	Calcium chloride
Cav	=	Calveolin 1
cDNA	=	complementary DNA
CL-AML	=	cytogenetically normal acute myeloid leukemia
C-terminal	=	Carboxyl-terminus
cm	=	centimeter
CoR	=	Corepressor
CO_2	=	Carbon dioxide
CSC	=	Cancer stem cell
CSF-1	=	Macrophage colony-stimulating factor
Cyclin D1	=	Cyclin protein D1
°C	=	Degree Celsius

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

DCIS	=	ductal carcinoma in situ
DEME	=	Dulbecco's modified eagle medium
DEPC	=	Diethyl pyrocarbonate
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
DNTM3A	=	DNA methylationtransferase 3A
dNTP	=	deoxynucleotide triphosphate
dsRNA	=	double-stranded RNA
DTT	=	D, L-dithiotreitol
DW	=	Distilled water
E. coli	=	*Escherichia coli*
*Eco*RI	=	restriction endonuclease I isolated from *Escherichia coli*
EDTA	=	Ethylenediaminetetraacetic acid
e.g.	=	for example
EGF	=	Epidermal growth factor
EGFR	=	Epidermal growth factor receptor
Egr-1	=	Early growth response protein 1
ER	=	Estrogen receptor
ERBB/HER	=	human epidermal growth factor receptors
ERE	=	Estrogen responsive element
ERK	=	Extracellular signal-regulated kinases
ER-α	=	Estrogen receptor alpha
et al.	=	Et ali (Latin) and others
EtBr	=	Ethidium bromide
FBS	=	Fetal Bovine Serum
g	=	gram
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

GF	=	Growth factor
GPCR	=	G protein coupled receptor
G6PD	=	Glucose-6-phosphate dehydrogenase deficiency
h	=	hour (s)
Ham's F-12	=	medium containing putrescine, hypoxanthine, thymidine and serum-free growth single-cell plating of Chinese Hamster Ovary (CHO) cells
HB-EGF	=	Harpin binding epidermal growth factor-like growth factor
HCl	=	Hydrochloric acid
HER2/neu	=	Human epidermal growth factor receptor 2
i.e.	=	id. Est, for example
HLA	=	Human leukocyte antigen
IDC	=	Invasive ductal carcinoma
IGFB	=	Insulin- like growth factor-binding protein
IGF-1	=	Insulin-like growth factor 1
IGFII	=	Insulin-like growth factor 2
IGFIR	=	Insulin-like growth factor receptor 1
IgG	=	Immunoglobulin G
ILC	=	Invasive lobular carcinoma
KCl	=	Potassium chloride
KDa	=	kilo Dalton (s)
KH_2PO_4	=	potassium dihydrogen phosphate
*Kpn*I	=	restriction endonuclease I isolated from *Klebsiella pneumonia*
KTS	=	Lysine, Threonine, serine
L	=	liter
LIQ	=	Lower inner quadrant
LOQ	=	Lower outer quadrant
Loqs	=	Loquacious

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

LB	=	luria-Bertani (medium)
LCIS	=	lobular arcinoma in situ
M	=	Molar
mA	=	milliampare
MAPK	=	Mitogen-activated protein Kinases
MEK	=	MAPK/ERK kinase
mg	=	milligram
$MgCl_2$	=	Magnesium chloride
min	=	minute (s)
miRNA	=	microRNA
MISS	=	Membrane-initiated steroid hormone
ml	=	milliliter
mM	=	milimolar
MMPs	=	Matrix metalloproteinase
MNAR	=	Modulator of non-genomic action of estrogen receptor
mRNA	=	Messenger RNA
MTA1s	=	Metastasis associated gene 1
NaCl	=	Sodium chloride
$NaHCO_3$	=	Sodium hydrogen carbonate
Na_2HPO_4	=	Disodium phosphate
NaOH	=	Sodium hydroxide
NCBI	=	National cancer for biotechnology information
NISS	=	Nuclear-initiated steroid signaling
nM	=	Nanomolar
nm	=	Nanometer
nmole	=	Nanomole
No.	=	Number
nt	=	nucleotide

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

N-terminal	=	amino-terminus/ amine-terminus
OD_{260}	=	Optical density at 260 nm
OD_{280}	=	Optical density at 280 nm
OD_{600}	=	Optical density at 600 nm
OD_{620}	=	Optical density at 620 nm
PAGE	=	Polyacrylamide gel electrophoresis
Par-4	=	Prostate apoptosis response-4
Ras	=	rat sarcoma viral oncogene homolog
PBS	=	Phosphate buffer saline
PDGF-A	=	Platelet-derived growth factor subunit A
pH	=	power of hydrogen
		(measurment of the hydrogen ion concentration)
PI3K	=	Phosphoinositide 3-kinase
P53	=	Protein 53
RAF	=	Rapidly accelerated fibrosarcoma
RAS	=	Rat sarcoma
Rb	=	Retinoblastoma gene
RICS	=	RNA induced silencing complex
RITS	=	Transcriptional gene Silencing complex
RNA	=	Ribonucleic acid
RNAi	=	RNA interference
RNase A	=	Ribonuclease A
RNP	=	ribonucleoprotein complex
rpm	=	Revolutions per minute
RPMI	=	Roswell Park Memorial Institute (medium)
rRNA	=	ribosomal RNA
RT	=	Room temperature

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

RT-PCR	=	Reverse transcriptase-polymerase chain reaction
Scr	=	Sacroma
SD	=	Standard deviation
SDS	=	Sodium dodecyl sulfate
sec	=	second
Ser	=	Serine
shRNA	=	Short hairpin RNA
siRNA	=	Small interference RNA
siRNAs	=	interfering RNAs
SNP	=	Single Nucleotide Polymorphisms
snoRNA	=	small nucleolar RNA
STET	=	Sodium choride-Tris-EDTA-Triton
TAM	=	Tamoxifen
Taq polymerase	=	thermostable DNA polymerase isolated from *Thermus aquaticus*
TBE	=	Buffer containing Tris base, Boric acid and EDTA
TBS	=	Tris-buffered saline
TEMED	=	N, N, N′, N′-tetramethylethylenediamine
TGF-1	=	Transforming growth factor 1
TGFα	=	Transforming growth factor alpha
TNF	=	Tumor necrosis factor alpha
Tm	=	melting temperature
Tris	=	Tris (hydroxymethyl) aminomethane
Tris-HCl	=	Tris (hydroxymethyl) aminomethane hydrochloric acid
tRNA	=	transfer RNA
TTBS	=	Tris-bufferd saline with Tween20
U	=	Unit (s)
UIQ	=	Upper inner quadrant

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

UOQ	=	Lower inner quadrant
UV	=	Ultraviolet
V	=	Volt
v-Myc	=	myelocytomatosis viral oncogene homolog
v/v	=	volume per volume
Vegf-a164	=	Vascular endothelial growth factor a164
WNT7B	=	Wnt signaling protein 7B
WT1	=	Wilms' tumor 1
w/v	=	weight per volume
*Xho*I	=	restriction endonuclease I isolated from *Xanthomonas campestris*
µl	=	microliter
µg	=	microgram
µM	=	micromolar
11p13	=	chromosome region at the short arm of chromosome 11

CHAPTER 1

INTRODUCTION

1.1 Backgound and Rationale

Cancer is a common malignant tumor. The total number of cancer cases between 2000 to 2020 is predicted to increase by 73% in developing country and 29% in developed country (Parkin et al., 2001; WHO, 2008). According to the American Cancer Society, there is new cases of invasive breast cancer approximately 192,370 persons who are diagnosed in women of U.S., and breast cancer deaths are estimated about 40,170 cases in 2009 (American Cancer Society, 2009). In Thailand, breast cancer is the first common cancer in women, and the expected incidence rate of breast cancer in 2007 is 20.5 per 100,000 cases. (Hugo and Jianjun, 2006; Khuhaprema et al., 2007; Bureau of Health Policy and strategy, 2008).

Breast cancer is one of disease that has been the cause from abnormal cells resulting in uncontrolled cell growth. Normally, mutation of one or more genetic material is the common feature of most tumors including breast cancer that enhance abnormal cell proliferation (Garrett, 2001). WT1 gene plays an oncogenes properties. Interestingly, breast cancer exhibits high level of WT1 protein and mRNA. The previous report demonstrated that WT1 mRNA and protein are markedly found in nearly 90% of breast cancers but not in normal breast tissues (Loeb et al., 2001). Silberstein et al. studied the WT1 expression by immunohistochemistry. Their investigation showed the correlation of the expression of WT1 protein with a biologically aggressive phenotype of breast cancer as ER status (Silberstein et al., 1997). Moreover, high WT1 mRNA expression involve in the poor prognosis of breast cancer patients (Miyoshi et al., 2002). These basic observations are steady with the conclusion that high WT1 expression is aberrant evidence in breast cancer. Therefore, this work underline to study how is different WT1 isoform expresstion in breast cancer and its function correlating with ER-α and HER2. Our work will address the possiblility that the different WT1 isoforms are an important function in breast cancer development.

In our previous study, Navakanit and coworker (2007) concludes that WT1 is necessary in breast cancer survival, and it might be a target for gene therapy. Moreover, Graidist

and research team (2010) found that WT1+/+, WT1+/- isoforms play anti-apoptotic function in breast cancer. Another associated studies shows that estrogen receptor (ER) has been essential molecule for an intimate crosstalk between the ER and HER family signaling pathways (Arpino et al. 2008). Moreover, WT1 induced estrogen-independent growth and anti-estrogen insensitivity in ER-positive breast cancer MCF-7 cells through activation of EGFR and HER2 expression (Wang et al., 2010). These previous observation underlines to explore oncogenic role of WT1 in breast cancer. Since there are rare reports involved the role of WT1 isoform in the genesis of breast still unclear, Also the molecular mechanisms of WT1 isoforms underlying of switch from estrogen-dependent to estrogen-independent growth that remains unclear. Therefore, more study of the WT1 isoform and function need to be insight explored. The present study involves the study of WT1 isoforms in human breast cancer and the correlation between WT1 expression and HER2, ER-α expression. Results from this study may be important for the studying function of WT1 isoforms and useful as a new diagnostic marker for choosing the most appropriate treatment and prognosis factor in breast cancer patients under endocrine therapy in the future.

1.2 Breast anatomy

The lobules (milk-producing grands), ducts (small tubes that carry milk from lobules to the nipple) and stroma (fatty and connective tissue surrounding blood vessels, lymphatic vessels, the lobules and the ducts) are main components of breast. The normal breast component consist of subcutaneous tissue, skin and breast tissue. Breast tissues includes both epithelial and stromal elements. The epithelial elements is made up 10% to 15% of the breast mass, with the remainder being stroma. Lobes of glandular tissue are supported by fibrous connective tissues, and each breast presents approximately 15 to 20% lobes of glandular tissue supported by fibrous connective tissues. The space between lobes is filled with adipose tissues, and the breast size depends on the density of adipose tissue and the layer of fatty tissue surrounding the breast grand that extends through the breast. Most breast cancers has original source to be in the ducts, some begin in the lobules, and a small number in start in other tissues. The normal structure of breast were shown in Figture 1 (Junqueira et al., 1995; American cancer society, 2013).

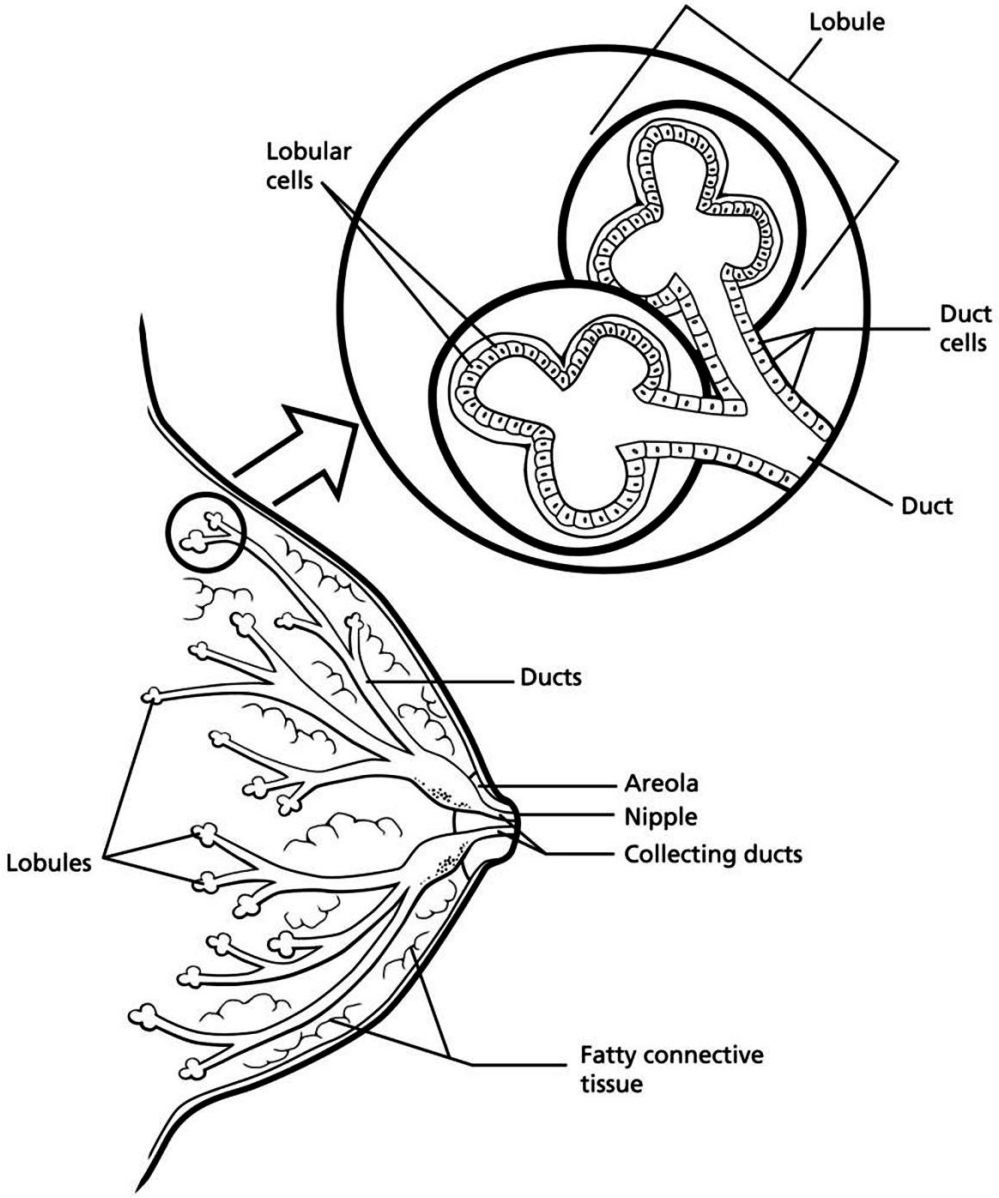

Figure 1. Schematic illustration of breast anatomy (American Cancer Society, 2013)

1.3 Breast cancer

Cancer is a group of diseases that arises from normal cell change to abnormal cell inside the body leading to uncontrolled abnormal cell growth. There are several risk factors that causes of breast cancer by both external factors such as tobacco, infectious organisms, chemicals, and radiation and internal factors including inherited mutations, hormones, immune conditions, and mutations that occur from metabolism. Breast cancer causality from many factors may play together to initiate or promote cancer development. Mostly, all common cancer cells ultimately form a lump or mass called a tumor, and are named following original source inside region of body (American cancer society, 2013).

Breast cancer is a malignant tumor that arises from cells of the breast, and it may originate in the ducts or the lobules and metastasizes beyond the breast duct or lobule wall. Breast cancer can spread to distance organs via the lymp system since the lymph nodes are small, and its bean-shaped collections of immune system cells that are circulated by lymphatic vessels. Breast cancer cells are carried to lymphatic vessels and it then begins to grow in lymph nodes. The lymph nodes can be found under the arm called axillary nodes, which is the main lymphatic vessels connecting with the breast. Another lymphatic vessels is called internal mammary nodes, which is lymphatic vessels connecting to lymph nodes inside the chest, and those either above called supraclavicular or below the collarbone called infraclavicular nodes, shown in Figure 2 (American cancer society, 2013).

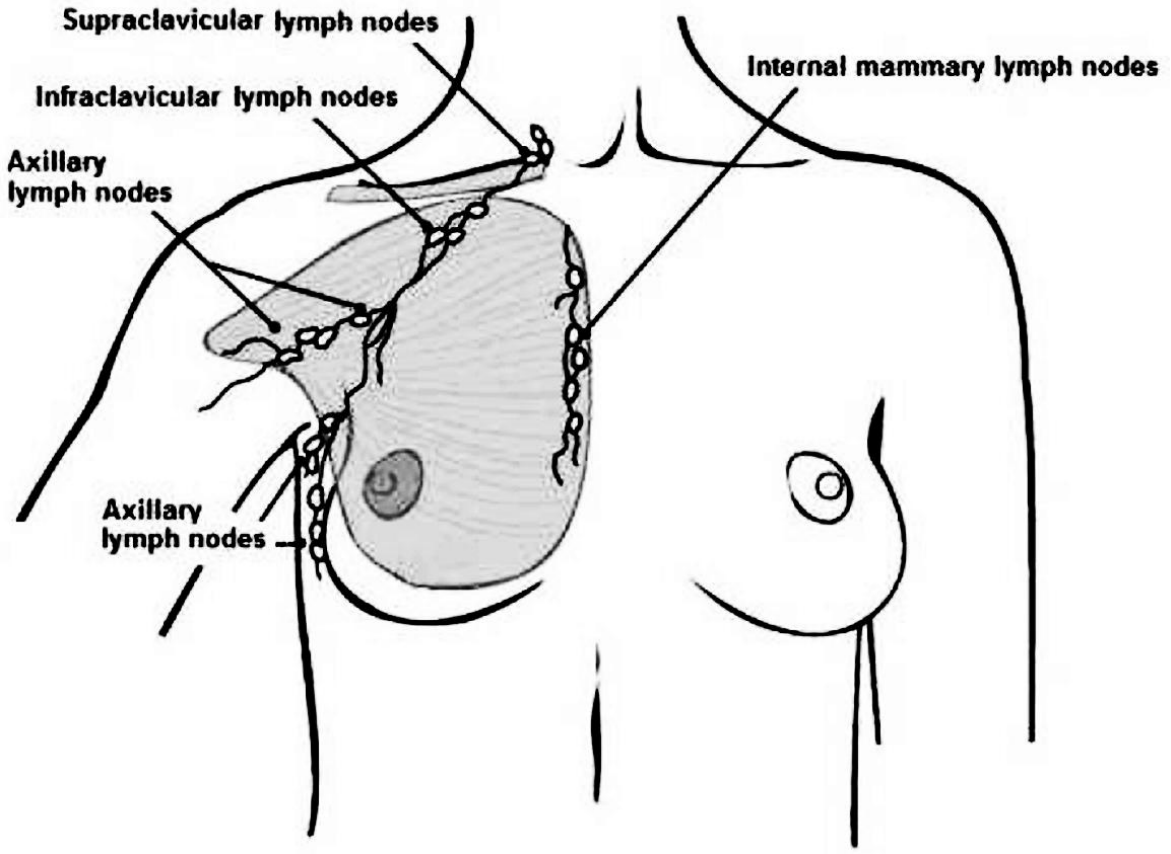

Figure 2. Lymphatic surrounding of the breast (American Cancer Society, 2013)

The breast is conventionally divided into four quadrants, upper outer quadrant (UOQ), upper inner quadrant (UIQ), lower outer quadrant (LOQ), lower inner quadrant (LIQ), and the axillary tail (Figure 3). The percentage of breast cancers is classified by region shown in Figure 4 (Joseph, 2013).

- Posterior to the nipple (34%),
- Upper outer quadrant UOQ (41%),
- Upper inner quadrant UIQ (14%),
- Lower outer quadrant LOQ (6%)
- Lower inner quadrant LIQ (5%)

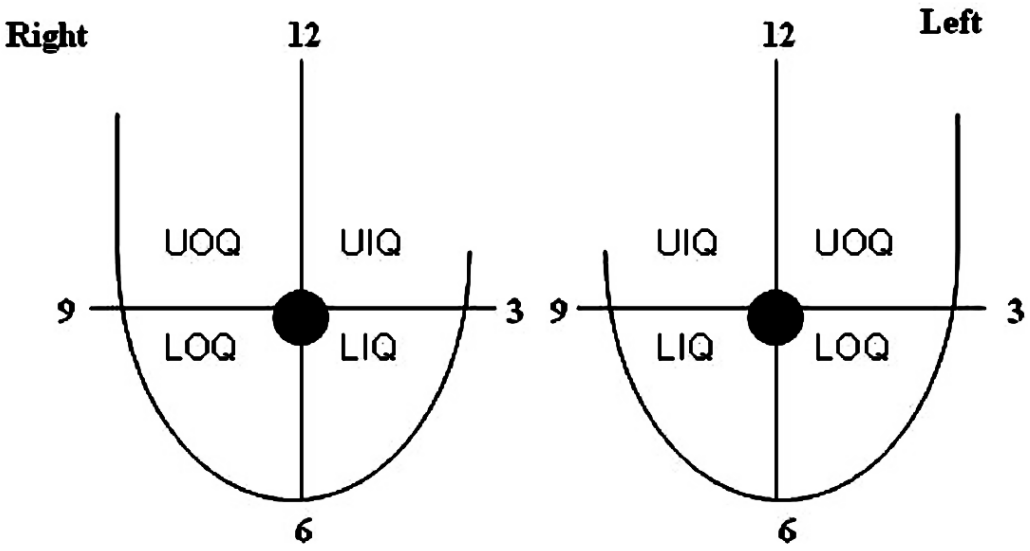

Figure 3. Schematic drawing demonstrates the four quadrants of breast. upper outer quadrant (UOQ), upper inner quadrant (UIQ), lower outer quadrant (LOQ), lower inner quadrant (LIQ) (American Cancer Society, 2013)

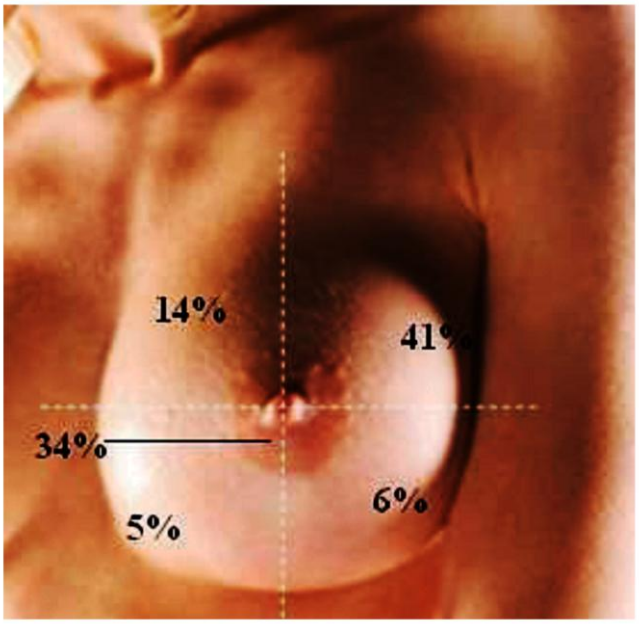

Figure 4. The relative locations and percents of cancers classified by regions (American Cancer Society, 2013)

1.3.1 Common breast cancer terms

Breast cancer can be divided into five major groups classified by origin source.

1.3.1.1 Carcinoma

The carcinoma is breast cancer that there is origin inside the lining layer of breast tissues. The carcinomas was named either ductal carcinomas or lobular carcinomas depending on origin region of breast.

1.3.1.2 Adenocarcinoma

An adenocarcinoma is a type of carcinoma that starts in glandular tissue. The glandular tissues consists of the ducts and lobules, so breast cancers originate from these tissues that is called adenocarcinomas.

1.3.1.3 Carcinoma *in situ*

The carcinoma *in situ* term is used for an early stage of cancer that means breast cancer cells localizing inside breast tissues. Breast cancer is called carcinoma *in situ* have not grown outside and not found spreading distance organ. The breast cancer cells locate limited to ducts that is called ductal carcinoma *in situ*. Whereas breast cancer cells are located only lobules region, it is named lobular carcinoma *in situ*.

1.3.1.4 Invasive (infiltrating) carcinoma

An invasive breast cancer is spreading of breast cancer cells form original source. The breast cancer cells spread from ducts that is called invasive ductal carcinoma. Whereas breast cancer cells are spread from lobules, it is called invasive lobular carcinoma.

1.3.1.5 Sarcoma

Breast cancer starting in connective tissue like muscle tissues, fat tissues or blood vessels, which is called Sarcomas.

1.3.2 Classification of breast cancer

The classification of breast cancer is divided by spreading of cancer cells. There are two major groups of breast cancer including noninvasive (*in situ*) carcinoma and invasive (infiltrating) carcinoma. Noninvasive carcinoma cancer cells are limited localized to the ducts and not found the invading of breast cancer cells surrounding fatty and connective tissues, whereas invasive carcinoma cancer cells invade from the duct and lobular and grow surrounding fatty and connective tissues of the breast (Rubin et al., 2005; Panomwan et al., 2005).

1.3.2.1 Carcinoma *in situ*

There are two types of carcinoma *in situ* composting of ductal carcinoma *in situ* and lobular carcinomar *in situ*.

1.3.2.1.1 Ductal carcinoma *in situ* (DCIS)

Ductal carcinoma *in situ* (DCIS) is one of a heterogeneous disease that defined as a neoplastic proliferation within the ductal structures of the breast. It is non-invasive or pre-invasive breast cancer, and DCIS cells locate inside and lined the ducts that change to look like breast cancer cells. It composed of very large, plemorphic cells that have abundant eosinophilic cytoplasm and irregular nuclei, also DCIS does not spread to the walls of duct into surrounding breast tissues.

1.3.2.1.2 Lobular carcinoma *in situ* (LCIS)

Lobular carcinoma in situ (LCIS) is one of breast cancer type, which originated and located in lobule of breast. It is not spread to other organ. The LCIS arises in the terminal duct units, and the form of LCIS cells present small and round with regular nuclei and minute nucleoli (Jones, 2006; Katz et al., 2007; Eheman et al., 2009).

1.3.2.2 Invasive carcinoma

Invasive breast cancer carcinoma is divided into two types containing invasive ductal carcinoma and invasive lobular carcinoma.

1.3.2.2.1 Invasive ductal carcinoma (IDC)

Invasive ductal carcinoma is the most common type of breast cancer, since the incidence of this breast cancer types was found up to 80% by clinical diagnosis, according to statistics from the United States in 2004. On a mammogram, it is usually visualized as a mass with fine spikes radiating from the edges. IDC is form in the milk ducts of the breast and penetrates to the wall of the duct and invades the fatty tissues of the breast also spread to other part of the body. On the gross examination, IDS exhibitss irregular margin, and the section surface is pale gray, gritty, flecked with yellow and chalky streaks. Histologically, the lesion is composed of dense fibrous stroma of tumor cells, dark nuclei with few mitosis.

1.3.2.2.2 Invasive lobular carcinoma (ILC)

Invasive lobular carcinoma is the second frequent histological breast cancer diagnosis that starts in milk-producing glands of the breast (lobules), and it often metastasizes to other region of the body. ILC contains the single strands of malignant cells infiltrating between stromatic fibers. There are approximately 1 invasive breast cancer in 10 that is an ILC, and ILC may be difficultly detected by a mammogram comparing to IDS.

1.3.3 Stage of breast cancer

The stageing system for breast cancer was updated in 2002. The American Joint Committee on Cancer (AJCC) TNM system is used as standard staging system for breast cancer diagnosis including designations for micro-metastases and isolate tumor cells, and separate classification of node (N) status was based on the number of involved lymph nodes. Metastases (M) to the supraclavicular nodes were reclassified as N3 rather than M1. Definitions for classifying the primary tumor (T) are the same for clinical and for pathologic classification. Stage IIIC breast includes patients with any T stage and they have pN3 disease. Whereas patients with pN3a and pN3b disease are classified as stage I, II, IIIA, and the patient was characterized as IIIC breast cancer. Patients with pN3c disease concluded stage IIIB or IIIC or inflammatory breast cancer. Patients in stage IV may or may not have spread to the axillary lymph nodes and there are any size of tumor (up to 2 cm, or 2 to 5 cm, or larger than 5 cm), also breast cancer cell can other organ of body. The TNM staging system and the anatomic stage of breast cancer is summarized and shown in Table 1 and Table 2, respectively (Greene et al., 2002; Woodward et al., 2003; American Joint Committee on Cancer, 2010).

Table 1. The TNM staging system by The American Joint Committee on Cancer (AJCC) (American Joint Committee on Cancer, 2010)

Primary tumor (T)	**Regional lymph nodes (N)**	**Distant metastasis (M)**
TX is primary tumor that cannot be assesed **T0** is no evidence of primary tumor **Tis** is intraductal carcinoma, lobular carcinoma *in situ*, or Paget's disease of the nipple with no associated invasion of normal breast tissues. **Tis** (DCIS) is ductal carcinoma in situ **Tis** (Pagets's) is Paget's disease of the nipple with no tumor. **T1** is tumor not larger than 2.0 cm in greatest dimension **T1mic** is microinvasion not larger than 0.1 cm in greatest dimension **T1a** is tumor larger than 0.1 cm but not larger than 0.5 cm in greatest dimension **T1b** is tumor larger than 0.5 cm but not larger than 1.0 cm in greatest dimension **T1c** is tumor larger than 1.0 cm but not larger than 2.0 cm in greatest dimension **T2** is tumor larger than 2.0 cm but not larger than 5.0 cm in greatest dimension **T3** is tumor larger than 5.0 cm in greatest dimension **T4** is of any size with direct extention to (a) chest wall or (b) skin, only as described below **T4a** is an extension to chest wall, not including pectoralis muscle **T4b** is edema or ulceration of the skin of the breast, or satellite skin nodules confined to the same breast **T4c** is T4a and T4b **T4d** is inflammatory carcinoma	**NX** is regional lymph nodes that can not be assessed **N0** is no regional lymph node metastasis **N1** is metastasis to movable ipsilateral axillary lymph node (s) **N2** is metastasis to ipsilateral axillary lymph node (s) that fixed or matted, or in clinically apparent[a] ipsilateral internal mammary nodes in absence of clinically evident lymph node metastasis. **N2a** is metastasis in ipsilateral axillary lymph node that fixed to one another (matted) or to other structure **N2b** is metastasis only in clinically apparent[a] ipsilateral internal mammary nodes and the absence of clinically evident axillary lymph node metastasis **N3** is metastasis in metastasis in ipsilateral infraclavicular lymph node (s) with or without axillary lymph node involvement, or in clinically apparent[a] ipsilateral internal mammary lymph node (s) and in the presence of clinically evident axillary lymph node metastasis; or, metastasis in ipsilateral supraclavicular lymph node (s) with or without axillary or internal mammary lymph node involvement **N3a** is metastasis in ipsilateral infraclavicular lymph node (s) **N3b** is metastasis in ipsilateral internal mammary lymph node (s) and axillary lymph node (s) **N3c** is metastasis in ipsilateral supraclavicular lymph node (s)	**MX** is presence of distant metastasis that cannot be assessed **M0** is no distant metastasis **M1** is distant metastasis

Table 2. The anatomic stage of breast cancer by The American Joint Committee on Cancer (AJCC) (American Joint Committee on Cancer, 2010)

Anatomic Stage/Prognotic Groups			
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T0	N1	M0
	T1	N1	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any T	M1

1.3.4 Grading of breast cancer

According to World Health Organization (WHO), breast cancer is precisely diagnosed, and the accuracy of prognosis are crucial to decrease the high death rate (Jelen et al. 2008). Grading system is factors for breast cancer prognosis. Breast cancer patients are characterized and evaluated by histological grade, and genome-wide microarray-based expression profiling studies (Rakha et al., 2010). For example, the Nottingham Grading System (NGS) is classified breast cancer by histological classification, and nuclear grade evaluation (World Health Organi zation (WHO), American Joint Committee on Cancer (AJCC), European Union (EU), and the Royal College of Pathologists (UK RCPath) (Elston and Ellis, 1991; Tavassoli and Devilee, 2003; Pathology Reporting of Breast Disease, 2005). In NGS histological classification, tumor grade is classified depending on the differentiated of breast tumor. The histological grade can evaluate into three morphological features by hematoxylin-eosin-staining composting of (a) degree of tubule or gland formation, (b) nuclear pleomorphism, and (c) mitotic count (Figure 5) (Rakha et al., 2010). In NGS nuclear grade evaluation, tumor grade is evaluated by size, shape, nucleus in the tumor cells, mitotic rates and cytological images. Grading of breast cancer is divided into four grades. Firstly, grade X is called undetermined grade that cannot be assed. Secondly, grade one is called low grade containing well differentiated cells, which look similar to normal cells and grow very slowly. Thirdly, grade two is called moderate or intermediated grade containing moderately differentiated cells, which are slightly faster growth. Next, grade three is called high grade that composts of poor differentiated cells. Lastly, grade four is called high grade consisting of undifferentiated cells (Jelen et al. 2008; American Joint Committee on Cancer, 2010; Rakha et al., 2010).

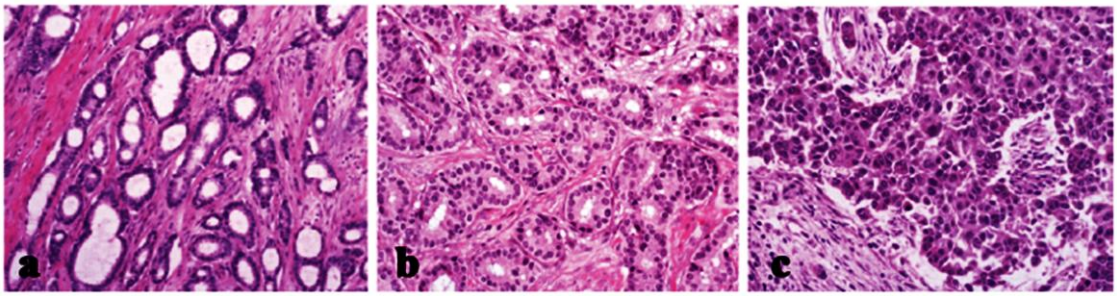

Figure 5. Immunohistochemistry for classification grading of breast cancer (Nothingham Grading System) (a) Breast cancer grade 1 exhibits a well-differentiated characteristic that the tissues presents high homology to duct lobular of normal breast tissue. Breast cancer potentially form the tubule up to 75%. (b) Breast cancer grade 2 exhibits a moderately differentiated characteristic comparing to normal breast tissues. (c) Breast cancer grade 3 exhibits a poorly differentiated tumor characteristic that dominantly presents the degree of cellular pleomorphism. Breast cancer grade 3 have a low capacity for tubule formation (Rakha et al., 2010).

1.3.5 Breast cancer subtypes

Breast cancer includes various distinct characteristics with different biological properties and clinical behavior. The morphological heterogeneity of breast cancer is classified into 6 main groups including two hormone receptor positive (luminal A and B), three hormone receptor negative groups (narmal breast-like, HER2+, and base-like) and claudin-low groups (Perou et al., 2000, Sorlie et al., 2003; Ibrahim et al., 2009). These classification is based on histological type, tumour grade, lymph node status and the presence of predictive markers such as human epidermal growth factor receptor 2 (HER2) and estrogen receptor (ER) (Holliday DL and Speirs V, 2011). The identification of luminal, basal, HER2 and claudin-low clusters in breast tumours was classified by using DNA microarrays to prove heterogeneity and molecular profiling, and ER-α, progesterone receptor (PR) and HER2 expression by immunohistochemical method. Molecular characteristics of these subtypes are shown in Table 3 (Perou et al., 1999; Perou et al., 2000) and the different morphology showing in Figure 6 (Neve et al., 2006; Kao et al., 2009; Mackay et al., 2009; Hollestelle et al., 2010; Keller et al., 2010; Prat et al., 2010). Moreover, the morphology of cell is also used for classification of breast cancer. In two

dimensions, the classic cobblestone morphology clearly found in luminal-like epithelial cells, and there is the expression of E-cadherin, which is cell-cell adhesion molecules. Whereas basal epithelial cells expresses vimentin protein that is markers of epithelial–mesenchymal transition. In contrast, cells grows in three-dimensional culture that shows characteristic as mass , round, stellate and grape-like (Kenny et al., 2007). MCF12A is normal human breast epithelial cells that forms round polarised acini-like structures. Breast cancer cell lines are identified as luminal A subtype such as T47D and MCF-7 cells, while BT474 cells is breast cancer cell lines that indicates in luminal B subtypes. Both luminal A and B subtypes can form tightly cohesive structures for displaying robust cell-cell adhesions. On the contrary, breast cancer cells are classified in basal subtype like MDA-MB-468 and in claudin-low subtype like MDAMB-231 and in HER2-positive subtype such as MDA-MB-453 and SKBR3 that forms loosely cohesive grape-like structures (Neve et al., 2006). The morphology of breast cancer cell lines in different breast cancer subtypes growing in two-dimensional and three-dimensional cultures are shown in Figure 6. T47D cells exhibits a tightly cohesive cobblestone appearance, whereas MDA-MB-453 cells shows an elongated and spindly appearance. Growing tree dimension, T47D cells form tightly cohesive mass structures displaying robust cell-cell adhesions, whereas MDA-MB-453 cells form loosely cohesive grape-like structures consistent with morphology observed by Kenny and colleagues (Kenny et al., 2007; Holliday DL and Speirs V, 2011).

Table 3. Molecular subtype of breast cancer (Holliday DL and Speirs V, 2011; Eroles et al., 2012).

Molecular subtype	Immunoprofile	Characteristrics genes	Frequency	Histological grade	Prognosis
Luminal A	ER+ PR+ HER2-	ESR1, GATA3, KRT8, KRT18, XBP1, FOXA1, TFF3,CCND1 , LIV1	50-60%	Low	Excellent
Luminal B	ER+/- PR+/- HER2+/-	ESR1, GATA3, KRT8, KRT18, XBP1, FOXA1, TFF3, SQLE, LAPTM4B	10-20%	Intermediate/High	Intermediate/Bad
HER2-enriched	ER- PR- HER2+	ERBB2, GRB7	10-15%	High	Bad
Basal-like	ER- PR- HER2-	KRT5, CDH3, ID4, FABP7, TRIM29, LAMC2	10-20%	High	Bad
Normal breast-like	ER-/+ HER2-	PTN, CD36, FABP4, AQP7, ITGA7	5-10%	Low	Intermediate
Claudin-low	ER- PR- HER2-	CD44, SNAI3	12-14%	High	Bad

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

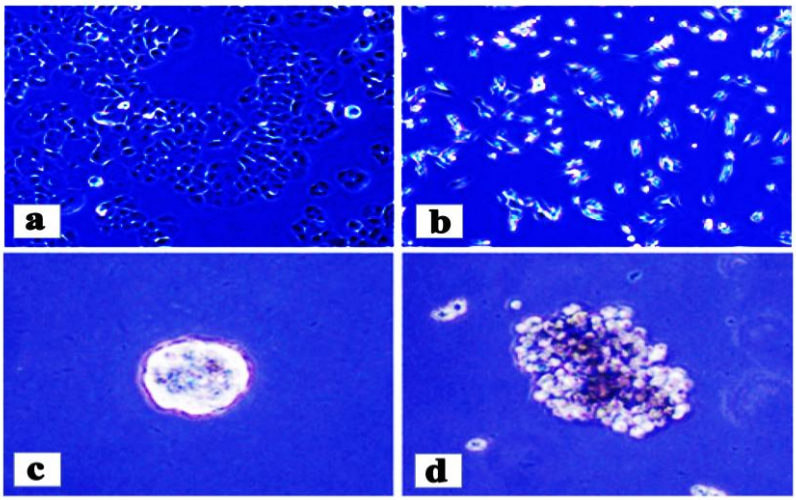

Figure 6. The morphology of breast cancer cell lines growing in two dimensional and three-dimensional cultures. Two-dimensional culture of (a) luminal A T47D and (b) HER2-positive MDA-MB-453 cell lines grown on tissue culture plastic. (c) T47D and (d) MDA-MB-453 cell lines cultured in three dimensions as previously (Holliday DL and Speirs V, 2011).

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1.3.5.1 Luminal A

The common of breast cancer in luminal A subtype was mostly found up to 50-60% of the total. Breast cancer in luminal is classified by detection of genes that were activated by ER transcription factor. Since there are high expression of ER in the mammary ducts, Breast cancer in luminal A is low expressed of genes correlated to the growth of cell (Perou et al., 2000; Sorlie et al., 2001). According to the gene profiles, breast cancer is classified as luminal A composting of lobular carcinoma *in situ* and infiltrating lobular carcinoma. All cases expressed ER, PGR, Bcl-2 and cytokeratin CK8/18. In addition, The absence HER2 protein is characterized by immunohistochemistry (IHC) method referring to luminal A subtype. Also breast cancer in luminal A exhibits the low expression of Ki67 to indicate that decrease of cell proliferation classifying low histological grade (Table 3). Breast cancer patients in this subtype have a good prognosis, and the recurrent rate is markedly lower comparing to other subtypes (Kennecke et al., 2010). Furthermore, survival of most patients from the recurrent time is also longer (median 2.2 years). Normally, the recurrence of breast cancer has an obvious pattern in patients. The most of breast cancer cells spreads to bone also localizes in other parts of body. There are several methods for treatment this breast cancer subgroup. For example, the postmenopausal patients were treated with aromatase inhibitors (AI), some patients were obtained tamoxifen or fulvestrant treatment (Guarneri et al., 2009; Eroles et al., 2012).

1.3.5.2 Luminal B

The luminal B breast cancer subtype is rarely detected between 10% and 20% of all breast cancers observing by molecular profiles. Breast cancer patients in this subtype have a more aggressive phenotype. They are classified in high grade of breast cancer, and there are proliferative index and worse prognosis. The most patients in luminal B have be recurrent of tumor in liver site. Additionally, the survival of patients is lower approximately 1.6 years of relapsed time (Kennecke et al., 2010). Luminal B patients dominantly express ER that is used as biomarker, and breast cancer cells in this subtype expresse proliferative genes, such as cyclin B1,

EGFR and HER2 (Table 3). Based on the immunohistochemical method, breast cancer cells with luminal B subtype express ER, high Ki67 or ER, and breast cancer cells has present or absent of HER2 expression. Patient in luminal B have a poor prognosis comparing to breast cancer patients in luminal A, who was carry the tamoxifen 9 and aromatase treatment (Eroles et al., 2012).

1.3.5.3 HER2 positive

The HER2 breast cancer is found 15-20% that was characterized by molecular pattern. The most patients in HER2 sub type highly expressed HER2 and gene related in HER2 signalling and cell growth. According to morphology, breast cancer cells highly proliferate leading to cancer progression, the most of breast patients with HER2 subtype was found as advance histological grade and present p53 mutations. This tumors poorly diagnosed with a 12% 10 year survival, whereas luminal A and luminal B groups are found the 50–55% survival (Staaf et al., 2010). In addition, expression of HER2 is used as strong prognostic marker for breast cancer diagnosis. There is over-expresses HER2 inside the cells leading to increasing of the breast cancer invasion. and worse immunity (Eroles et al., 2012).

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1.3.5.4 Basal-like

The common of breast carcinoma is classified as basal-like subtype approximately 10-20%. Gene profiles of basal-likes subtype is presented in myoepithelial cells of normal breast including cytokeratins CK5 and CK17, P-cadherin, caveolin 1 and 2, nestin, CD44 and EGFR, whereas luminal epithelium express CK8/18 and Kit (Table 3) (Burwell et al., 2007). Basal-like tumors are most frequent found in infiltrating ductal carcinoma. The distinct characteristic of basal-like subtype composts of the dominant of mitotic index, necrosis margins and a marked respons of lymphocyte (Livasy et al., 2006). The expression of ER, PR and HER2 is not found in these tumor, therefore it is called basal-like (Table 3). The basal-like breast cancer subtype expressed ER, PGR, HER2, EGFR and CK5/6 that these proteins were detected using immunohistochemistry. These tumors have a worse prognosis, and the patients is high recurrencein first 3 years (Dent et al., 2007) despite their presenting a high treatment by

chemotherapy (Sorlie et al., 2001; Sorlie et al., 2003; Rouzier et al., 2005). There is high p53 mutations in basal-like patients, and they display aggressiveness and poor prognosis (Sorlie et al., 2001). In addition, the basal-like subgroup is found that presents the mutation of BRCA1 classification by intrinsic subtypes (Sorlie et al., 2003; Eroles et al., 2012).

1.3.5.5 Normal breast-like

Normal breast-like tumors is found approximately 5-10% in breast carcinomas. It difficultly characterized, and have been classified with fibroadenomas and normal breast (Perou et al., 2000). The normal breast-like subtype express genes in adipose tissue that these subtype may grouped between basal-like and luminal. Breast cancer with normal breast-like subtype does not respond to neo-adjuvant chemotherapy, and it lacks the expression of ER, HER2, PR, CK5 and EGFR (Eroles et al., 2012).

1.3.5.6 Claudin-low

The Clandin-low subtype firstly identified in 2007 (Van Cutsem et al., 2011). It is characterized by a low expression of genes involved in tight junctions and intercellular adhesion including claudin-3, -4, -7 cingulin, ocludin, and E-cadherin hence the name claudin-low. Breast cancer in Claudin-low subtype is located in the hierarchical clustering near the basal-like tumors, and it shares some characteristic gene expression with the basal-like tumors. So both claudin-low and basal-like tumors slightly express HER2 and luminal gene cluster. Claudin-low tumors have a poor prognosis, albeit they present proliferative gene expression. Otherwise, they overexpress several genes related mesenchymal differentiation and epithelial-mesenchymal transition. These tumors rarely correlate with subset of tumors approximately 12-14%, and correspond to high grade infiltrating ductal carcinomas that present metaplastic or medullary differentiation (Hennessy et al., 2009; Prat et al., 2010). These tumors are grouped as poor long-term prognostic cancer (Prat et al., 2010) and difficult to treat with neoadjuvant chemotherapy (Ma et al., 2004; Eroles et al., 2012).

1.4 Wilms' Tumor 1 (WT1)

Wilms' tumor suppressor gene (WT1) was first detected and displays tumor suppressor properties, which plays a function in organ development, particularly differentiation and progression of the genitourinary system and mesothelial tissues and maintenance of the kidneys and heart (Lee and Haber, 2001; Scharnhorst et al., 2001; Scholz and Kirschner, 2005). Although the WT1 gene has been regarded as a tumor suppressor gene, but the wild-type of WT1 gene is significantly found in primary human leukemia (Inoue et al., 1994) also wide variety of solid cancer, including lung (Oji et al., 2002), colon (Oji et al., 2003), esophageal (Oji et al., 2004), breast (Loeb et al., 2001; Miyoshi et al., 2002), thyroid (Oji et al., 2003), pancreatic ductal cancer (Oji et al., 2004), head and neck sqaumous tumors (Oji et al., 2003), astocytic tumor (Oji et al., 2004), and bone and solf tissue sarcoma (Ueda et al., 2003). In addition, the wild-type of WT1 gene plays an oncogenic roles rather than tumor suppressor properties in tumorgenesis of several cancers (Sugiyama, 2001).

WT1 mutation cause the several syndromes such as Denys-Drash syndrome (Pelletier et al., 1991; Patek et al., 1999), Frasier syndrome (Barbaux et al., 1997; Klamt et al., 1998), and Wilms' tumor-Aniridai-Genitourinary malformaticns-Mental retardation (WAGR) syndrome (Gessler et al., 1990). The mutation of WT1 also found in variety malignancies including leukemia, lung cancer, rentinoblastoma and breast cancer (Wagner et al., 2003). The hypermethylation of WT1 gene by DNA methylationtransferase 3A (DNTM3A) leads to upregulation of WT1 expresion in human embryonal kindey-delived cell lines and Wilms't tumor cells (Szemes et al., 2012).

The most acute myeloid leukemia (AML) patients present WT1 wild type mutation that lead to worse treatment (Virappane et al., 2008; Hollink et al., 2009; Owen et al., 2010; Cunningham et al., 2013). WT1 play a role in hematopoietic development therefore WT1 mutation in embryonic stem cells affects to decrease of hematopoietic differentiation (Wagner et al., 2002). In addition, highly expression of Vascular endothelial growth factor a164 (Vegf-a164) can protect apoptotic cell death evidence in embryonic stem cells. Endogenous of Vegf-a164 are produced from wild-type embryonic stem cells, which is cleavage by WT1 regulation (Cunningham et al., 2013).

The different single nucleotide polymorphisms (SNP) that occur in WT1 gene at exon 1 and exon 7 were found in breast cancer patients. First SNP in exon 1, the nucleotide was changed from C to T nucleotide however a proline42 residule (Pro42) does not change. Second SNP in exon 7, the nucleotide was changed from A to G however an arginine residule (Arg300) does not change (Oji et al., 2004). Moreover, SNP is also found in WT1 gene at rs16754 ($WT1^{AG/GG}$) in exon 7 in cytogenetically normal acute myeloid leukemia (CL-AML). The minor allele of WT1 SNP rs16754 is a prognostic marker and may be associated with drug sensitive in CN-AML patients (Damm et al., 2010).

1.4.1 Structure Wilms' Tumor 1

The Wilms' tumor gene (WT1) was first identified as a tumor suppressor gene responsible for Wilms' tumor, a kidney neoplasm of childhood (Call et al., 1990; Gessler et al., 1990). The expression of WT1 was found during mammalian embryonic development in many tissues, including the genitourinary system, spleen, certain area of the brain, spinal cord, mesothelial organs, diaphragm and limb (Wagner et al., 2003; Scholz et al., 2005). The WT1 gene spans about 50 kb and locates at chromosome 11p13 (Call et al., 1990). This gene encodes for 10 exons and generates a 1.5 kb mRNA. There are two predominant alternative splicing events including the splicing of exon 5 (17 amino acids), and of a stretch of nine nucleotides (three amino acids: lysine (K), threoine (T), and serine (S)) in the 3´ end of exon 9 (Yang et al., 2007). Alternative splicing of these two sites gives the major four different protein isoforms designed A (-/-), B (+/-), C (-/+) and D (+/+), respectively (Haber et al., 1991). This occurence as a result of gene transcription initiating within a promoter in intron 1 (Dallosso et al., 2004). The N-terminal domain of WT1 comprises of proline-glutamine-rich sequences, which involved in RNA and protein interactions (Figure 7). This domain is an important region for transcription process in both transcriptional repression and activation domains. Whereas the C-terminal domain of WT1 composes of four Cysteine2-Histidine2 zinc fingers, which can bind directly to target DNA sequences. But this region are also interacts to RNA and protein (Yang et al., 2007).

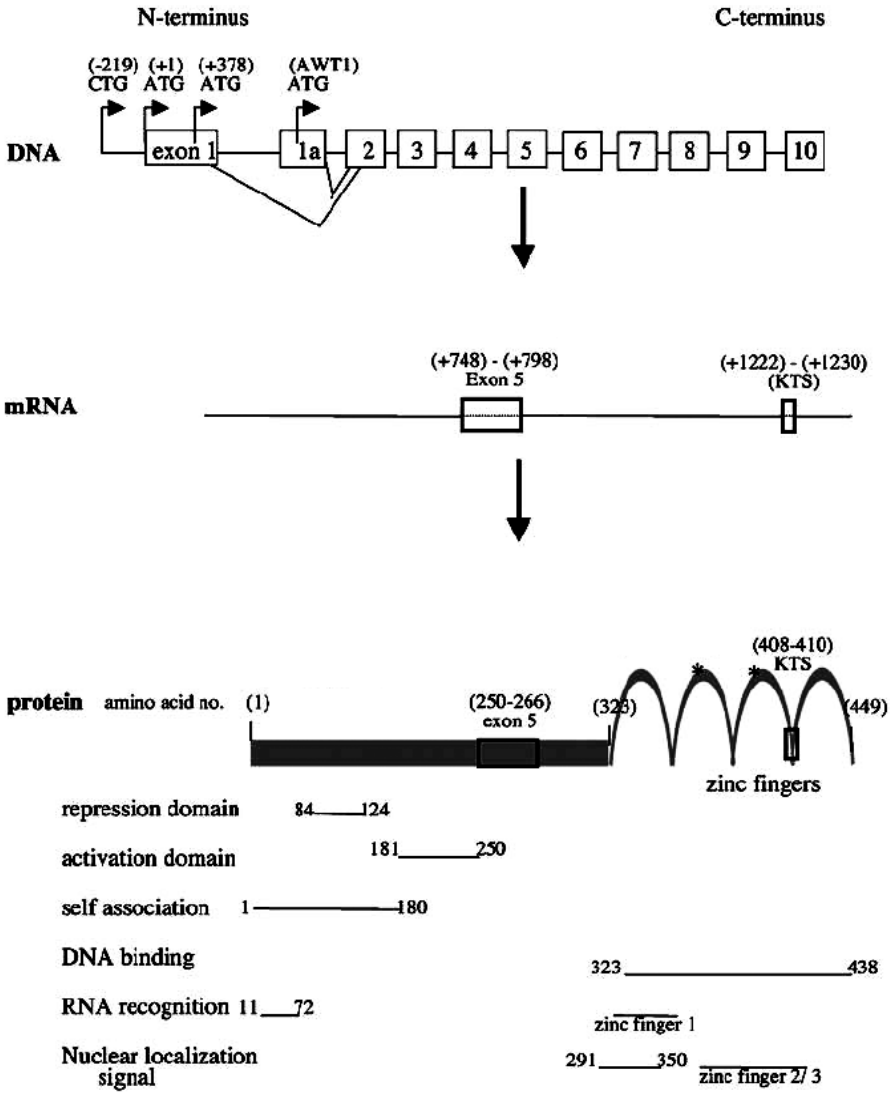

Figure 7. Schematic diagram of WT1 structure at the DNA (exon only), mRNA and protein level. All numbers represent the human WT1 (+/+) isoform and the lines shown above the WT1 protein schematic indicate reported regions of WT1. (*) indicates reported phosphorylation sites, Serine 365 and Serince 393, located in zinc-fingers 2 and 3. (Yang et al., 2007).

1.4.2 WT1 function in kidneys development

The main function of WT1 in mammalian kidney development involves in formation of two cellular compartments comprising the mesodermal mesenchyme and the ureteric bud, which is an outgrowth of the Wolffian duct. The glial-derived neurotrophic factor can directly induced kidney cell proliferation and invasion leading to the ureteric bud formation, which is produced from metanephric (Kuure et al., 2000), and the mesenchyme cells. After ureteric bud formation, The epithelial located nearly the ureteric bud tips and branches forms complex like duct tree and induced the condensation of mesenchymal cells (Figure 8). Next, the metanephric condensates transform to the S shaped bodies resulting in the formation of ducts. Also the nephrons will be conducted that is the functional excretory units of the kidneys. The expression of WT1 is high arisen in the metanephric mesenchyme (Pritchard-Jones et al., 1990; Armstrong et al., 1993) Previous finding shows that WT1-/- does not lead to induce mesenchymal apoptotic cell death in embryonic kidney. WT1 rescues the metanephric mesenchyme from apoptosis by activation of Bcl-2 transcription, which encodes a major anti-apoptotic proteins (Kreidberg et al., 1993; Mayo et al., 1999) leading to inhibition of mesenchymal cell proliferation (Kreidberg et al., 1993; Scholz and Kirschner, 2005).

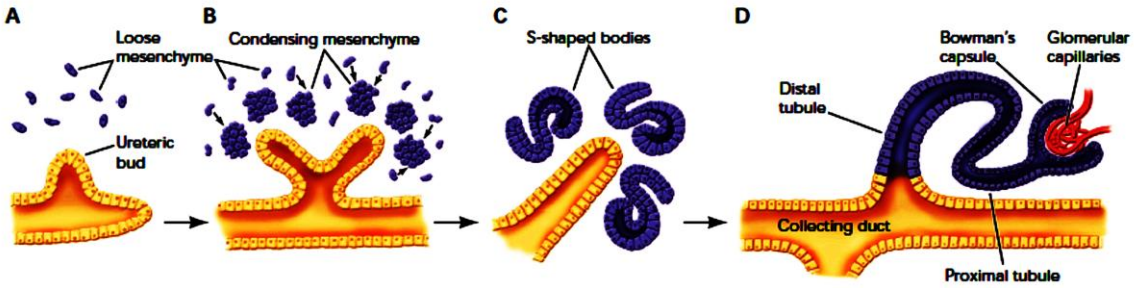

Figure 8. WT1 function during kidney development. (A) and (B) The mesenchyme is activated by glial-derived neurotrophic factor. Next, activated mesenchymal will be formed to epithelial condensates around the ureteric bud tips. (C) In the absence of WT1, WT1 acts as a survival factor for populations of embryonic kidney cells. (C) and (D) During the later stages of renal development, WT1 inhibits mesenchymal cell proliferation and forms S-shaped bodies. Next, the S bodies can extend resulting in the formation of branching duct tree (Scholz and Kirschner, 2005).

1.4.3 WT1 function in heart formation

The epicardium is a mesothelial tissue that originates from the septum transversum of the proepicardial organ (Manner et al., 2001), and it plays important roles for normal cardiac development. During development of the heart, WT1 is initially detected in the proepicardial mesenchymal villi on the cranial surface of the septum transversum (Moore et al., 1997). Cells presets WT1 expression that can migrate to pericardial cavity and spread over the surface of the myocardium, leading to the epicardial layer formation. The erythropoietin and retinoids can promote the release of mitogenic factors from the epicardium, which stimulate the proliferation of cardiac myocytes (Figure 9) (Stuckmann et al., 2003). Next, WT1 directly upregulates the mitogenic expression in the epicardium, or it activates the epicardial receptors resulting in proliferlation and migration of myocyte (Scholz and Kirschner, 2005).

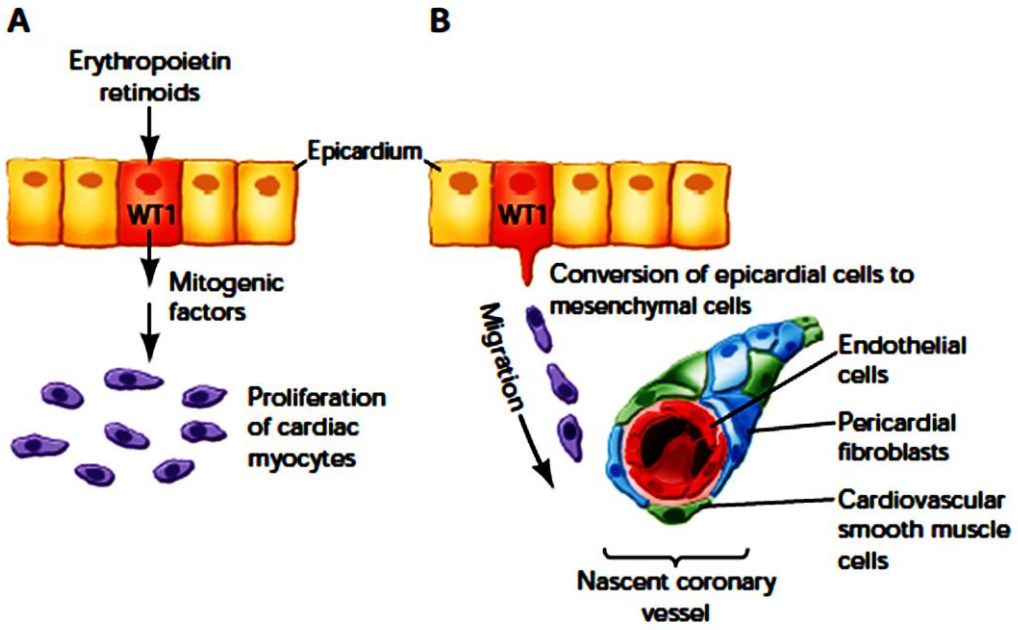

Figure 9. The functions of WT1 during heart development. (A) WT1induces the formation of myocardium and myocardial vessels. The cardiac myocytes can proliferate by mitogenic factor activation. (B) The cellular components of nascent coronary vessels originate from the epicardium through the conversion of epicardial to mesenchymal cells. The cardiac myocytes migrate into nascent coronary vessels and differentiated into the endothelial cells and vascular smooth muscle cells of the coronary vessels, as well as to perivascular fibroblasts (Scholz and Kirschner, 2005).

1.4.4 WT1 function in cancer

Since C-terminal region of WT1 protein composts of zinc finger domain that acts as a transcriptional regulator for transcriptional process of variety genes involved in the cell differentiation, proliferation, and apoptosis. The functions of WT1 has been exceedingly explored in various human solid tumor. There are different functions of WT1 as either a transcriptional activator or repressor, depending on the cellular contexts and experimental partner proteins. For example, WT1 lacks KTS acts as a transcriptional repressor of Egr-1 promoter in NIH3T3 cells (Maheswaran et al., 1993) whereas it correctly functions as a transcriptional activator for the

similar promoter in both Saos-2 (Maheswaran et al., 1993) and U2OS (Englert et al., 1995). The different function of each WT1 isoforms in the regulation of gene expression has been examined in many studies. Under transient transfection condition, WT1(KTS-) and (KTS+) have been exhibited the regulating IGFII promoter (Drummond et al., 1992), and PDGF-A chain (Wang et al., 1995). These findings support the idea that both KTS splicing variants have different particularity on regulation of same target gene. Therefore, various putative target genes of WT1 have been characterized mainly by transient transfection assays, and the consensus sequences of WT1 for interaction with target gene are clearly identified by *in vitro* binding assays. The most of target genes are involved in cellular proliferation, differentiation and apoptosis (Wang et al., 1995; Hohenstein et al., 2006).

1.4.4.1 Transcription factor

WT1 has been granted to act as a transcriptional regulator potency. The targets of WT1 are the crucial genes inducing cell proliferation and cellular mechanism such as extracellular matrix components, growth factors and other transcription factor (Yang et al., 2007). Whether WT1 also functions as a repressor or activator depending on WT1 protein levels, the isoforms of WT1 (primarily either +KTS or -KTS), and the location of the transcriptional start site in relationship to the WT1 DNA binding site (Laity et al., 2000). For instance, the recently report demonstrated that the exogenous expression of WT1 in the human breast cancer cell lines, MDA-MB-468 and MCF-7 and in human leukemia K562 cells, can activate the c-Myc promoter and stimulate cellular proliferation (Han et al., 2004) while WT1 in Hela cells can suppress the c-Mcy promoter (Hewitt et al., 1995).

1.4.4.2 Growth

WT1 has been previously found to present transcriptional activator domain that its region involves in regulation of CSF-1, amphiregulin, syndecan-1, E-cadherin, human vitamin D receptor and Bcl-2 expression (Mayo et al., 1999; Scharnhorst et al., 2001), Therefore, WT1 exhibits a growth potential in the regulation of differentiation and cellular proliferation.

Moreover, knocking down WT1 by antisense methodologies leads to loss of proliferation in lung cancer and leukemia cells while dictated overexpression of WT1 in leukemia cells inhibits cellular differentiation. Additionally, WT1 protein regulates breast cancer cell proliferation using WT1 antisense oligodeoxynucleotides, and down-regulation of WT1 protein decreases breast cancer growth also reduces cyclin D1 protein levels. These findings indicate that WT1 protein promotes breast cancer progression and development (Zapata-Benavides et al., 2002). There are current investigation that the malignant melanoma expresses WT1 +/+ isoform. Also WT1 +/+ has been shared an overlapping expression with proliferating nuclear cell antigen including Nestin and Zyxin, which are proliferative molecules in melanoma.(Wagner et al., 2007).

1.4.4.3 Tumor suppressor

There are several studies show that WT1 plays tumor suppressor properties in various cancerous cells. For instance, WT1 is able to directly regulate the apoptotic signalling by activation of bax proapototic protein. Moreover, it also blocks growth factor receptor expression composting of the epidermal growth factor receptor (EGFR) and insulin receptor (Englert et al., 1995; Menke et al., 1997). Other studies exploring the tumor suppressor roles of WT1 demonstrate that an activation of WT1 -/- isoform increases p21 expression. The cyclin dependent kinase inhibitor also knows as p21 that blocks several cyclin kinase molecules, and leads to a G2-phase cell cycle arrest and slow cellular proliferation. Whereas, WT1 +/+ and -/+ isoform induces apoptotic cell death in HepG2 cell (Oji et al., 2004; Loeb et al., 2006). WT1 +/- isoform induces p53-independent apoptosis in HepG2, Hep3B, U20S, Saos-2 and osteosarcoma cell lines. The WT1 serves as tumor suppressor role by regulation of many genes such as p53, Bax, Bak and Par-4 (Hartkamp et al., 2010). However, the cancerous properties of WT1 in breast cancer still debates about evidences of both tumor-promoting and tumor-suppressing functions (Burwell et al., 2007).

1.4.4.4 Oncogene

In recently years, there are many supported reports that unexpectedly reveal the oncogenic role of WT1. For example, WT1 induces cytoskeletal changes and promote *in vitro* invasion via upregulation of actin binding protein (Leech et al., 2000). WT1 -/- isoform also activates Bcl-2 promoter in Saos-2 and CV1 cell lines (Loeb, 2006). Interestingly, WT1 -/- isoform induces morphology changes in ZR-75 and SKBR3 cell lines since these cancer cells acquires invasive phenotypes *in vitro*. Furthermore, WT1 -/- modulates the cytoskeletal dynamics through decreasing of α-actinin 1 and cofilin expression and increasing gelsolin expression in WT1 -/- isoform-transduced TYK cells (Jomgeow et al., 2006). While, WT1 -/+ and +/+ isoforms suppress proapoptotic protein Bax and intrinsic casspase-9 and casspase-3 (Loeb, 2006). Additionally, WT1 +/+ isoform is not inhibit on p21 or proliferation, but it has effect on epithelial–mesenchymal transition, also it redistributes E-cadherin from the cytoplasmic membrane into cytoplasm (Burwell et al., 2007).

1.5 The relation of WT1 in breast cancer

There are several studies found that WT1 has presented an oncogenic properties ant its functions correlates in wide variety regulating tumorgenesis and breast cancer development. In previous reports, WT1 is detected in a variety of solid tumors in different origins where it is not normally expressed, including carcinomas of the breast (Loeb et al., 2001), lung (Oji et al., 2002), colon (Koesters et al., 2004), pancreas (Oji et al., 2004), brain tumors (Oji et al., 2004) and desmoid (Amini et al., 2005). Other report shows that there are some reports to explore the functions of WT1 in breast cancer. For example, Silberstein and research team show that WT1 protein is an oncogene in breast cancer by regulation of insulin-like growth factor (IGF) and TGF-ß molecules resulting in mammary cell growth and tumorigenesis, and it is expressed in both normal and cancerous human breast (Silberstein et al., 1997). Miyoshi and coworkers demonstrate that WT1 mRNA is detected by real-time RT-PCR method that the high expression of WT1 is associated with poor prognosis in breast cancer patients, and it may be key molecule for detection of breast cancer (Miyoshi et al., 2002). Additionally, Zapata-Benavides et al. shows

that cyclin D1 protein is up-regulated by WT1 leading to breast cancer cell proliferation (Zapata-Benavides et al., 2002). These occurrence support the oncogenic roles of WT1 in various tumours. For instance, the exogenous expression of WT1 in the human breast cancer cell lines including MDAMB-468 and MCF-7 and in human leukemic K562 cells can activate the c-Myc promoter and stimulate cellular proliferation (Han et al., 2004; Yang et al. 2007). Additionally, down-regulation of WT1 was associated with the decreasing of Bcl-2 and cyclin D1 mRNA and protein levels (Tuna et al., 2005). Recently, The bcl-2 gene has been identified as a particular target of WT1 that it also regulated with context specific molecules (Hartkamp et al., 2008). Promoter hypermethylation is very common found in both male and female breast cancer. Also promoter hypermethylation of WT1 is frequently found in ductal carcinoma *in situ* and invasive duct cancer (Herman and Baylin, 2003; Kornegoor et al., 2012). These observation show similar results in previous report that the promoter of WT1 has been found the hypermethylation approximately 32% of breast cancer (Loeb et al., 2001; Kornegoor et al., 2012). In 2013, Choi and coworker investigate new RSPO1 and SDC1 genes associating with breast cancer grade. The strong expression of SDC1 exhibits positively correlation with tumor-prone proteins containing WT1 and WNT1 in invasive ductal carcinoma. Accumulating evidence indicates that WT1 might be further used as an important prognostic biomarkers in breast cancer, and it plays oncogenic activities in several mechanisms regulating breast cancer development (Choi et al., 2013).

1.6 The relation between estrogen receptor (ER) and HER2 in breast cancer

Previous reports study the mechanism of ER and HER2 in breast cancer. It was found that estradiol directly binds to membrane ER-α, also interacts with HER2 and activate its dimer inducing activation of PI3K/Art. These crosstalk evidence leads to amplify down-stream molecule signaling of many growth factor signaling cascades (Franke et al., 1995; Andjelkovic et al., 1996) including EGF (Burgering and Coffer, 1995; Martin et al., 2000), IGF-I, and Heregulin (Alessi et al., 1996; Martin et al., 2000) resulting in breast cancer cell survival and proliferation (Stoica et al., 2003). Additionally, Martin and coworker have demonstrated previously that EGF and IGF-I regulates ER-α expression via Akt hormone-dependent in MCF-7 cell lines, and induces breast cancer cell growth (Martin et al., 2000; Stoica et al., 2003). Moreover, High levels

of HER2 increases WT1 expression to stimulate S-phase cell proliferation in BT-474 and MDA-MB-453 breast cancer cell lines, and inhibits Bcl-2 resulting in inhibition of intrinsic apoptosis pathway (Tuna et al., 2005). In recent year, Chaudhri and researcher team demonstrate that ERα36 promotes breast cancer cell survival by inhibition of Taxol induced apoptosis through membrane ER signaling, and increase metastatic factors via the decressing of E-cadherin and RANK ligand, and the increasing of CXCR4 and IL-6. These finding conclude that ERα36 localizes to plasma membrane, and it is activated by estradiol leading to enhancement of proliferation, protection against apoptosis, and enhancement of metastatic factors. ERα36 might be possible target for breast cancer development and therapy (Chaudhri et al., 2012).

1.6.1 ER signaling pathway

Estrogen receptor also knows as a nuclear protein (Osborne et al., 2001). It is a major nuclear protein that shares a common structural and functional organization with many other nuclear receptor, and it is recognized as a ligand-dependent transcription factor and promotes gene that involves in proliferation, surviaval, antiapoptosis and angiogenesis leading to aggressive of breast cancer (Osborne et al., 2005). There are two main different ways of ER function that compost of genomic ER action and non-genomic rapid ER activity.

In the genomic ER action, the signal transduction is occurred inside nucleus, and its activity is divided to classical nuclear action and non-classical nuclear action that also names as nuclear-initiated steroid signaling (NISS) showing in Figure 10. The genomic ER action produces several proteins (Osborn et al., 2001), There are many gene products that are directly promoted via genomic ER action composting of the IGF-I receptor (IGFR), the cell cycle regulator cyclin D1, the antiapoptotic factor Bcl-2 (Lee et al., 2001; Klinge, 2001 and Sanchez et al., 2002) and the proangiogenic vascular endothelial growth factor (Klinge et al., 2001 and Schiff et al., 2004). These situation leads to promoting of breast cancer cell proliferation and survival and tumor progression (Arpino et al., 2008). Genomic ER action also induces the expression of different HER and other growth factor receptor ligands including TGFα and amphiregulin (Saeki et al., 1991), which binds and activates EGFR (Salomon et al., 1995). In contrast, ER is also able

to inhibit expression of subclass of genes (Frasor et al., 2003) resulting in exhibition of anti-proliferative or pro-apoptotic function.

In classical nuclear action, ER firstly binds to DNA sequences that is called estrogen response elements (EREs) residing in the promoter region of target genes, and recruiting coregulatory proteins resulting in gene transcription. Under presenting estrogen (E2) condition, ER generally recruits coactivator (CoA) complexes to induces gene transcription, In lacked E2 condition with presenting tamoxifen (Tam), Tam is estrogen antagonists, which mostly leads to ER association with corepressor (CoR) complexes, thereby turning off gene transcription (Figure 10A) (Arpino et al., 2008).

In Non-classical nuclear action, ER regulates gene transcription via protein-protein interation between Fos/Jun family members with activating protein 1 (AP-1) or specific protein 1 (SP-1). After binding, E2 activates association of coactivator (CoA) and ER for established tether complex leading to gene regulation (Figure 10B).

In the non-genomic rappid ER activity, ER signailing is occurred in cytoplasm through ER membrane signaling, and it is also called membrane-initiated steroid hormone (MISS)(Nemere et al., 2003). These signaling exerts rapid stimulatory effects on variety molecules and transduction pathways. ER functions as a initially independent of gene transcription. Cytoplasmic signaling molecules related to growth factor signaling (Figure 11). The membrane bounded ER are activated by ligand estrogen (E2), and it forms to inner plasma membrane via binding membrane protein of lipid rafts such as carviolin 1 (Cav) and flotillin 2. While cytoplasmic ER first binds at cytoplasm. It turns through multiple interaction with signaling intermediated molecules such as Sch and modulator of non-genomic action of estrogen receptor (MNAR) (Wong et al., 2002) and metastasis associated gene 1 (MTA1s) leading to activation of growth factor (GF), tyrosine kinase receptor EGFR, HER2, IGFR, cellular kinase c-Src, MMPs. Subsequently, hairpin binding epidermal growth factor (HB-EGF) is cleavaged and released, also it then turn activates EGFR down-stream kinase cascades, promotes crosstalk of genomic and non-genomic ER signaling leading to gene transcription, cell proliferation and endocrine resistance. Alternatively, E2 can activate G protein coupled receptor (GPCR) and triggers various signaling processes such as activation of PLC-PKC-PKA and MAPK/AKT resulting in cell growth (Arpino et al., 2008). Importantly, the intracellular signal of ER pathway

is potentially extended by phosphorylation of various factors. As an example, kinase-induced phosphorylation of nuclear ER on serine 305 enhances cyclin D1 transcription in breast cancer (Balasenthil et al., 2004; Rayala et al., 2006 and Zwart et al., 2007). ER and coregulatory protein phosphorylation are an essential component of the ordinary regulation and function of genomic ER activity. However there is the hyperactive growth factor signaling, that often occurs in breast cancer (e.g., HER2 overexpression), and appears in an excessive phosphorylation of ER leading to severely weaken the inhibitory effects of various endocrine therapies and endocrine resistance (Arpino et al., 2008).

1.6.2 HER2 signalling pathway

The human epidermal growth factor receptors (HER) undertakes as ERBB receptors that are a family of signal transduction proteins. There are 4 family members in humans containing HER1, HER2, HER3, and HER4. Each of which is composed of an extracellular ligand binding domain, is a transmembrane domain, and an intracellular tyrosine kinase except HER3, which lacks a tyrosine kinase domain (Hudis, 2007; Ross et al., 2009). All HER proteins interact with receptor specific ligands except HER2 receptor that has no known activating ligands (Hudis, 2007). For example, HER1 is able to interact with 6 different ligands composting of epidermal growth factor (EGF), transforming growth factor 1 (TGF 1), amphiregulin, heparin-binding EGF-like growth factor, betacellulin, and epiregulin (Rubin and Yarden, 2001). Whereas HER2 is names as ERBB2, NEU, or HER2/neu that functions as a dimerization partner with the other HER receptor proteins (Rubin and Yarden, 2001). Upon activation, the tyrosine kinase domain of each of the HER-family receptors can activate downstream signaling molecules, such as those in the PI3K/Akt and RAS/RAF/MEK/MAPK pathways as show in Figure 12. The overexpression of HER2 in cancer cells leads to increasing of cell proliferation and decreasing of cell death, as well as changes in cell motility (Hudis, 2007).

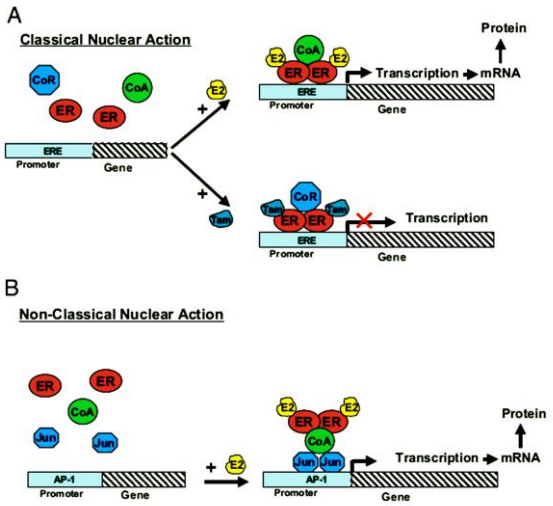

Figure 10. Nuclear genomic ER activity. Two different nuclear genomic signalling consists of (A) classical nuclear action is activates through direct binding between ER and estrogen responsive element (ERE), and (B) non-classical nuclear action is stimulates via protein-protein interaction between Fos/Jun family members with AP-1 or Sp-1 (Arpino et al., 2008).

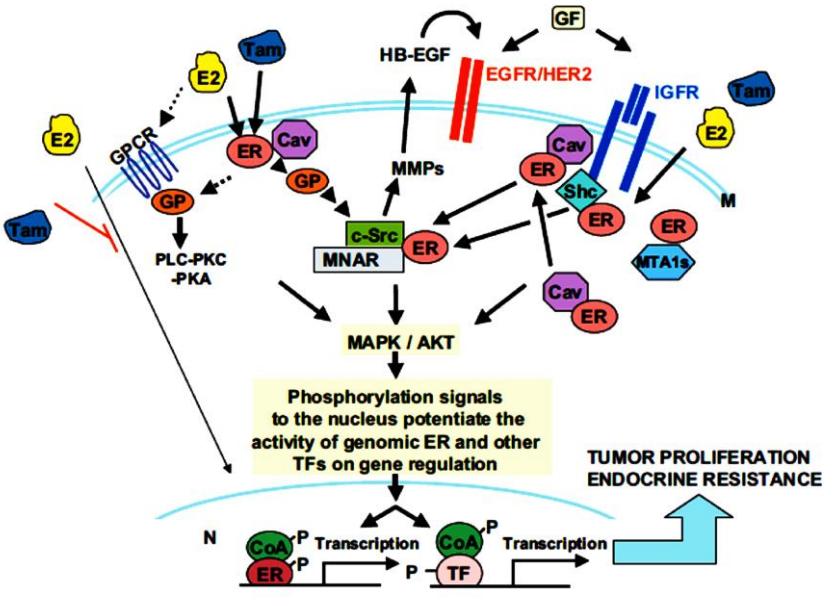

Figure 11. Non-genomic rapid ER signaling (Arpino et al., 2008).

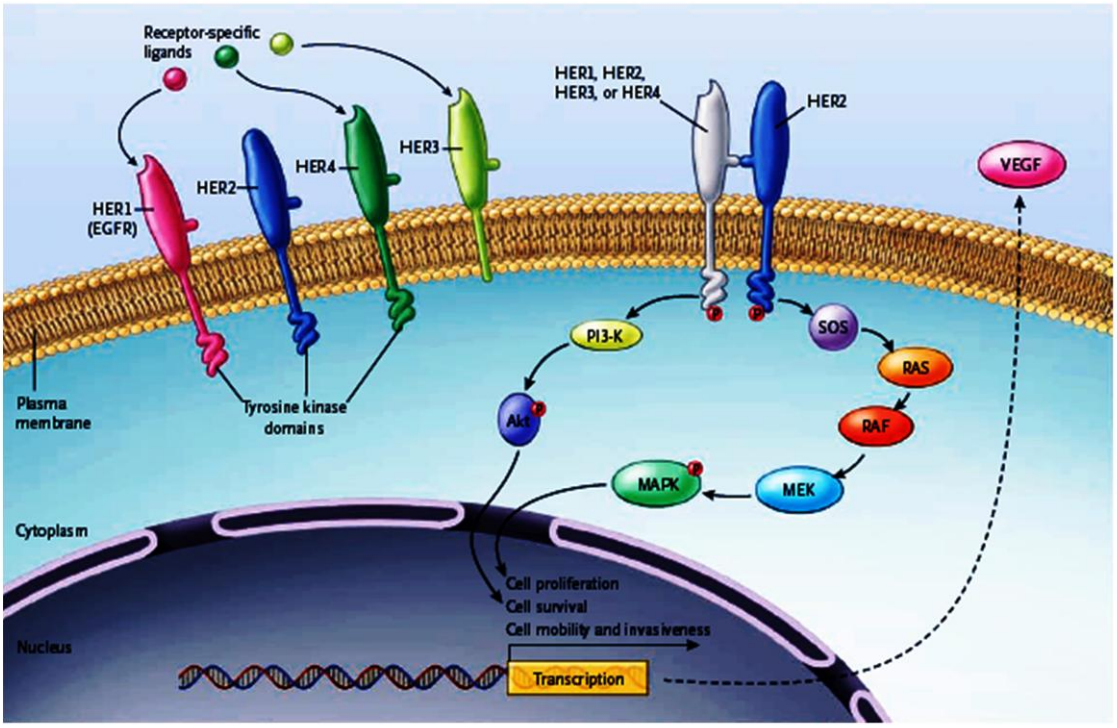

Figure 12. Signal transduction by HER family promotes proliferation, survival and invasiveness (Hudis, 2007).

1.6.3 The correlation between WT1, ER and HER2 in breast cancer

There are several reports to explore the correlation between WT1 and ER. In previous studies, high expression of WT1 and focal adhesion kinase (FAK) is stimulated by growth factor receptor bound protein-2 (Grb2). These activation leads to regulated kinase 1, 2 (Erk1, 2) pathway (Zang et al., 2004; Zang et al., 2008) that is an intracellular signaling of non-genomic rapid ER pathway (Arpino et al., 2008) promoting the proliferation of MCF-7 breast cancer cells (Zang et al., 2008). Surprisingly, WT1 down regulates ER-α expression and mediated anti-estrogen resistance (Han et al., 2008). For instant, WT1 induces estrogen-independent growth and anti-estrogen insensitivity in ER-positive breast cancer MCF-7 cells through MAPK/ERK pathway, and it also involves in regulating ER-α expression during development of acquired tamoxifen (TAM) resistance (Yang et al., 2007; Wang and Wang, 2010). In addition, down-regulation of WT1 in tamoxifen resistant MCF-7 cells (MCF-7^{TAM}) is a

cause of the decreasing ER-α that leads to the decressing MCF-7^{TAM} cells (Wang and Wang, 2010). Recently, Qi and researcher team investigate correlation of WT1 with clinicopathological factors and molecular subtype for prognosis of breast cancer patients using microarray and bioinformatic analysis. An overexpression of WT1 is found in ER-negative, basal-like and ERBB breast cancer subtypes, which have poor prognosis than luminal subtypes (Camei et al., 2011). Also it is detected in high grade of breast cancer (Qi et al., 2012). Accumulating data indicated that WT1 correlates with estrogen signaling pathway, however the further investigation still requires to confirm mechanism of WT1 in human breast cancer.

The recent report studies WT1 function on regulating HER in breast cancer progression. For example, forced overexpression WT1 in MCF-7 cells induced up-regulation of HER2 and EGFR (Wang and Wang, 2010). WT1 also displays oncogenic potential to elevate the expression of HER2 and EGFR in tamoxifen resistant MCF-7 cells (MCF-7^{TAM}). Additional experiments indicate that down-regulation of WT1 in MCF-7^{TAM} is a cause of the decreasing HER2 resulting in inhibition of cell proliferation (Wang and Wang, 2010). These finding show that high WT1 expression and p-AKT, p-ERK 1/2, EGFR and HER2 protein levels are found in high passage MCF7 cells, and WT1 induces estrogen-independent growth that produces cyclin-D1 and c-Myc molecules via stimulation of EGFR and HER2 expression. These observation indicate that WT1 involves on regulation of EGFR and HER2. However the mechanism of WT1 on regulating HER2 in breast cancer is still unclear, therefore it should be extending explored for clarify its molecular mechanism in breast cancerous development.

1.7 Stable and transient transfection

Transfection is a process that introduces foreign nucleic acids into cells to produce genetically modified cells. This process is common used for study of gene function and regulation and protein function. To introduce genetic materials, DNAs and RNAs exist inside the cells either stably or transiently depending on the nature of genetic materials (Recillas-Targa 2006; Kim and Eberwine, 2010). In stable transfection process, genetic materials usually have a marker gene for selection (transgenes) that are integrated into host genome and it sustains transgene expression even after replication of host cells (Figure 13a). In transient transfection

procedure, transient transfected genes are only expressed for limited period of time and are not integrated into the genome (Figure 13b). Whence transiently transfected genetic materials can be lost by environmental factors and cell division. To perform transfection for studying gene functions, interested genes can be enhanced or inhibited and to produced recombinant proteins in cells (Wurm, 2004; Kim and Eberwine, 2010). As examples, gene therapy is the delivering a interested gene into cells for disease treatment, or for improving symptoms. Human tissue plasminogen activator is produced in immortalized Chinese hamster ovary (CHO) cells for therapy (Hamilton and Baulcombe, 1999; Pfeifer and Verma, 2001; Wurm, 2004; Takahashi and Yamanaha, 2006; Kim and Eberwine, 2010).

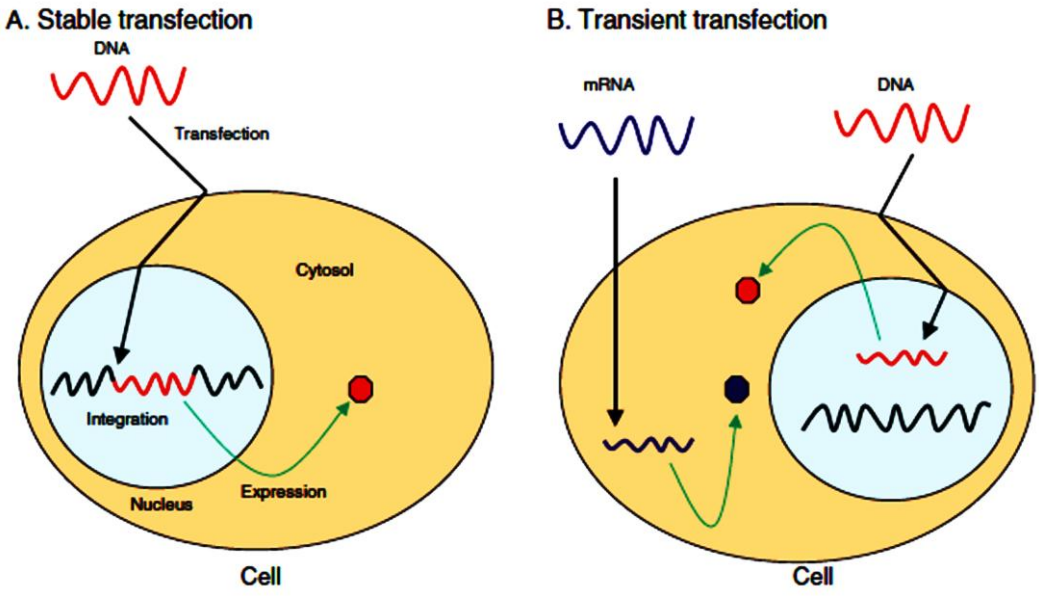

Figure 13. Schematic diagrams of two different transfections. (a) Stable transfection. Foreign DNA (red wave) is delivered to nucleus by passage through the cell and nuclear membranes. Foreign DNA is integrated into the host genome indicating in black wave and expressed sustainably. (b) Transient transfection. Foreign DNA is delivered into nucleus but is not integrated into the genome. Foreign mRNA (blue wave) is also delivered into the cytosol, where it is translated. Hexogons are expressed protein from transfected nucleic acids. Black arrows indicate delivery of foreign nucleic acids (Kim and Eberwine, 2010).

1.8 Biogenesis and mechanism of RNA interference

RNA interference (RNAi) is the sequence-specific gene silencing that mediated by small interfering RNAs (siRNAs), and it is a cellular process in post-transcripional gene silencing mediated by either degradation or translation arrest of target RNA. This process is firstly generated from long double-stranded RNA (dsRNA) of exogenouse or endogenous origin (Hamilton and Baulcombe, 1999; Zomore et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001; Kim, 2003; Ryther et al., 2005). The small endogenous RNA molecules consist of many classes such as small transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA), small interfering RNA (siRNA) and microRNA (miRNA). They are small RNA of 21-28 nucleotide length that play a crucial role in the regulation of gene expression controlling various cellular and metabolic pathways, and they is classified as two classes of small RNAs in which small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Kwak et al., 2010).

Biogenesis of siRNA is introducing of double stranded RNA either naturally or artificially inside the cells, and the process of siRNA biogenesis firstly cleavages the long dsRNA into small pieces of RNA by endoribonuclease enzyme. The small pieces can be miRNA or siRNA depending upon the origin of long dsRNA such as endogenous or exogenous, respectively. A double stranded RNA may be generated by either RNA dependent RNA polymerase or bidirectional transcription of transposable elements or physically introduced (Das et al., 2011).

The mechanism of RNA interference consists of four stages (Figure 14). Firstly, long dsRNA is mediadted by type III endonuclease Dicer to generates RNA duplex with 2-nt overhang at each 3′ end. In Drosophila, Dicer, which is a large multidomain RNase III enzyme has been identified in existence into two forms, Dcr-1/Loquacious (Loqs)-PB (also known as R3D1-L(long)) and Dcr-2/R2D2 (Jiang et al., 2005, Saito et al., 2005; Forstemann et al., 2005). Drc-1 is an enzyme that shows ATP independent function and it generates miRNA, and it shares a structural homology with Dcr-2. Drc-2 exhibits ATP dependent activity with substrate specificity to double strand RNA for generation siRNA (Jiang et al., 2005). Secondly, siRNA that contains a 5′ phosphate and 3′ hydroxyl group in each strand, forms complex with members of the Argonaute family proteins into ribonucleoprotein complex (RNP). After

association RNP complex, siRNA is incorporated into a nuclease complex that called RICS (RNA induced silencing complex). This complex still exists inactive form, and it is called pre-RISC complex (Elbashir et al., 2001; Nykanen et al., 2001; Kim, 2003; Das et al., 2011). The third step of interference mechanism involves loss of one strand of duplex by an RNA helicase activity. Ago protein is one of member of the Argonaute clade that consists of four major domains including N-terminal, PAZ domain, Middle and PIWI domains. Ago plays active form when it is consistently in two functional domains PAZ and PIWI. Additionally, Ago protein presents the catalytic subunit of RISC (Liu et al., 2004). In Eukaryotes, Ago protein acts as RNase H. For example, Ago2 is found to be responsible for conversion of pre-RISC to holo-RISC, and it can effectively remove passenger strand. The pre-RISC complex contains duplex siRNA whereas holo-RISC contains guide strand of the siRNA. The guide strand in holo-RISC presents disassociated activity leading to separating passenger strand. The 5 prime end of siRNA acts as a target recognition guide which is required for the efficient RNA interference mechanism (Nykanen et al., 2001; Schwarz et al., 2003; Das et al., 2011). Last step of siRNA silencing process involves in specific mRNA targeting and degradation for repression of translation. RISC cleaves mRNA or represses their translation by homology dependent mRNA degradation, and RNA-induced Initiation of Transcriptional gene Silencing complex (RITS) regulates heterochromatin assembly. After removal of passenger siRNA strand, the siRNA guide strand can bind with complementary mRNA target, also RISC is transformed into holo-RISC that is active form (RISC*) to cleave the mRNA backbone by catalytic activity of PIWI domain. In human, elF2c that is Ago2 protein, composts of two isoforms (eLF2C1 and elF2C2). Ago in human contains two common domains, PAZ and PIWI domain (Nykanen et al., 2001; Makeyev and Bamford, 2002; Kim, 2003; Das et al., 2011).

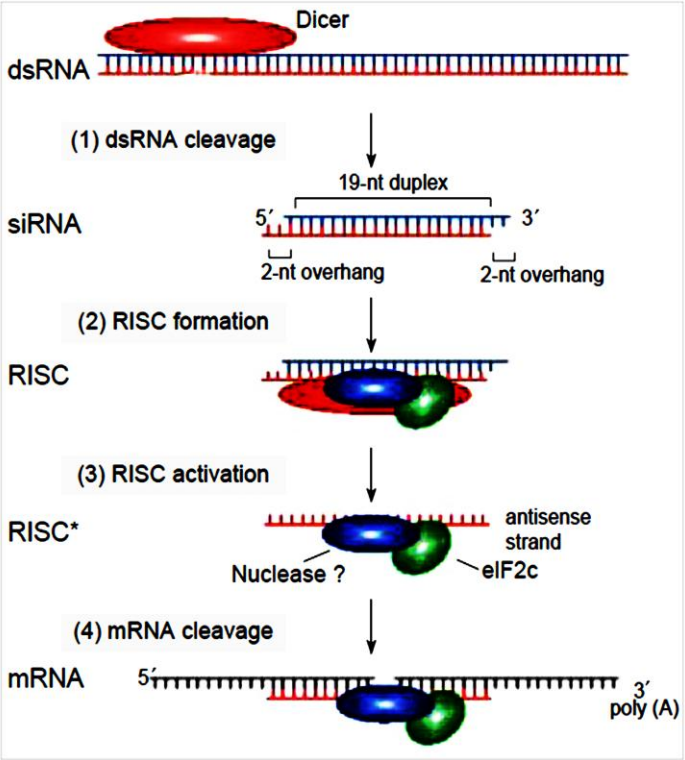

Figure 14. Mechanism of RNA silencing. RNAi process can be divided into four stages, (1) ds RNA cleavage by Dicer and generation of siRNA duplex, (2) recruitment of RNAi factors and formation of RICS (RNA-induced silencing complex), (3) siRNA unwinding and RICS activation, and mRNA degradation (Kim, 2003).

1.9 Objectives

1. To study WT1(17AA) and WT1(KTS) isoform expression in human breast cancer.

2. To evaluate the correlation between WT1 with HER2 and ER-α in human breast cancer tissues and breast cancer cell lines.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals were obtained from Sigma-Aldrich, USA; PIERCE, USA; Amersham Biosciences, UK; QIAGEN, Germany; BIO-RAD, USA; Therno Fisher Scientific, USA; Calbiochem, Germany; Santa Cruz Biotechnology, USA and Cell Signaling Technology, USA.

2.1.2 Primers

Primers for detection of WT1(17AA), WT1(KTS) and GAPDH transcripts are shown in Table 4 and for construction of full length WT1s variants are shown in Table 5.

2.1.3 RT-PCR reaction and condition

The reaction components for RT-PCR method and condition of one step RT-PCR reaction are shown in Table 6 and Table 7, respectively.

Table 4. Primers for detection of WT1(17AA) and WT1(KTS) using RT-PCR technique

Gene	Primers	Size
WT1(17AA)	e4-6C-Forward: 5'-CCAGCTTGAATGCATGAC-3' e4-6C-Reverse: 5'-CCAGCTTGAATGCATGAC-3'	185 bp for 17AA+ 134 bp for 17AA-
WT1(KTS)	e6-10C-Forward: 5'-CCACAGCACAGGGTAGGA-3' e6-10C-Reverse: 5'-TCAAAGCGCCAGCTGGAGTTT-3'	553 bp for 17AA+ 544 bp for 17AA-
GAPDH	GAPDH-Forward: 5'-GAAGGTGAAGGTCGGAGT-3' GAPDH-Reverse: 5'-GAAGATGGTGATGGGATTTC-3'	230 bp

Table 5. Primers for construction of WT1(+/+), WT1(+/-), WT1(-/+) and WT1(-/-) isoforms

Gene	Primers	Size
WT1	WT6-KpnI-Forward: 5'-GGGTAGCATGGGTTCCGAC GTTCGT-3' WTRp6-SalI-Reverse: 5'-GCGTCGACACAAGCGCCAG CTGGACT-3'	1,363 bp for WT(+/+) 1,354 bp for WT(+/-) 1,312 bp for WT(-/+) 1,303 bp for WT(-/-)

Table 6. The reaction components for amplification of WT1s by RT-PCR technique

Components	Volume/reaction	Final concentration
RNase-free water	12 µl	-
5X QIAGEN OneStep RT-PCR buffer containing 12.5 mM $MgCl_2$	10 µl	1X
dNTP Mix containing 10 mM of each dNTP	2 µl	400 µM of each dNTP
6 µM Forward primer	5 µl	0.6 µM
6 µM Reverse primer	5 µl	0.6 µM
RNA template	16 µl	1 µg - 500 ng
Total volume	50 µl	-

Table 7. One-step RT-PCR condition of WT1(17AA), WT1(KTS) and GAPDH genes.

Gene	RT-PCR condition	Company
WT1(17AA)	50°C/ 50 min, 95°C/ 15 min, 95°C/ 30 sec 40°C/ 30 sec 72°C/ 45 sec for 35 cycles and 72°C/ 10 min	QIAGEN
WT1(KTS)	50°C/ 50 min, 95°C/ 15 min, 95°C/ 30 sec 40°C/ 30 sec 72°C/ 45 sec for 35 cycles and 72°C/ 10 min	QIAGEN
GAPDH	50°C/ 50 min, 95°C/ 15 min, 95°C/ 30 sec 40°C/ 30 sec 72°C/ 45 sec for 35 cycles and 72°C/ 10 min	QIAGEN

2.1.4 siRNA

The siRNA for knockdown of WT1 gene, is shown in Table 8.

Table 8. The $siRNA_{WT1}$ sequences.

Name	Target	$siRNA_{WT1}$
WT1-HSS111388	exon 7	5′-AAAUAUCUCUUAUUGCAGCCUGGGU-3′
WT1-HSS111390	exon 8	5′-UUUCACACCUGUAUGUCUCCUUUGG-3′

2.1.5 Primary antibody

All primary antibodies were diluted in 1% skim milk with Tris-buffered saline containing Tween 20 (8 mM Tris-Base, 0.125 M NaCl, 4.028 M Tris-HCl, 0.1% Tween 20). Each of WT1, ER-α, HER2 and GAPDH proteins were detected in different dilution that demonstrate in Table 9.

Table 9. Primary antibodies and dilution for detection of WT1, ER-α, HER2 and GAPDH proteins.

Primary antibody	Dilution	Protein sizes (kDa)	Company
Anti-WT1 rabbit polyclonal antibody	1:200	53	Santa Cruz Biotechnology (CA, USA
Anti-ER-α rabbit polyclonal antibody	1:500	66	Santa Cruz Biotechnology (CA, USA
Anti-HER2 rabbit polyclonal antibody	1:1,000	185	Cell Signalling technology (CA, USA
Anti-GAPDH mous monoclonal antibody	1:10,000	36	Calbiochem (Hofheim, Germany)

2.1.6 Secondary antibody

All secondary antibodies were diluted in Tris-buffered saline with Tween 20 (8mM Tris-Base, 0.125 M NaCl, 4.028 M Tris-HCl, 0.1% Tween 20). Horse reddish peroxidase (HRP) labeled Anti-mouse IgG and anti-rabbit IgG for Western blot analysis were used in different dilution as shown in Table 10.

Table 10. The dilution of secondary antibodies for WT1, ER-α, HER2 and GAPDH detection.

Primary antibody	Secondary antibodies	Dilution	Company
WT1	Anti-rabbit IgG	1:10,000	Cell Signalling Technology (CA, USA)
ER-α	Anti-rabbit IgG	1:5,000	Cell Signalling Technology (CA, USA))
HER2	Anti-rabbit IgG	1:2,500	Cell Signalling Technology (CA, USA)
GAPDH	Anti-mouse IgG	1:10,000	Amersham, GE Healthcare (Buckinghamshire, UK)

2.2 Methods

2.2.1 Study of WT1 mRNA in breast cancer tissues

2.2.1.1 Patients and tissues specimens

All tissues samples were kindly consented to use in this study and obtained from Thai patients who underwent surgery at Songklanakarin hospital, Prince of Songkla University, Thailand. The medical Ethics Committee of Songklanakarin Hospital approved these sample that presents the approval number as EC 55-014-04-1-3 and EC 53-072-04-2-3. After tissues collection, the tissues containing adjacent normal breast abd breast cancer tissues were examined by one pathologist and the grading of each samples was performed by an experienced gynecologic pathologist according to Scarff-Bloom-Richardson system (Bloom and Richardson, 1957; Scarff and Torloni, 1968). The adjacent normal breast tissues (30 samples) and breast cancer tissues (23 samples) were obtained at surgery and then chopped. Small tissues were washed with 1XPBS for 2 times and kept at -70°C until use. The clinical and histopathological characteristics of these samples are shown in Table 11. All breast cancer patients were not treated with chemotherapeutic drugs that were classified as breast carcinoma grade 1, 2 and 3.

Table 11. The clinical and histopathological characteristics of breast cancer patients.

Parameter	Number of patients	Total cases	Percentage of patients
Age of diagnosis (years)			
≤ 40	3	13	23
41-50	7	31	23
51-60	9	39	23
61-70	4	17	24
Tumor size (cm)			
≤ 2	14	61	23
>2	9	39	23
Histologicaltypes			
Invasive ductal carcinoma	23	100	23
Tumor grade			
1	4	17	24
2	11	48	23
3	8	35	23
Lymph node metastasis			
Positive	11	48	23
Negative	12	52	23

Note: All breast cancer patients who admitted for surgery, were not treated with chemotherapeutic drugs.

2.2.1.2 Primer design for detection of WT1(17AA) and WT1(KTS)

RT-PCR primer sequences for detection of WT1(17AA) splice variants were designed using Vector NT1 software. NM024426 is an accession number of the homo sapiens Wilms' Tumor 1 (WT1), transcript variant D, which was used as mRNA template for designed 17AA and KTS primers. Primers for identification of WT1(17AA-) and WT1(17AA+) spanned between exon 4 and exon 6 which generated the PCR products at 185 and 134 bp for WT1(17AA+) and WT1(17AA-), respectively. Detection of WT1(KTS+) and WT1(KTS-) used specific primers that located between exon 6 and exon 10. The WT1(KTS) primers amplified two different PCR product sizes at 553 bp for WT1(KTS+) detection and 544 bp for WT1(KTS-) detection. Whereas internal control primers are designed from homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 1 (NM002046), which was used as mRNA template. After RT-PCR performance, the PCR product was sequenced for confirmation of collected WT1(17AA) and WT1(KTS) variants.

2.2.1.3 RNA extraction

Five hundred milligram of frozen adjacent normal breast and breast cancer tissues were chopped on steriled glass plate, and transferred to centrifuge tube containing 500 µl Trizol reagent (Invitrogen, CA, USA). Tissue was homogenized and incubated at room temperature (25°C) for 5 min. After incubation, 200 µl of chloroform was added and vigorously vortex for 15 sec. The mixture was incubate at room temperature for 3 min, and centrifuged at 12,000 xg 15 min. After centrifugation, the aqueous phase on top solution was transfered into new centrifuge tube, and 500 µl of isopropanol was added. The centrifuge tube was inverted for 5-6 times and incubated at room temperature for 10 min. After incubation, the mixture was centrifuged at 12,000 xg for 10 min and the supernatant was discarded. Next, excess isopropanol was removed by place in an inverted position on a paper tower. The RNA was dried for 30 min and dissolved with RNase-free deionized water by incubation at 55°C for 10 min.

2.2.1.4 Reverse transcription polymerase chain reaction (RT-PCR)

After RNA extraction, the mRNA was first determined using OneStep RT-PCR kit (Qiagen, Hilden, Germany) according to the instructions from the manufacturer. In brief, PCR was performed in a total volume of 25 µl with 500 ng of total RNA. The PCR reaction components and One-step RT-PCR condition are shown in Table 6 and Table 7. Primers for RT-PCR amplification consisting of WT1(17AA) and WT1(KTS) are shown in Table 4. GAPDH primers were used as an internal control. In addition, PCR products were run onto gel electrophoresis and visualized by ethidium bromide staining. The specific PCR bands were purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany). The DNA sequence was assessed using ABI Prism 377 DNA sequencer.

2.2.1.5 Gel electrophoresis

After amplification of WT1 gene, the PCR product was separated by gel electrophoresis including agarose gel electrophoresis Single Strand Conformation Polymorphism (SSCP) and high resolution metaphor gel electrophoresis.

2.2.1.5.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation of WT1(17AA+) and WT1(17AA-). The 185 bp and 134 bp represented for WT1(17AA+) and WT1(17AA-), respectively. WT1(17AA) PCR products were separated in 2% agarose gel, 100 volts for 40 min at room temperature. Two percentage agarose gel mixture contained 1.6 g of agarose gel and 80 ml of 1X TBE buffer (90 mM Tris-base, 0.5 M EDTA). After setting up agarose gel mixture in a glass container, the mixture was heated in the microwave oven on high power until the gel was melted, and the mixture was then placed at room temperature until the mixture temperature decrease to 60°C. The volume of 10 µl ethidium bromide at concentration 1% w/v was added to the mixture, and gently swirled before agarose gel setting to gel tray. As the gel setting up, a comb was placed in form and poured cooled agar solution into the form. The gel solution was stand at room temperature for 1 h. After gel polymerization, the comb was carefully removed and

the gel was place in the electrophoresis chamber that contain with 1XTBE buffer. For DNA loading, the DNA samples were mixed with gel-loading buffer (25% v/v of glycerol, 60 mM EDTA, 0.25% w/v of bromophenol blue). The DNA samples were loaded into each wells. The bands were observed and photographed under gel doc (Gel Doc™ XR+, Bio-Rad).

2.2.1.5.2 Single Strand Conformation Polymorphism (SSCP)

SSCP was used to separate WT1(KTS+) and WT1(KTS-). The 553 bp and 544 bp represented for WT1(KTS+) and WT1(KTS-), respectively. The PCR product were denatured in denaturing solution (99% formamide, 1% xylene cyanol, 0.4 mg/ml of bromophenol blue) at 95°C for 5 min. After denaturing, the PCR products directly placed on ice to prevent re-annealing of the single stranded product. Then, 6 µl of samples were apply to the 15% acrylamide gel (GeneGel Clean Kit, GH Healthcare) and run at 200 volts for 15 min followed by 600 volts for 80 min. The band were detected by Silver staining (PlusOne DNA Staining Kit, GE Healthcare).

2.2.1.5.3 High resolution metaphor gel electrophoresis

High resolution metaphor gel electrophoresis was used to separate WT1(KTS+) and WT1(KTS-). The 553 bp and 544 bp represented for WT1(KTS+) and WT1(KTS-), respectively. PCR products were separated in 3% metaphor agarose gel electrophoresis. According to instructions from manufacturer, the empty beaker at 50 ml was measured, and the same size of beaker containing buffer at 80 ml was measured. Two point four gram of metaphor gel was added slowly to 60 ml of 1XTBE buffer (90 mM Tris-base, 0.5 M EDTA). The mixture were gently mixed by stirer. After dissolving, the beaker with gel solution was measured again, and added the buffer for giving the final weight equal as the weight of beaker containing buffer 80 ml. The mixture was then melted in the microwave oven until the gel was complete dissolved. The 10 µl of ethidium bromide at concentration 1% w/v was added into the agar solution and gently swirled the gel solution to re-suspend any settled agar. As the gel setting up, a comb was placed in form and poured cooled agar solution into the form. The gel

solution was stand at room temperature for 1 h. After gel polymerization, the comb was carefully removed and the gel was place in the electrophoresis chamber that contain with 1XTBE buffer. For DNA loading, the DNA samples were mixed with gel-loading buffer (25% v/v of glycerol, 60 mM EDTA, 0.25% w/v of bromophenol blue). The DNA samples were loaded into each wells. After gel loading, the DNA samples were was then run under 75 volts for 5 h at 4°C. Also the DNA band was visualized under UV light and photographed by gel doc (Gel Doc™ XR+, Bio-Rad).

2.2.1.6 Gel purification

The PCR product was purified by QIAquick gel extraction kit (QIAGEN). The manufacture's instruction was followed. The DNA fragment was excised from the agarose gel with a clean scalpel and transferred to a microcentrifuge tube. Three volumes of gel and buffer QG was added to 1 volume of the gel (100 mg of gel ~ 100 µl). Next, the mixture was incubated at 50°C for 10 min or until the gel slice had completely dissolved and mixed the mixture by vortexing the tube every 2-3 min during incubation. After gel dissolution, an isopropanol was added a 1 gel volume to the sample mixture. A QIAquick spin column was placed in a provided 2.0 ml collection tube and the sample was applied to the QIAquick column and centrifuge at 10,000 xg for 1 min. The flow-through solution was discarded and the QIAquick column back were placed in the same collection tube. The QIAquick column was washed by adding 0.5 ml of buffer QG and centrifuged at 10,000 xg for 1 min. After centrifugation, the flow-through was discarded and the QIAquick column was washed by adding 0.75 ml of buffer PE containing ethanol and centrifuged at 10,000 xg for 1 min. Next, the flow-through was discarded and the QIAquick column was additionally centrifuged at 13,000 xg for 1 min to remove the residual ethanol from buffer. After washing, the QIAquick column was placed into a clean microcentrifuge tube and the elution of DNA was performed by addition 30 µl of deionized water, then left standing at room temperature for 1 min. The DNA was eluted by centrifugation at 14,000 xg for 1 min. Finally, the eluted DNA was determined by running in agarose gel electrophoresis.

2.2.1.7 DNA ligation

The PCR products from step 2.2.1.6 were and then ligated with pGEM®-T Easy vectors (Promega) according to the instructions from the manufacturer. Briefly, The pGEM®-T Easy vectors were aliqouted, spun down to collect contents at the bottom of the tube. The ligation reaction contained 5 µl of 2X Rapid ligation buffer T4 DNA ligase (Promega), 4 µl of purified PCR products, 0.5 µl of pGEM®-T Easy vectors (25 ng), followed by 0.5 µl of T4 DNA ligase (1 units/µl). The ligation mixture was mixed gently, spun down and incubated the reactions overnight at 4°C.

2.2.1.8 Preparation of *Escherichia coli* (*E. coli*) competent cells

The preparation of *E. coli* TOP 10F competent cells by $CaCl_2$ method. Briefly, the frozen glycerol stock of *E. coli* TOP 10F was streaked on to Luria-Bertani (LB) agar plate containing with 10 µg/ml of tetracycline at 37°C for 24 h. After isolation, the single colony of was picked and cultured in 25 ml of fresh LB broth with 10 µg/ml of tetracycline by shaking at 37°C for 16-20 h. The saturated overnight culture was continually cultured in 225 ml of LB broth containing 10 µg/ml of tetracycline by shaking at 37°C for 3 h, or until the turbidity at OD_{600} equal 0.5-0.6. In cooling steps, this point the cells should never touch anything that was warm. The two hundred milliliter of cell suspension were aliquot to sterile centrifuge tube, and the cell pellets were harvested by centrifugation at 4,500 xg for 6 min at 4°C. After centrifugation, the supernatant was discarded, and cell pellets were washed with 80 ml of ice-cold 0.1 M $CaCl_2$ by swirling on ice gently. The pellets was resuspend in 40 ml of ice-cold 0.1 M $CgCl_2$, then incubated on ice for 30 min to establish competency. After stand on ice, the cell suspension was centrifuged at 4,500 xg for 6 min at 4°C. Additionally, the supernatant was removed, and cell pellets were resuspended in 6.8 ml of ice-cold 0.1 M $CgCl_2$ by swiriling on ice gently. The cell suspension was combined with 1.2 ml of glycerol to give concentration of 15% (v/v) and swirled the cell mixture. Final step, 200 µl of the cell suspension was aliquot and stored at -80°C.

2.2.1.9 Transformation of recombinant DNA into *E. coli* competent cells

A volume of 200 µl of competent cells was thawed on ice and gently mixed by adding 0.1-1 µg of a supercoiled plasmid DNA. The mixture was incubated on ice for 30 min, incubate in water bath at 42°C for 90 sec, and placed on iced immediately for an additional 3 min. 800 µl of LB broth without antibiotics were added into mixture and incubated at 37°C for 1 h. Finally, mixture was spread on LB selective plate containing 100 µg/ml of ampicillin and incubated at 37°C for 16 h.

2.2.1.10 Plasmid DNA extraction

Plasmid DNA was extracted using a rapid protocol based on the alkaline lysis method (extraction pH 8.0). Briefly, a positive clone from step 2.2.1.9 was inoculated into 3.0 ml of LB broth medium containing 100 µg/ml of ampicillin and incubated overnight at 37°C with vigorous shaking. The cell was collected using centrifugation at 14,000 xg for 1 min at room temperature. After centrifugation, a supernatant was discarded, the cell pellets was resuspended in 350 µl of STET buffer (8% (w/v) glucose, 5% (v/v) Triton X-100, 50 mM EDTA and 50 mM Tris-HCl, pH 8.0) by vortexing. After resuspending, the mixture was incubated at room temperature for 5 min and boiled in hot water for 40 sec, then incubated immediately on ice for 5 min. Next, the cell lysate was centrifuge at 14,000 xg for 10 min at room temperature to precipitate cell debris. After centrifugation, the cell debris was removed from the microcentrifuge tube by fishing it out with a sterile wooden toothpick. An equal volume of isopropanol approximately 350 µl was added to this tube and the mixture was incubated at -80°C for 1 h. The mixture was centrifuged at 14,000 xg for 10 min at 4°C and, the supernatant was discarded. Next, excess isopropanol was removed by place in an inverted position on a paper tower, and the DNA pellet was died for 30 min. Plasmid DNA was resuspended in 30 µl of sterilized water, and then vortex to ensure complete dissolution.

2.2.1.11 Restriction endonuclease digestion by *Eco*RI

In this study the pGEM®-T Easy vectors were ligated with PCR products (WT1(17AA+), WT1(17AA-), WT1(KTS+), or WT1(KTS-)). The recombinant plasmid was digested by *Eco*RI. The digestion reaction contained 2 µl of 10X of *Eco*RI buffer, 1 µl of RNase A (10 mg/ml), 0.5 µl of *Eco*RI (20,000 units/ml) and 16.5 µl of of recombinant DNA. After setting up the digestion mixtures, a total volume of 20 µl of digestion mixture was incubated at 37°C overnight. After digestion, the digested products were analyzed by 1.8% agarose gel electrophoresis, 100 volts for 35 min.

2.2.1.12 Purification of plasmid DNA

Plasmid DNA was purified using the QIAprep spin miniprep kit (QIAGEN). According to instruction of plasmid purification, a single bacteria colony from a freshly streaked selective plate was picked and inoculated into 3 ml of LB broth medium containing 100 µg/ml ampicillin and incubated 12-16 h at 37°C with vigorous shaking. Next, the bacteria culture was aliquot to microcentrifuge tube at a volume 1.5 ml, and harvested cell pellets using centrifugation at 14,000 xg for 1 min at room temperature. A supernatant was discarded and the bacteria pellet was resuspended in 125 µl of buffer P1 containing RNase A. Additionally, cell suspention was pulled in same microcentrifuge tube. Next, the shearing of genomic DNA was perform by adding 350 µl of buffer P2 and mixed thoroughly by inverting the tube for 4-6 times. Next, 350 µl of N3 buffer were at the volume of 350 µl was added to the suspension and mixed immediately by inverting the tube for 4-6 times. The proteins and white cell debris were precipitated by centrifugation at 14,000 xg for 10 min. After centrifugation, the supernatants was applied to QIAprep spin column and centrifuged at 14,000 xg for 1 min. Next, the flow-through was discarded and the QIAprep spin column was washed by adding 500 µl of PB buffer and centrifuged at 14,000 xg for 1 min, then the flow-through was discarded. Next, the QIAprep spin column was washed once again by washing with 750 µl of buffer PE and centrifuged at 14,000 xg for 1 min. The flow-through was discarded and the QIAprep spin column was additionally centrifuged at 14,000 xg for 1 min to remove of the residual wash buffer. After centrifugation, the

QIAprep spin column was placed in a clean microcentrifuge tube, then left standing at room temperature for 5 min. The DNA was eluted with 50 µl of detionized water treated DEPC by centrifugation at 14,000 xg for 1 min. Finally, the eluted DNA was observed by running in agarose gel electrophoresis and measurement using spectrophotometer.

2.2.1.13 Automated DNA sequencing

The specific PCR bands were purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany) and sequenced using ABI Prism 377 DNA sequencer at scientific equipment center, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

2.2.2 Study of WT1, ER-α and HER2 proteins in breast cancer tissues

2.2.2.1 Protein extraction from frozen tissues

Adjacent normal breast and breast cancer tissues were chopped on sterilized plate. The tissue was ground in liquid nitrogen-cooled mortar then tissues powder was suspend in RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate and 1 mM EDTA) (Sigma-Aldrich, MO, USA). After incubation, the mixture was incubated in liquid nitrogen for 10 min followed by thawing in water bath at 42°C. This thawing step was repeated for 2-3 times. Solution was cleared by centrifugation at 14,000x g for 30 min at 4°C. After centrifugation, supernatant from tissues was measured protein concentration by Bradford's method (Bio-Rad, CA, USA).

2.2.2.2 Protein determination

Protein quantification was determined by Bradford dye-binding method. Protein standard BSA was diluted in deionized water to concentration of 3.125, 6.25, 12.5, 25, 50, 100 µg/ml. A volume of 400 µl of either protein standard or protein sample was mixed with Biorad Protein Assay (Bio-Rad, CA, USA). The absorbance at 620 nm was determined from

protein standard. The concentration of protein sample was calculated by referring to the the concentration of protein standard.

2.2.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The method of SDS-PAGE was performed as described by Graidist, 2010. The resoution gel was prepared as shown in Table 12. The acrylamide gel was prepared by O' Farrell system (ATTO, Japan), and gel size is 13.5 x 13 cm (Wide x Length). The stab gel cast (Catalog number AE-6210) and stab EP chamber (Catalog number AE-6200) from ATTO product catalog 2005 to 2007 (ATTO, Bioscience and Technology, Japan) were used for large size acrylamide gel preparation. The compatible gel size 8.6 x 6.8 cm (Wide x Length) was used for mini acrylamide gel preparation (Bio-Rad, CA, USA) using mini-PROTEAN® 3 cell electrophoresis system. Electrophoresis was carried out the descending direction on the Tris-glycine buffer (25 mM Tris-Hcl, pH 6.8, 192 mM glycine and 0.1% (w/v) SDS) using a constant 30 mA for 1.30 h and 180 volts for 3 h until the tracking dye reached the edge of the gel.

2.2.2.4 Western analysis

Equal amounts of protein lysates were subjected to 12% SDS-PAGE and transferred to a nitocellulose membrane (Bio-Rad), which were then blocked with 5% non-fat milk in TBST (0.5% Tween 20, 154 mM NaCl, 40 mM Tris-HCl, 48 mM Tris-base) for 1 h. After membrane blocking, Membranes were incubated with primary antibodies against WT (1:200, Santa Cruz Biotechnology, CA, USA), ER-α (1:500, Santa Cruz Biotechnology, CA, USA), HER2 (1:1000, Cell Signaling Technology, CA, USA) and GAPDH (1:1000, Calbiochem, Darmstadt, Germany). Bound antibodies were detected by donkey anti-rabbit or sheep anti-mouse HRP conjugated antibodies and visualized with chemiluminescence detection using the SuperSignal West Dura substrate (Pierce, IL, USA). The intensity of the band was calculated analyzed by Scion image software.

Table 12. Preparation of SDS-polyacrylamide gel

Solution	Preparation of 8.6 x 6.8 cm acrylamide gel using (Bio-Rad, CA, USA)		Preparation of 13.5 x 13 cm acrylamide gel using ATTO (Japan)	
	4% Stacking gel	12% Resolution gel	4% Stacking gel	12% Resolution gel
Deionized water	2.17 ml	3.28 ml	5.09 ml	7.67 ml
40% Acrylamide (Acrylamide; N,N′-methylenebisacrylamide, 37.5:1)	0.37 ml	2.13 ml	0.88 ml	5.40 ml
1.5 M Tris-HCl, pH 8.8	-	1.95 ml	-	4.56 ml
0.5 M Tris-Hcl, pH 6.8	375 µl	-	0.88 ml	-
10% SDS (Sodium Dodecyl Sulfate)	30 µl	76.92 µl	70.28 µl	179.9 µl
10% APS (Ammonium Persulfate)	30 µl	76.92 µl	70.28 µl	179.9 µl
TEMED (N, N, N, ′N′-tetramethylenediamine)	3 µl	3.08 µl	7.028 µl	7.2 µl
Total	2.973 ml	7.608 ml	6.998 ml	17.997 ml

2.2.3 Study of the correlation between WT1, ER-α and HER2 in breast cancer cell lines

2.2.3.1 Cell lines and culture conditions

Cell lines that were used in this study, were obtained from Aamerican Type Culture Collection (ATCC) containing three breast cancer cell lines, MDA-MB-231 and MDA-MB-468, were cultured in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) supplement with 10% of fetal bovine serum (Invitrogen, CA, USA), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen, CA, USA) and 2 mM of L-glutamine (Invitrogen, CA, USA) and MCF-7, breast cancer cell line, was maintained as previously described (Graidist et al., 2010). In addition, one normal breast cell line, MCF-12A, was grown in medium containing 1:1 ratio mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (JR Scientific, CA, USA) supplemented with 20 ng/ml of human epidermal growth factor (Calbiochem, Darmstadt, Germany), 100 ng/ml of cholera toxin (Sigma-Aldrich, MO, USA), 0.01 mg/ml of bovine insulin (Sigma-Aldrich, MO, USA) and 500 ng/ml of hydrocortisone (Sigma-Aldrich, MO, USA) and 5% of horse serum (Invitrogen, CA, USA). All cells were incubated at 37°C in an incubator with humidified atmosphere of 5% CO_2.

2.2.3.2 Construction of recombinant plasmid DNA containing WT1s (WT1-/+ or WT1-/-) cDNA

Since WT1+/+ and WT1+/- recombinant plasmid were previously constructed and kindly provided from Asst. Prof. Dr. Potchanapond Graidist, Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Songkhla, Thailand (Graidist et al., 2010), the construction of plasmid DNA for production of WT1-/+ and WT1-/- proteins was performed in this study. There are three steps for construction of recombinant plasmid DNA.

2.2.3.2.1 Inserted gene and vector preparation

Full length of WT1(-/+) and WT1(-/-) were previously constructed into pcDNA3 vector. Then the inserted WT1(-/+), WT1(-/-) were amplified using RT-PCR method. The forward and reverse primers were used as shown in Table 5. The primers were designed from homo sapiens Wilms tumor 1 (WT1), transcript variant A (WT1(-/-)) and C (WT1(-/+)) as shown in Figure 15 and 16. The nucleotide region in each 10 exons of wild type WT1(+/+) gene was summarized as shown in Table 13. The PCR products (WT1(-/+), or WT1(-/-)) was digested with *Kpn*I. The digestion reaction contained 2 µl of 10X of NEB buffer, 1 µl of RNase A (10 mg/ml), 0.5 µl of *Kpn*I (10,000 units/ml), and 16.5 µl of of PCR product. After setting up the digestion mixtures, a total volume of 20 µl of digestion mixture was incubated at 37°C overnight. Next, the digestion mixture was purified, and the purified DNA was digested again with *Sal*I and purified. The digestion reaction for *Sal*I contained 2 µl of 10X of NEB buffer, 1 µl of RNase A (10 mg/ml), 0.5 µl of *Sal*I (20,000 units/ml), and 16.5 µl of PCR product.

In vector preparation, the pcDNA™6/V5-His B vector was used as an expression vector for generation of WT1(-/+) and WT1(-/-) proteins (Figure 17). The pcDNA™6/V5-His B vector was digest by *Kpn*I and *Xho*I restriction enzymes. After digestion, the digestion mixture was purified by QIAprep spin miniprep kit (QIAGEN, USA), and the purification method followed as step 2.2.1.11.

2.2.3.2.2 Ligation and digestion

The WT1s inserted gene was ligated with pcDNA™6/V5-His B vector by T4 ligase enzyme. The ligation reaction contained 5 µl of 5X ligation buffer T4 DNA ligase (Invitrogen), 0.5 µl T4 DNA ligase (1 units/µl), 6 µl of purified PCR products, and 11.5 µl of the mixture was added in 0.5 µl of pcDNA™6/V5-His B vector (25 ng). The ligation mixture was mixed gently, spun down and incubated at 16°C for 3 h.

2.2.3.2.3 Transformation

After ligation, the recombinant plasmid was transform into *E. coli* TOP10F´ as described in step 2.2.1.9. The positive clone was selected using 100 µg/ml of amplicilin. Then, recombinant plasmid was extracted from positive clones using the QIAprep spin miniprep kit (Step 2.2.1.12). After plasmid extraction, the recombinant plasmid was recleaved with *Taq*I and *Kpn*I to confirm size. The digestion reaction for *Taq*I and *Kpn*I contained 2 µl of 10X of NEB buffer, 1 µl of RNase A (10 mg/ml), 0.5 µl of *Kpn*I (10,000 units/ml), 0.5 µl of *Taq*I (10,000 units/ml) and 16 µl of of PCR product. Then, the digestion product was analyzed by 1.5% gel electrophoresis at 100 volts for 40 min and sequenced using ABI Prism 377 DNA sequencer at Scientific equipment center, Prince of Songkla University, Hat-Yai, Songkhla, Thailand.

A

Homo sapiens Wilms tumor 1 (WT1), transcript variant A, mRNA

LOCUS NM_000378 1494 bp mRNA linear PRI 11-APR-2010
DEFINITION Homo sapiens Wilms tumor 1 (WT1), transcript variant A, mRNA.
ACCESSION NM_000378 REGION: 197..1690
VERSION NM_000378.3 GI:65507713
KEYWORDS .
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
ORIGIN

```
   1 CTGCAGGACC CGGCTTCCAC GTGTGTCCCG GAGCCGGCGT CTCAGCACAC GCTCCGCTCC
  61 GGGCCTGGGT GCCTACAGCA GCCAGAGCAG CAGGGAGTCC GGGACCCGGG CGGCATCTGG
 121 GCCAAGTTAG GCGCCGCCGA GGCCAGCGCT GAACGTCTCC AGGGCCGGAG GAGCCGCGGG
 181 GCGTCCGGGT CTGAGCCGCA GCAAATGGGC TCCGACGTGC GGGACCTGAA CGCGCTGCTG
 241 CCCGCCGTCC CCTCCCTGGG TGGCGGCGGC GGCTGTGCCC TGCCTGTGAG CGGCGCGGCG
 301 CAGTGGGCGC CGGTGCTGGA CTTTGCGCCC CCGGGCGCTT CGGCTTACGG GTCGTTGGGC
 361 GGCCCCGCGC CGCCACCGGC TCCGCCGCCA CCCCCGCCGC CGCCGCCTCA CTCCTTCATC
 421 AAACAGGAGC CGAGCTGGGG CGGCGCGGAG CCGCACGAGG AGCAGTGCCT GAGCGCCTTC
 481 ACTGTCCACT TTTCCGGCCA GTTCACTGGC ACAGCCGGAG CCTGTCGCTA CGGGCCCTTC
 541 GGTCCTCCTC CGCCCAGCCA GGCGTCATCC GGCCAGGCCA GGATGTTTCC TAACGCGCCC
 601 TACCTGCCCA GCTGCCTCGA GAGCCAGCCC GCTATTCGCA ATCAGGGTTA CAGCACGGTC
 661 ACCTTCGACG GGACGCCCAG CTACGGTCAC ACGCCCTCGC ACCATGCGGC GCAGTTCCCC
 721 AACCACTCAT TCAAGCATGA GGATCCCATG GGCCAGCAGG GCTCGCTGGG TGAGCAGCAG
 781 TACTCGGTGC CGCCCCCGGT CTATGGCTGC CACACCCCCA CCGACAGCTG CACCGGCAGC
 841 CAGGCTTTGC TGCTGAGGAC GCCCTACAGC AGTGACAATT TATACCAAAT GACATCCCAG
 901 CTTGAATGCA TGACCTGGAA TCAGATGAAC TTAGGAGCCA CCTTAAAGGG CCACAGCACA
 961 GGGTACGAGA GCGATAACCA CACAACGCCC ATCCTCTGCG GAGCCCAATA CAGAATACAC
1021 ACGCACGGTG TCTTCAGAGG CATTCAGGAT GTGCGACGTG TGCCTGGAGT AGCCCCGACT
1081 CTTGTACGGT CGGCATCTGA GACCAGTGAG AAACGCCCCT TCATGTGTGC TTACCCAGGC
1141 TGCAATAAGA GATATTTTAA GCTGTCCCAC TTACAGATGC ACAGCAGGAA GCACACTGGT
1201 GAGAAACCAT ACCAGTGTGA CTTCAAGGAC TGTGAACGAA GGTTTTCTCG TTCAGACCAG
1261 CTCAAAAGAC ACCAAAGGAG ACATACAGGT GTGAAACCAT TCCAGTGTAA AACTTGTCAG
1321 CGAAAGTTCT CCCGGTCCGA CCACCTGAAG ACCCACACCA GGACTCATAC AGGTGAAAAG
1381 CCCTTCAGCT GTCGGTGGCC AAGTTGTCAG AAAAAGTTTG CCCGGTCAGA TGAATTAGTC
1441 CGCCATCACA ACATGCATCA GAGAAACATG ACCAAACTCC AGCTGGCGCT TTGA
//
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B

Protein sequences

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1 AUG CAG GAC CCG GCU UCC ACG UGU GUC CCG GAG CCG GCG UCU CAG CAC ACG CUC CGC UCC 60
  M  Q  D  P  A  S  T  C  V  P  E  P  A  S  Q  H  T  L  R  S
61GGG CCU GGG UGC CUA CAG CAG CCA GAG CAG CAG GGA GUC CGG GAC CCG GGC GGC AUC UGG 120
   G  P  G  C  L  Q  Q  P  E  Q  Q  G  V  R  D  P  G  G  I  W
121 GCC AAG UUA GGC GCC GCC GAG GCC AGC GCU GAA CGU CUC CAG GGC CGG AGG AGC CGC GGG 180
   A  K  L  G  A  A  E  A  S  A  E  R  L  Q  G  R  R  S  R  G
181 GCG UCC GGG UCU GAG CCG CAG CAA AUG GGC UCC GAC GUG CGG GAC CUG AAC GCG CUG CUG 240
   A  S  G  S  E  P  Q  Q  M  G  S  D  V  R  D  L  N  A  L  L
241 CCC GCC GUC CCC UCC CUG GGU GGC GGC GGC GGC UGU GCC CUG CCU GUG AGC GGC GCG GCG 300
   P  A  V  P  S  L  G  G  G  G  G  C  A  L  P  V  S  G  A  A
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   Q  W  A  P  V  L  D  F  A  P  P  G  A  S  A  Y  G  S  L  G
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   T  F  D  G  T  P  S  Y  G  H  T  P  S  H  H  A  A  Q  F  P
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   N  H  S  F  K  H  E  D  P  M  G  Q  Q  G  S  L  G  E  Q  Q
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```

Figure 15. Homo sapiens Wilms tumor 1 (WT1), transcript variant A. A: mRNA sequences of WT1(-/-) provided from nucleotide data base, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). B: The WT1(-/-) protein translated sequences was analyzed by blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Blue letter = Start codon, Red letter = Protein abbreviation and * = Stop codon.

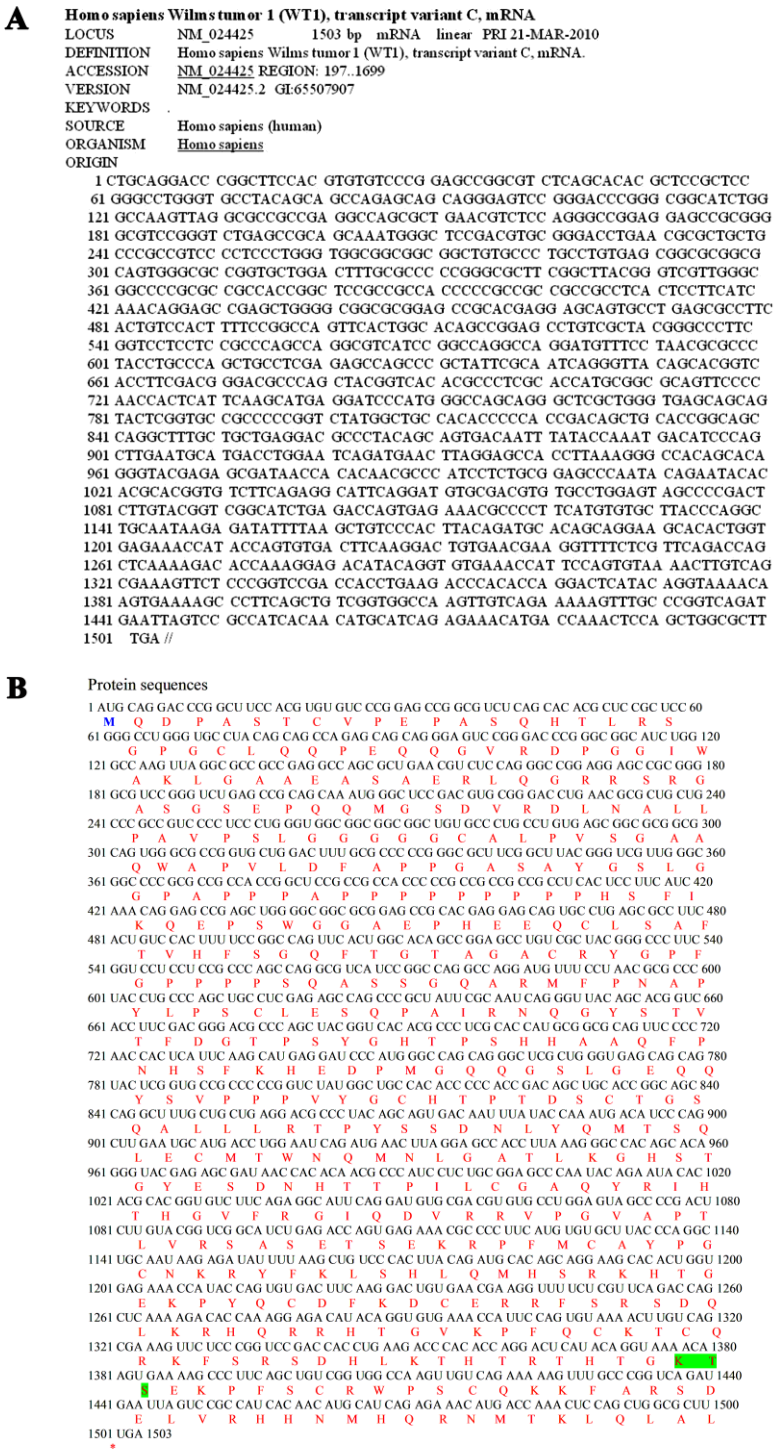

Figure 16. Homo sapiens Wilms tumor 1 (WT1), transcript variant C. A: mRNA sequences of WT1(-/+) provided from nucleotide data base, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). B: The WT1(-/+) protein translated sequences was analyzed by blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Blue letter = Start codon, Red letter = Protein abbreviation, * = Stop codon and Green highlight = KTS amino acid in exon 9.

Table 13. Nucleotide region in various exons and amino acid length of homo sapiens Wilms tumor 1 (WT1), transcript variant D or WT1(+/+).

Exon	Exon region	Amino acid length (aa)
1	1-646	251
2	647-769	41
3	770-872	34
4	873-950	26
5	951-1001	17
6	1002-1098	33
7	1099-1249	50
8	1250-1339	30
9	1340-1432	31
10	1433-1554	41
Total of amino acid		518

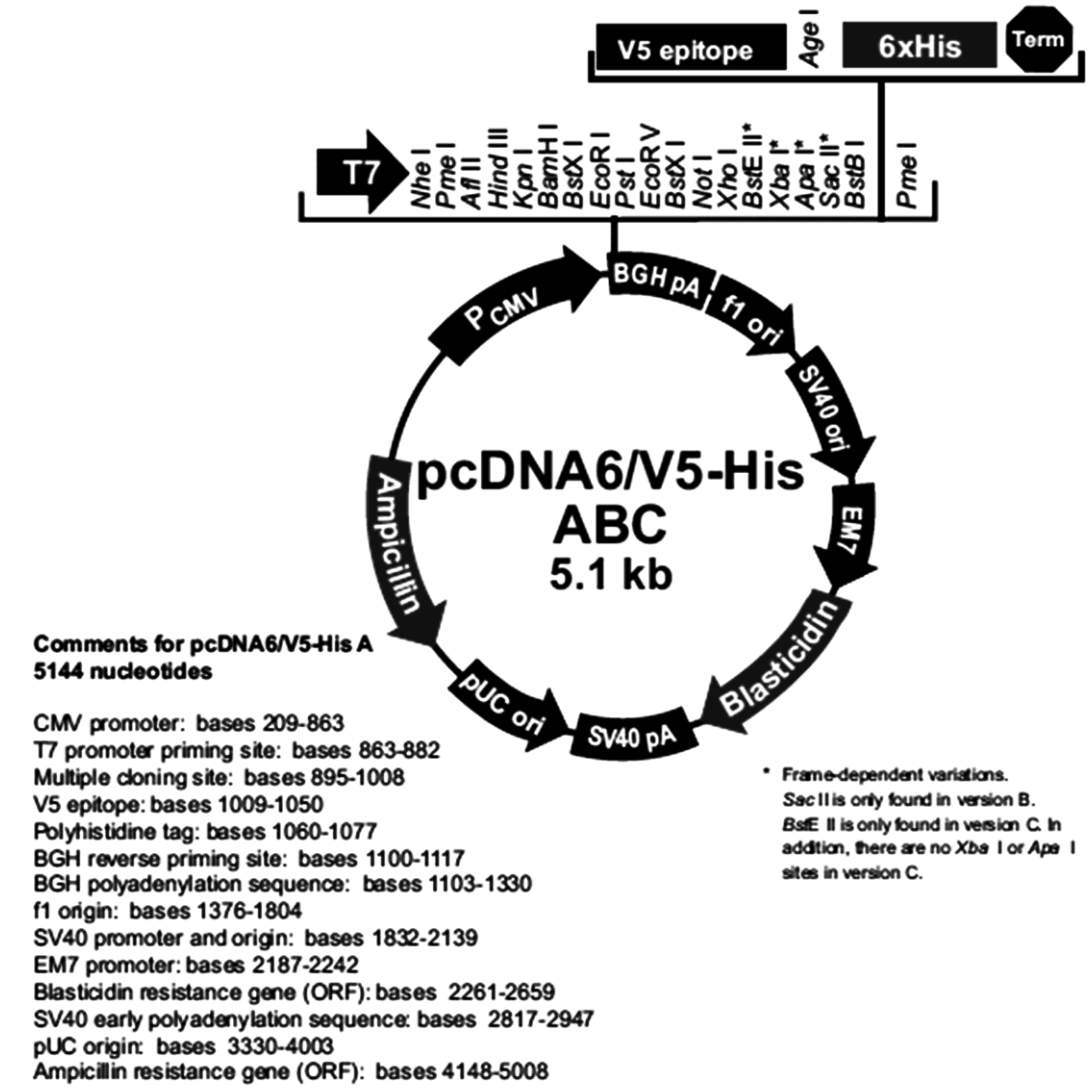

Figure 17. The feature of the pcDNA6™/V5-His vector (Invitrogen, CA, USA).

2.2.3.3 Establishment of cells stably overexpressing WT1 isoforms

The recombinant plasmid (step 2.2.3.2.3) containing WT1-/+ and WT1-/- gene were transfected into MCF-7 cells. The following protocol was optimized for MCF-7 cells, using a ratio of FuGENE® 6 reagent : DNA ratios of 3:1 and transfection mixture is shown in Table 14. One day before the transfection experiment, cells were seeded into 10 cm culture dish at the concentration of $1x10^6$ cells and incubated in a 37°C CO_2 incubator to achieve the desired density of 50–60% confluency. The transfection mixture was prepared in microcentrifugr tube. The serum free medium was added into centrifuge tube, and added FuGENE® 6 reagent into the culture medium without serum. Then, the recombinant plasmid from step 2.2.3.1 was added into the transfection mixture and gently mixed. The mixture was then incubated for 20 min at room temperature. The media in cell culture plate was removed and then cells were washed once with 1XPBS. Next, 5 ml of fresh complete medium were added into cell culture plate. The transfection mixture was added into 5 ml of culture medium and incubated at room temperature for 5 min. Then, the transfection mixture was wide dropped into cell culture plate and swirl the dish. After 48 h of incubation, the old medium was removed and washed once with 1XPBS. The medium including 10 μg/ml of Blasticidin was added, and cell culture dish was incubated in a CO_2 incubator at 37^oC until control cell die completely. The positive clones were selected using cloning ring, and cultured into 24 well plate.

2.2.3.4 Protein extraction for cell lines

Each positive clones from step 2.2.3.3 were coded as an aerobic number, and harvested. After harvesting, cell pellets were lysed in RIPA lysis buffer (Themo Scientific, Pierce) (150 mM NaCl, 50 mM Tris, pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate and 1 mM EDTA). The mixture was incubated on ice for 10 min and centrifuged at 14,000 xg for 10 min. After centrifugation, supernatant was measured to determine protein concentration by Bradford's method (Bio-Rad, CA, USA). The protein was determined as previously describes (2.2.2.2). The positive clone was selected with anti-WT1 antibody using Western blotting as previously describes (2.2.2.3 and 2.2.2.4).

Table 14. The Optimized transfection mixture for MCF-7 cells, using a ratio of FuGENE® 6 reagent : DNA ratios of 3:1.

Samples	**Culture medium without serum (µl)**	**FuGENE 6 (µl)**	**Plasmid DNA final concentration 10 µg (µl)**	**Culture medium without selective marker (µl)**
cDNA encoding WT1+/+ or WT1+/- or WT1-/+ or WT1-/-	176	18	6	5000
Empty pcDNA™6/V5-His B vector	176	18	6	5000

2.2.3.5 Small interference RNA (siRNA) transfection

The siRNA was transfected into mammalian cells using Lipofectamine™ 2000 (Invitrogen, CA, USA). Briefly, the number of 7.5 x10^4 overexpressing MCF-$7_{WT1+/+}$, MCF-$7_{WT1+/-}$, MCF-$7_{WT1-/+}$, MCF-$7_{WT1-/-}$ and MCF-7_{empty} cells were seeded into 24 well plates and incubated the culture plate in 37°C CO_2 incubator for 24 h. After cell grew to 50-60 % confluent, the control and siRNA transfection mixtures were prepared as shown in Table 15 and Table 16. $siRNA_{WT1}$ was specific with WT1 target and exon 7 and exon 8. The siRNA control is non specific sequence in human mammalian cells (siGENOME non-Targeting siRNA#2, Dharmacon product, Thermo Fisher Scientific, Co, USA). Solution 1 and solution 2 was prepared and incubated at room temperature for 5 min. After incubation, the solution 1 and solution 2 was combined. Then the transfection mixture was incubated at room temperature for 15 min. The culture medium was removed and cells were washed once with 1XPBS. The OptiMEM I solution were added into each 24 cell culture plate. Then, the siRNA control or $siRNA_{WT1}$ mixture was added into 24 cell culture plate and incubated at 37°C in a CO_2 incubator. After 6 h of incubation, the transfection reagent was removed, and cells were was once with 1XPBS. The RPMI complete medium was added and incubated in the 37°C CO_2 incubator for 4 days. After completed time incubation, cells were harvested and detected with ER-α, HER2 and WT1 proteins using Western blot analysis as previously describes (2.2.2.3 and 2.2.2.4).

Table 15. The solution 1 mixture for transfection of siRNA into MCF-7 cell lines.

siRNA Final concentration 100 nM	**Solution 1**		**Total volume (µl)**
	siRNA volume (µl)	**OptiMEM I (µl)**	
$siRNA_{WT1}$	1.25 µl of $siRNA_{WT1}$ 88 1.25 µl of $siRNA_{WT1}$ 90	47.5	50
siRNA control	2.5	47.5	50

Table 16. The solution 2 mixture and OptiMEM I for transfection of siRNA into MCF-7 cell lines.

siRNA Final concentration 100 nM	**Solution 2**		**OptiMEM I (µl)**	**Total volume (µl)**
	Lipofectamine™2000 (µl)	**OptiMEM I (µl)**		
$siRNA_{WT1}$	1	49	400	450
siRNA control	1	49	400	450

2.2.4 Statistic analysis

The different of intergroup was analyzed using Student's *t* test. Each experiments were repeated at least three times and data are represented as mean ± SD. A *P* value of less than 0.05 was considered to be statistically significant. The Spearman's correlation coefficient by rank test was used to analyze correlation of mRNA and protein expression.

CHAPTER 3

RESULTS

3.1 Study of WT1 mRNA in breast cancer tissues

3.1.1 Study of WT1(17AA) in breast cancer tissues

To examine the expression of WT1 at splicing variant 17AA at exon5 in breast cancer comparing to adjacent normal breast tissues, PCR primers for detection of WT1(17AA) splicing variants were designed to span over the exon 5 covering 17AA. Forward and reverse primers located on exon 4 and exon 6 of WT1 mRNA for amplification of two PCR product sizes, 185 bp for WT1(17AA+) and 134 bp for WT1(17AA-). Whereas WT1(KTS) primers were used to detect two PCR product sizes, 553 bp for WT1(KTS+) and 544 bp for WT1(KTS-). WT1(KTS) primers were specifically located on exon 6 and 10 of WT1 mRNA. Samples used in this study including 30 samples from adjacent normal breast tissues and 23 samples from breast cancer tissues were consented and approved from the ethic committee of Songklanakarin hospital. After sample collection, total RNA were isolated and the expression of WT1(17AA) and WT1(KTS) mRNA were then investigated using RT-PCR technique.

WT1(17AA-) mRNA was dominantly expressed in adjacent normal breast whereas the mixture of WT1(17AA+) and WT1(17AA-) mRNA was exhibited in both breast cancer carcinoma grade 1, grade 2, and grade 3 (Figure 18). WT1(17AA-) and WT1(17AA+) PCR products were randomly examined to confirm the sequence by Blastx data base and pair wise sequence alignment (Figure 19 and 20). The summary of WT1(17AA) mRNA expression is shown in Table 17. The WT1 mRNA was found in adjacent normal breast (83%, 25 of 30 samples), in breast carcinoma grade 1 and 2 (100%, 15 of 15 samples), and in breast carcinoma grade 3 (100% , 8 of 8 samples). However, WT1(17AA-) mRNA was strongly expressed at 53% (16 of 30 samples) in adjacent normal breast while low expressed in breast carcinoma grade 1 and 2, and 3. Interestingly, the mixture of WT1(17AA+) and WT1(17AA-) mRNA was highly detectable in breast carcinoma grade 3 at 88% and breast carcinoma grade 1 and 2 at 73%, but rarely detectable in adjacent normal breast at 13.33% (4 of 30 samples).

3.1.2 Study of WT1(KTS) in breast cancer tissues

The investigation of WT1(KTS) was first performed by SSCP analysis. The selected results of WT1(KTS) expression by SSCP analysis was shown in Figure 21. These results showed that control WT1(KTS+) and control WT1(KTS-) exhibited specific bands at 553 and 544 bp respectively. The control mixed WT1(KTS+) and WT1(KTS-) PCR products contained 553 and 544 bp bands. The mixed WT1(KTS+) and WT1(KTS-) was observed in two breast cancer cell lines (MCF-7, MDA-MB-468) and breast carcinoma grade 3 (12CA). While WT1(KTS-) was found in MCF-12A, which is normal breast cell lines. Since the GeneGel SSCP Starter kit was abolished to produce from the company (Amersham Biosciences, Pisacataway NJ, USA), the high resolution gel (metaphor agarose) was used for WT1(KTS) examination.

WT1(KTS) was separated by 3% metaphor agarose gel electrophoresis (Figure 22). The expression of WT1(KTS-) exhibited in adjacent normal breast tissues. DNA sequencing, Blastx data base and pair wise sequence alignment were used to confirm WT1(KTS) PCR products (Figure 23 and 24). The summary of WT1(KTS) splicing variants is shown in Table 18. WT1(KTS-) expressed in adjacent normal breast and was not found in breast carcinoma grade 1, grade 2, and grade 3. The mixture of WT1(KTS+) and WT1(KTS-) dominantly expressed in breast carcinoma grade 1 and grade 2.

The summary results for detection of WT1(KTS) PCR products only presented by 3% metaphor gel electrophoresis because SSCP analysis kit was not available from the origin of company (Life technologies coorporation, USA).

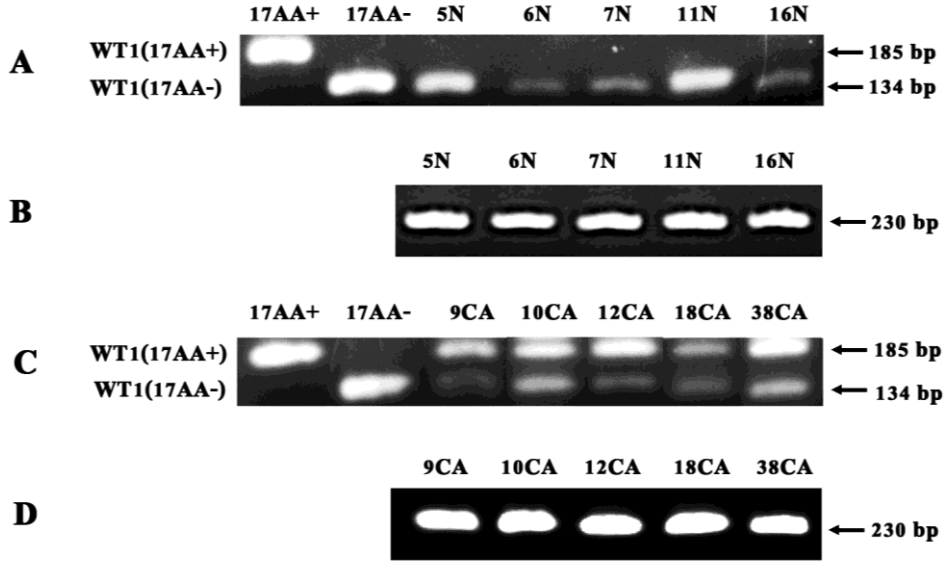

Figure 18. Expression of WT1(17AA+) and WT1(17AA-) in adjacent normal breast and breast carcinoma tissues. (A) Expression of WT1(17AA) in adjacent normal breast tissues was examined by RT-PCR. (B), (D) GAPDH PCR product was used as an internal control. (C) Expression of WT1(17AA) in breast cancer tissues was performed using primers that amplify regions surrounding exon 5. PCR products were observed under 2% agarose gel electrophoresis, 100 volt for 40 min at room temperature. (17AA+) and (17AA-) served as a positive control. The gel image presented is a representative of PCR product from 30 samples of adjacent normal breast tissues and 23 samples of breast cancer tissues. The result was performed in three independent experiments. N = adjacent normal breast tissues; CA = breast cancer tissues; 5N = adjacent normal breast tissue from fibroadenomas breast patient; 6N = adjacent normal breast tissues from phyllodes breast tumor patient; 7N = adjacent normal breast tissues from invasive ductal breast carcinoma patient; 11N = adjacent normal breast tissues from mucinous breast carcinoma patient; 16N = adjacent normal breast tissues from stromal breast fibrosis and sclerosing breast adenosis patient; 9CA, 10CA and 12CA = breast cancer tissues from infiltrating duct carcinoma grade 3 patient; 18CA = breast cancer tissues from invasive ductal carcinoma grade 2 patient; 38CA = breast cancer tissues from infiltrating duct carcinoma grade 3 patient.

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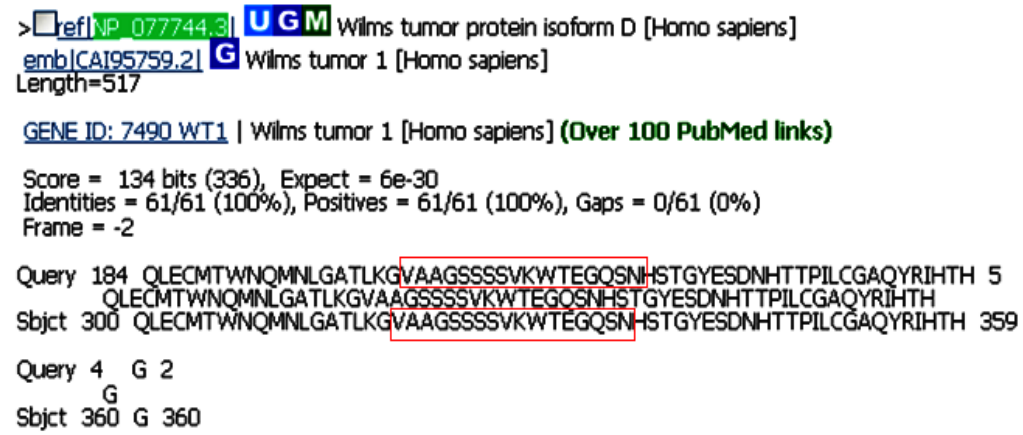

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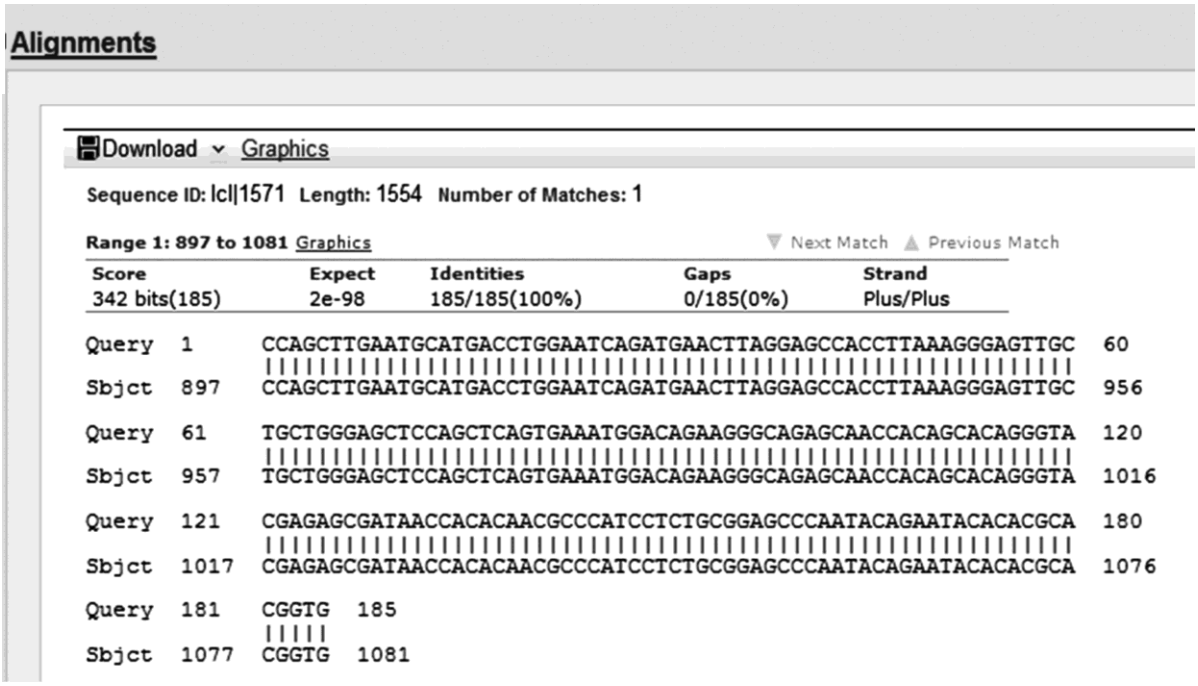

Figure 19. DNA sequences of WT1(17AA+) gene. (A) WT1(17AA+) PCR product was analyzed by blastx alignment comparing to full length WT1(+/+) protein (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). (B) Pair wise sequence between WT1(17AA+) PCR product and WT1(+/+) mRNA were aligned using align two sequence BLAST (bl2seq) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq).

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B

Alignments

Download Graphics Sort by: E value

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Figure 20. DNA sequences of WT1(17AA-) gene. (A) WT1(17AA-) PCR product was analyzed by blastx alignment comparing to full length WT1(+/+) protein (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). (B) Pair wise sequence between WT1(17AA-) PCR product and WT1(+/+) mRNA were aligned using align two sequence BLAST (bl2seq) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq).

Table 17. Summary of WT1(17AA+) and WT1(17AA-) mRNA expression in adjacent normal breast and breast cancer tissues using RT-PCR.

17AA	Adjacent normal breast	Invasive carcinoma grade 1 and 2	Invasive carcinoma grade 3
No bane	5 (17%)	0 (0%)	0 (0%)
17AA-	16 (53%)	2 (13%)	1 (14%)
17AA+	5 (17%)	2 (13%)	0 (0%)
17AA- and 17AA+	4 (13%)	11 (73%)	7 (88%)
Total cases	30	15	8

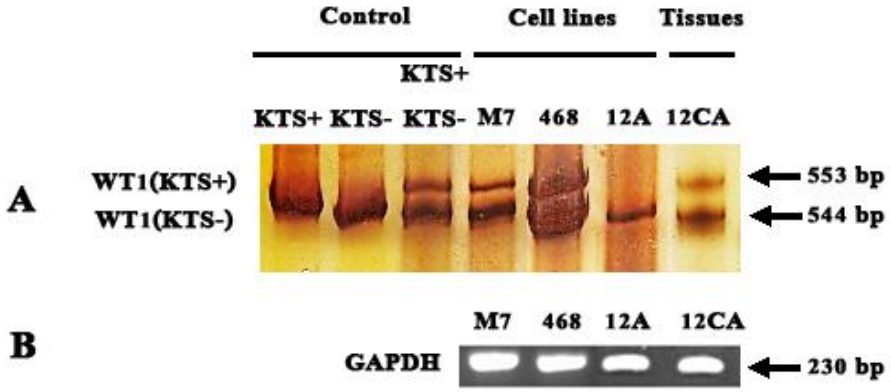


Figure 21. Expression of WT1(KTS) by SSCP analysis. (A) RT-PCR was performed using WT1(KTS) primers that amplify regions surrounding exon 9. Control consisted of KTS+, KTS-, and the mixture of KTS+ and KTS-. The KTS+ and KTS- exhibited PCR product sizes at 553 bp and 544 bp while the mixture of KTS+ and KTS- presented both bands 553 and 544 bp. (B) GAPDH was used as an internal control. M7: MCF-7 cell lines, 468: MDA-MB-468 cell lines, 12A: MCF-12A cell lines, 12CA: breast cancer tissues grade 3.

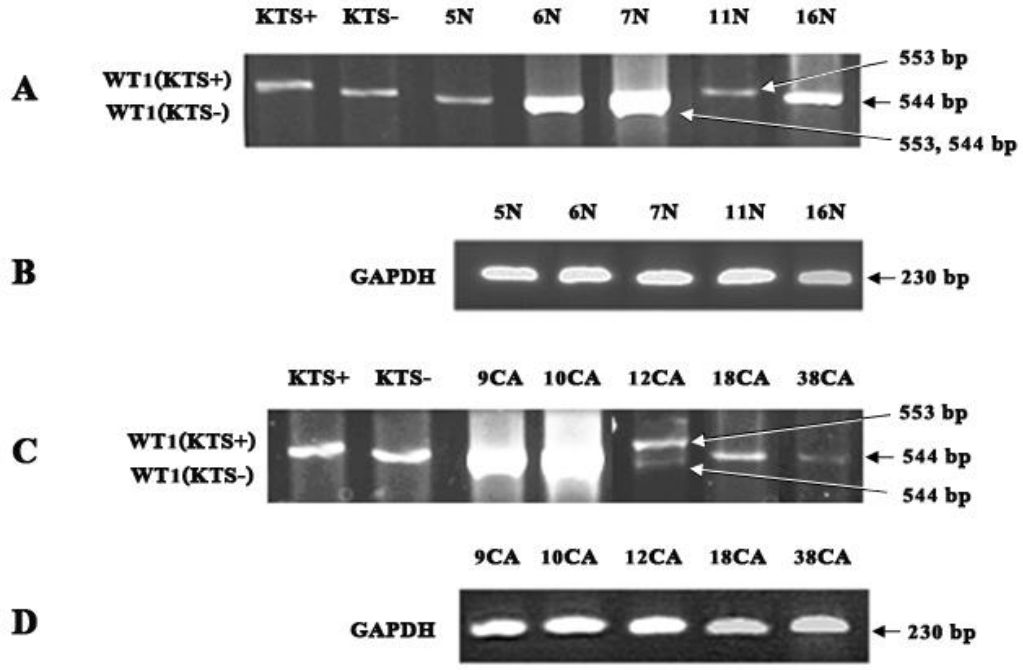
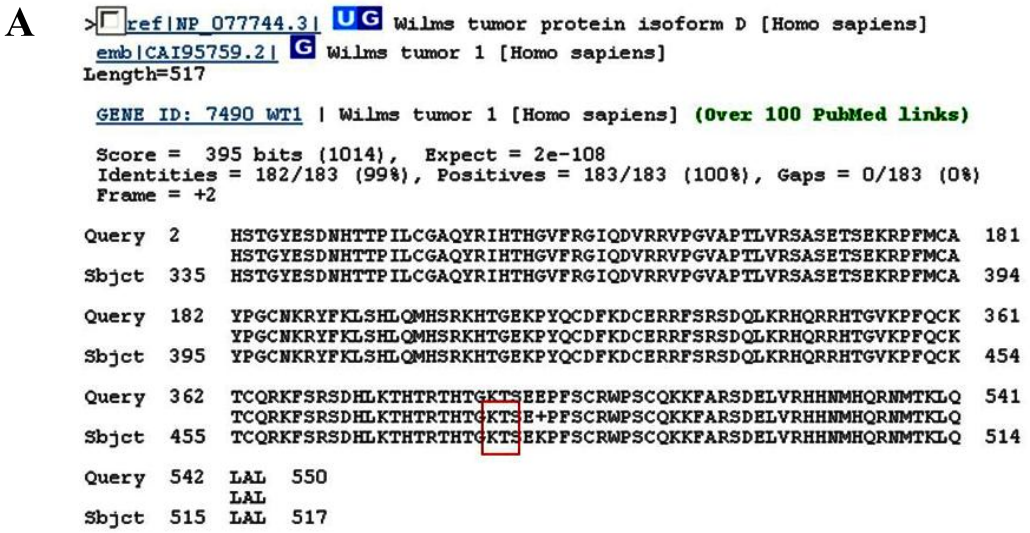


Figure 22. Expression of WT1(KTS+) and WT1(KTS-) in adjacent normal breast and breast cancer tissues. Expression of WT1(KTS) was examined in adjacent normal breast tissues (A) and breast cancer tissues (C). RT-PCR was performed using WT1(KTS) primers that amplify regions surrounding exon 9. KTS+ and KTS- at first and second lanes served as a positive control. PCR products were observed under 3% metaphor agarose gel electrophoresis, 75 volt for 5 h at 4°C. GAPDH gene was used as an internal control that was amplified in adjacent normal breast tissues (B) and breast cancer tissues (D). GAPDH was run in 2% agarose gel, 100 volt for 40 min at room temperature. Result was performed in three independent experiments. N = adjacent normal breast tissues; CA = breast cancer tissues grade 3; 5N = adjacent normal breast tissue from fibroadenomas breast patient; 6N = adjacent normal breast tissues from phyllodes breast tumor patient; 7N = adjacent normal breast tissues from invasive ductal breast carcinoma patient; 11N = adjacent normal breast tissues from mucinous breast carcinoma patient; 16N = adjacent normal breast tissues from stromal breast fibrosis and sclerosing breast adenosis patient; 9CA, 10CA and 12CA = breast cancer tissues from infiltrating duct carcinoma grade 3 patient; 18CA = breast cancer tissues from invasive ductal carcinoma grade 2 patient; 38CA = breast cancer tissues from infiltrating duct carcinoma grade 3 patient.

A



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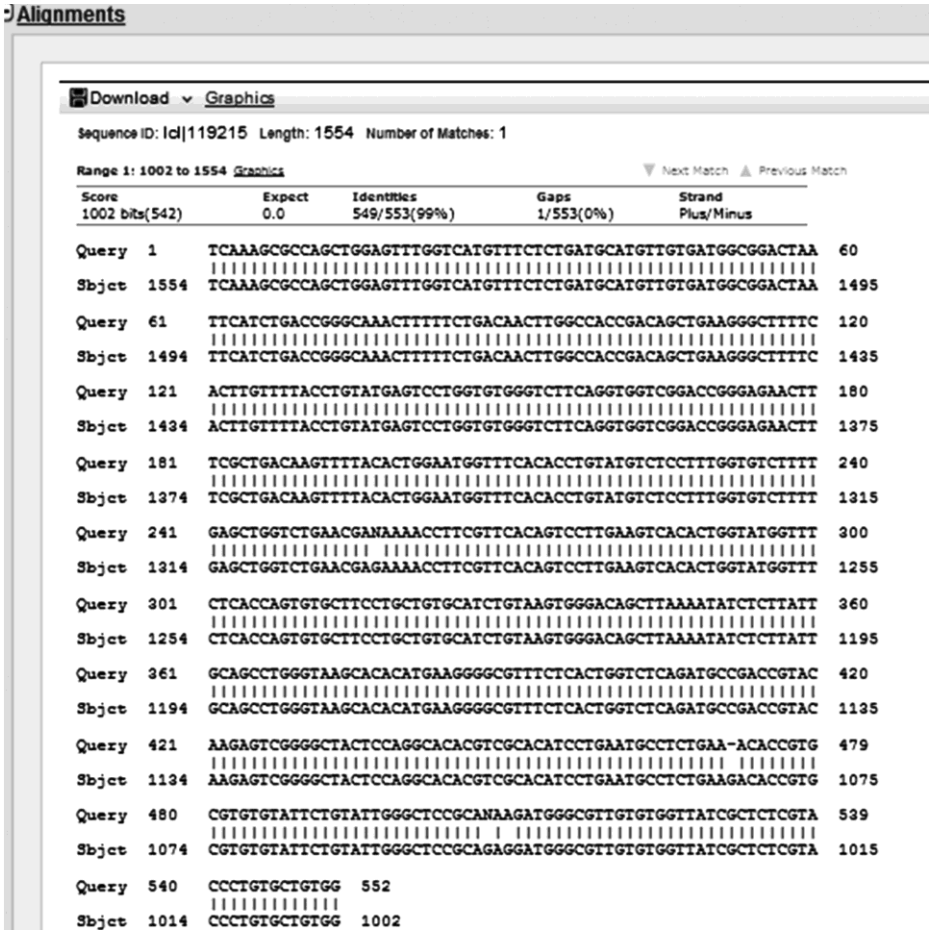

Alignments

Download Graphics

Sequence ID: lcl|119215 Length: 1554 Number of Matches: 1

Range 1: 1002 to 1554 Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1002 bits(542)	0.0	549/553(99%)	1/553(0%)	Plus/Minus

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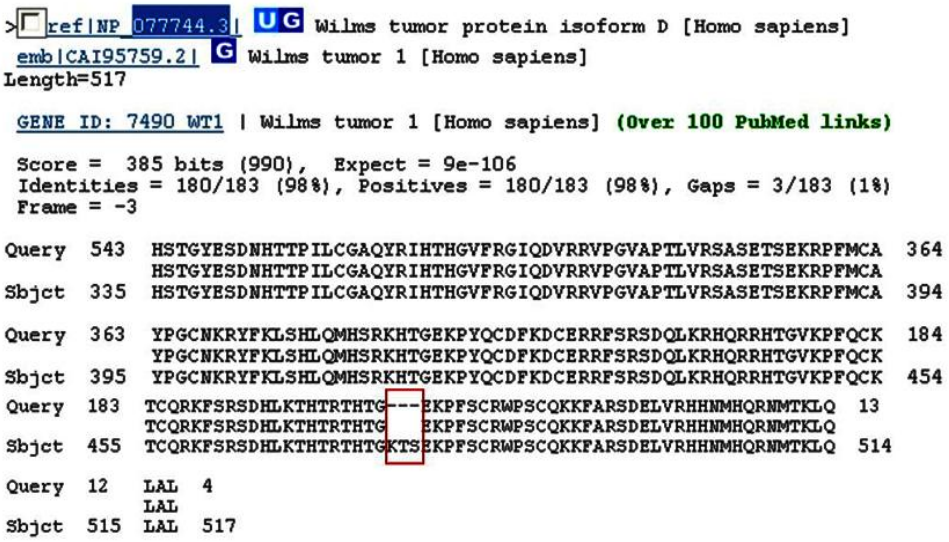
Figure 23. DNA sequences of WT1(KTS+) gene. (A) WT1(KTS+) PCR product was analyzed by blastx alignment comparing to full length WT1(+/+) protein (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). (B) Pair wise sequence between WT1(KTS+) PCR product and WT1(+/+) mRNA were aligned using align two sequence BLAST (bl2seq) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq).

A



B

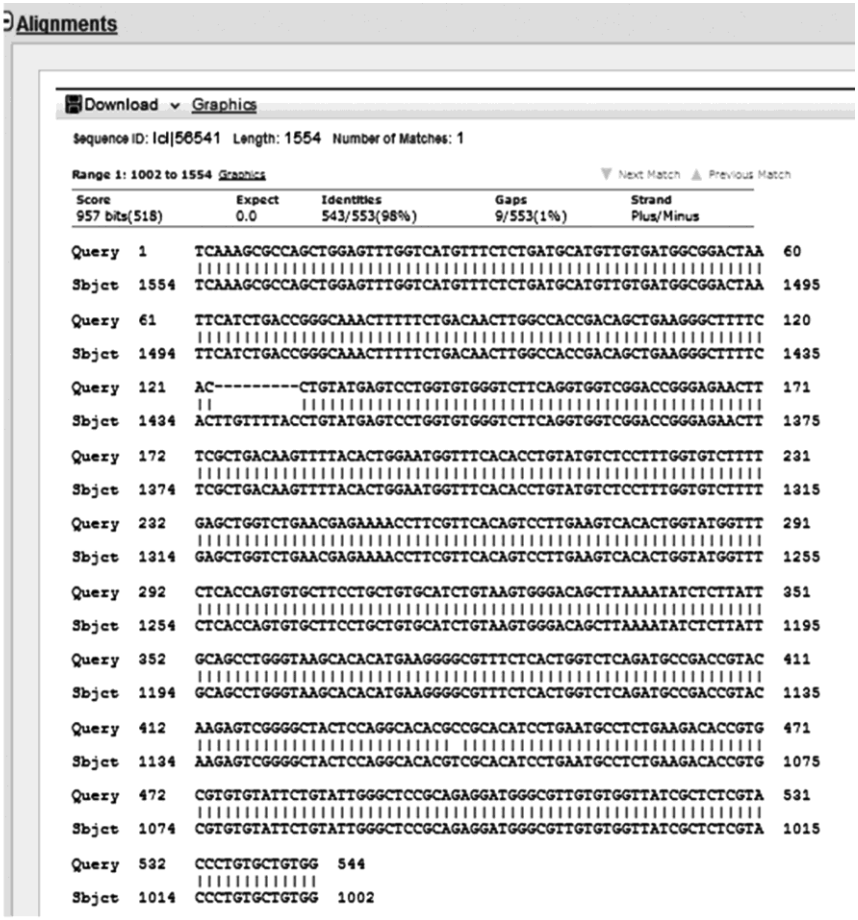


Figure 24. DNA sequences of WT1(KTS-) gene. (A) WT1(KTS-) PCR product was analyzed by blastx alignment comparing to full length WT1(+/+) protein (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). (B) Pair wise sequence between WT1(KTS-) PCR product and WT1(+/+) mRNA were aligned using align two sequence BLAST (bl2seq) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq).

Table 18. Summary of WT1(KTS+) and WT1(KTS-) splicing mRNA variant expression in adjacent normal breast and breast cancer tissues using RT-PCR.

KTS	Adjacent normal breast	Invasive carcinoma grade 1 and 2	Invasive carcinoma grade 3
KTS-	2 (17%)	0 (0%)	0 (0%)
KTS+	3 (25%)	1 (20%)	2 (33%)
KTS- and KTS+	7 (58%)	4 (80%)	4 (67%)
Total cases	12	5	6

3.2 Study of WT1, ER-α and HER2 proteins in breast cancer tissues

Here, the expression of WT1 isoforms was examined using Western blot analysis to confirm the RT-PCR results. WT1 protein bands at upper and lower represented 59 kDa for WT(17AA+) and 53 kDa for WT1(17AA-), respectively (Figure 25). WT1 protein was used to confirm WT1 mRNA expression. The summary of WT1 protein is shown in Table 19. WT1(17AA-) isoform alone was dominantly expressed in adjacent normal breast at 88% (15 of 17 samples) but was not detected in breast carcinoma grade 1 and 2, and 3. In addition, the mixed WT1(17AA+) and WT1(17AA-) isoform was significantly expressed in breast carcinoma grade 1, 2 and grade 3 (100% from 17 samples) but low expressed in adjacent normal breast (12% from 2 of 17 samples). This finding suggests that WT1(17AA+) may be a partner molecule of WT1(17AA-) that plays a function as an oncogene in breast cancer.

Since ER-α and HER2 involved in breast cancer progression via multi-molecular mechanism (Arpino et al., 2008). Therefore, this study investigated the correlation of WT1 isoforms on ER-α and HER2 regulation in adjacent normal breast and breast cancer tissues using Western blotting. The expression of ER-α and HER2 was not found in adjacent normal breast tissues. In contrary, ER-α and HER2 proteins were detected in breast cancer grade 1 and 2 and highly expressed in in breast cancer grade 3 (Figure 25). The expression of ER-α presented in breast carcinoma grade 1 and 2, and 3 at 60% and 100% respectively whereas ER-α was low expressed in adjacent normal breast tissues at 23%. HER2 expression was found in breast

carcinoma grade 1 and 2, and 3 at 80% and 71% respectively. HER2 was low expressed in adjacent normal breast tissues at 11% (Table 19).

The results of Spearman rank correlation showed the negative correlation between 17AA- and ER-α (Table 20), and the negative correlation between 17AA- and HER2 (Table 21). In contrast, the expression of the mixed WT1(17AA+) and (17AA-) isoform in adjacent normal breast and breast carcinoma tissues were moderately correlated with ER-α (r = 0.5, $P = 0.575$) (Table 22). The strong positive correlation between the mixed WT1(17AA+) and (17AA-) isoform and HER2 (r = 1, $P < 0.001$) is shown in Table 23. Additionally, the positive correlation of WT1 expression was observed between invasive carcinoma grade 1 and 2, and grade 3 (r = 0.87, $P = 0.33$) (Table 24) while the inverse correlation of WT1 was found in adjacent normal breast and invasive carcinoma grade 1 and 2 (r = -0.66, $P = 0.33$) (Table 25). The negative correlation of WT1 expression between adjacent normal breast and invasive carcinoma grade 3 (r = -0.5, $P = 0.67$) is shown in Table 26. These finding indicated that the expression of mixed WT1(17AA+) and (17AA-) isoform showed positively correlation with ER-α and HER2, but the *P* value of Spearman's rank correlation was not significant. However, the correlated tenor of WT1, ER-α, and HER2 expression might be useful to study in large sampling scale of breast cancer patients.

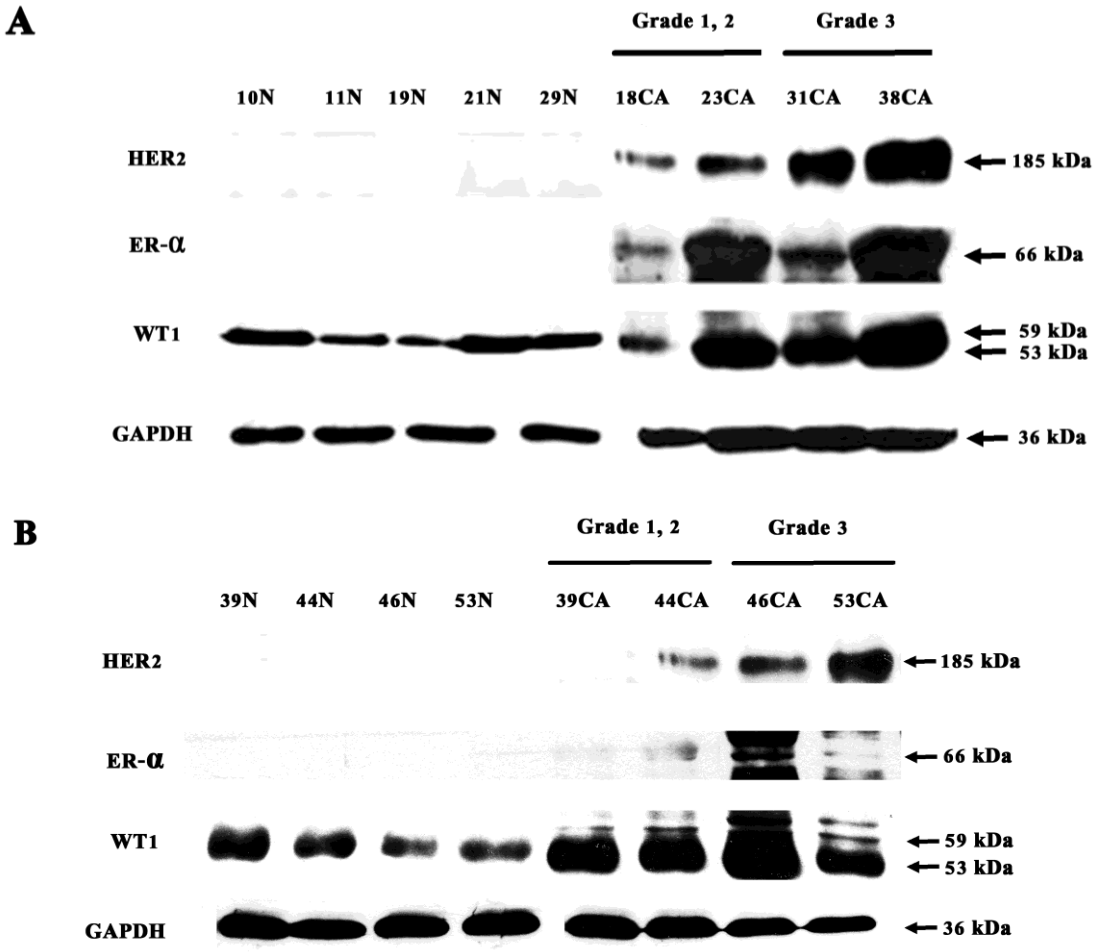


Figure 25. Expression of WT1, ER-α, and HER2 in adjacent normal breast and breast cancer tissues grade 1 and 2, and 3 tissues using Western blot analysis. Fifty micrograms of protein concentration from each sample were load and separated on 12% SDS-PAGE. (A), (B) Sampling western blot analysis results from adjacent normal breast and breast cancer tissues. GAPDH was used as an internal control. The case number of breast cancer patients represented in an Arabic number. N = adjacent normal breast tissues, CA = breast cancer tissues grade1 and 2, and 3. HER2, ER-α , WT1(17AA+), and WT1(17AA-) presented protein bands at 185, 66, 59 and 53 kDa respectively. The molecular weight was referred form MajicMark™ XP Western Protein Standard protein marker (Invitrogen, USA).

Table 19. Correlation of WT1(17AA+), WT1(17AA-), ER-α, and HER2 in adjacent normal breast and breast cancer tissues using Western blot analysis.

Proteins	Adjacent normal breast	Invasive carcinoma grade 1 and 2	Invasive carcinoma grade 3
17AA-	15 (88%)	0 (0%)	0 (0%)
17AA+	0 (0%)	0 (0%)	0 (0%)
17AA- and 17AA+	2 (12%)	10 (100%)	7 (100%)
ER-α	4 (23%)	6 (60%)	7 (100%)
HER2	2 (12%)	8 (80%)	5 (71%)
Total cases	17	10	7

Table 20. The correlation of WT1(17AA-) with ER-α protein in adjacent normal breast and breast carcinoma grade 1 and 2, and grade 3 tissues using Spearman rank correlation test.

Tissues	17AA-	ER-α	r	*P*
Adjacent normal breast	15/17 (88.24)	4/17 (23.53)	-0.87	0.33
Invasive carcinoma grade 1 and 2	0/10 (0)	6/10 (60)		
Invasive carcinoma grade 3	0/7 (0)	7/7 (100)		

Table 21. The correlation of WT1(17AA-) with HER2 protein in adjacent normal breast and breast carcinoma grade 1 and 2, and grade 3 tissues using Spearman rank correlation test.

Tissues	17AA-	HER2	r	*P*
Adjacent normal breast	15/17 (88.24)	2/17 (11.76)	-0.86603	0.333
Invasive carcinoma grade 1 and 2	0/10 (0)	8/10 (80)		
Invasive carcinoma grade 3	0/7 (0)	5/7 (71.43)		

Table 22. The correlation of mixed WT1(17AA+) and WT1(17AA-) with ER-α protein in adjacent normal breast and breast carcinoma grade 1 and 2, and grade 3 tissues using Spearman rank correlation test.

Tissues	17AA+ and 17AA-	ER-α	r	*P*
Adjacent normal breast	2/17 (11.76)	7/7 (100)	0.5	0.575
Invasive carcinoma grade 1 and 2	10/10 (100)	7/7 (100)		
Invasive carcinoma grade 3	7/7 (100)	7/7 (100)		

Table 23. The correlation of mixed WT1(17AA+) and WT1(17AA-) with HER2 proteins in adjacent normal breast and breast carcinoma grade 1 and 2, and grade 3 tissues using Spearman rank correlation test.

Tissues	17AA+ and 17AA-	HER2	r	*P*
Adjacent normal breast	2/17 (11.76)	2/17 (11.76)	1	< 0.001
Invasive carcinoma grade 1 and 2	10/10 (100)	8/10 (80)		
Invasive carcinoma grade 3	7/7 (100)	5/7 (71.43)		

Table 24. The correlation of breast cancer tissues grade 1 and 2, and grade 3 with the expression of WT1 mRNA using Spearman rank correlation test.

WT1 isoforms	Invasive carcinoma grade 1 and 2	Invasive carcinoma grade 3	r	*P*
17AA-	2	1	0.87	0.33
17AA+	2	0		
17AA+ and 17AA-	11	7		
Total	15	8		

Table 25. The correlation of adjacent normal breast tissues and breast cancer tissues grade 1 and 2 with the expression of WT1 mRNA using Spearman rank correlation test.

WT1 isoforms	Adjacent normal breast	Invasive carcinoma grade 1 and 2	r	*P*
17AA-	16	2	-0.66	0.33
17AA+	5	2		
17AA+ and 17AA-	4	11		
Total	25	15		

Table 26. The correlation of adjacent normal breast tissues and breast cancer tissues grade 3 with the expression of WT1 mRNA using Spearman rank correlation test.

WT1 isoforms	Adjacent normal breast	Invasive carcinoma grade 3	r	*P*
17AA-	16	1	-0.5	0.67
17AA+	5	0		
17AA+ and 17AA-	4	7		
Total	25	8		

3.3 Study of the correlation between WT1, ER-α, and HER2 in breast cancer cell lines

The expression of WT1, ER-α, and HER2 was examined by Western blotting in normal breast cell line (MCF-12A) and three breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-468) (Figure 26). WT1 expression was the highest in MDA-MD-488 comparing to MCF-7 and MDA-MB-231, but not detected in MCF-12A. In addition, the protein levels of ER-α were found in MCF-7 and slightly expressed in MDA-MB-231 while ER-α protein was not found in MDA-MB-468 and MCF-12A. HER2 protein detected in MCF-7 was higher than in

MDA-MB-231, but it was not detected in both MCF-12A and MDA-MB-468. Since MCF-7 represented high expression of ER-α and HER2 proteins and low expression of WT1, MCF-7 cells was suitable for being used as model for establishing WT1s stable cell lines. The full length of WT1(+/+), WT1(+/-), WT1(-/+), and WT1(-/-) genes were fused with pCDNA6 vector. Then, the recombinant plasmid was sequenced and transfected into MCF-7 cells. The positive clones were selected using 10 μg/ml of Blasticidin and Western blotting (Figure 27). Twenty four clones were obtained from. MCF-$7_{WT1-/+}$. The positive clone number 5 was selected for next experiment because this clone represented the highest WT1 expression. Clones were obtained from MCF-$7_{WT1-/-}$. Clone number 2 was highly expressed WT1 and selected for next study. The positive clone number 1 of MCF-7_{WT1++-} and clone number 8 of MCF-$7_{WT1+/-}$ kindly provided from Asst. Prof. Dr. Potchanapond Gradist that contained empty vector MCF-7_{Empty} were used as a negative control.

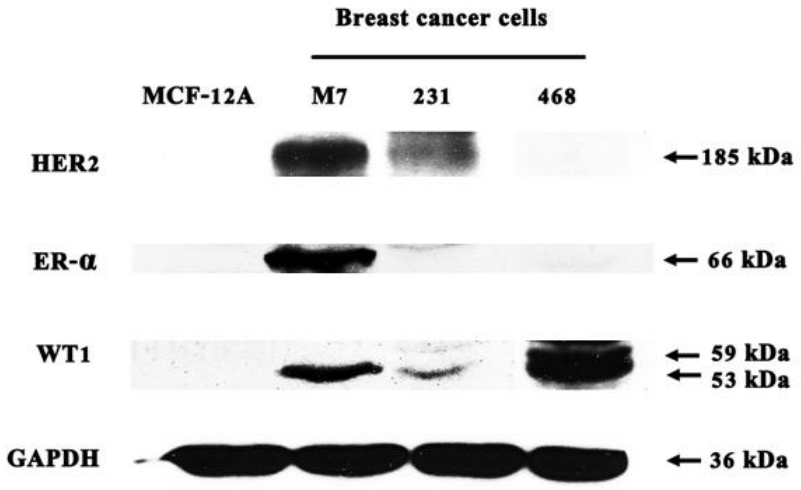


Figure 26. The expression of WT1, ER-α, and HER2 in normal breast and breast cancer cell lines. One hundred micrograms of protein from three breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-468) and one normal breast cell lines (MCF-12A) were separated on 12% SDS-PAGE. GAPDH was used as an internal control. Expression of WT1(17AA+) and WT1(17AA-) presents protein bands at 59 and 53 kDa respectively. The molecular weight was referred from MajicMark™ XP Western Protein Standard protein marker (Invitrogen, USA). M7 = breast cancer cell line MCF-7; 231 = breast cancer cell line MDA-MB-231; 468 = breast cancer cell line MDA-MB-468.

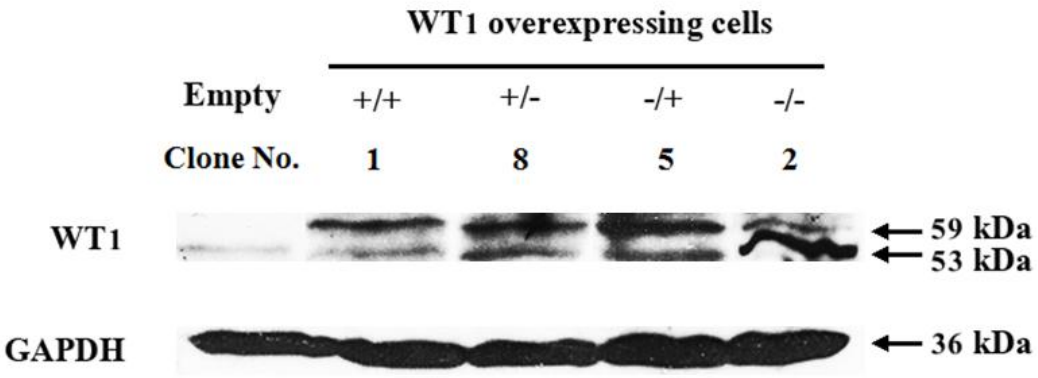


Figure 27. Expression of WT1 isoforms in MCF-7 cells. One hundred micrograms of protein were separated on 12% SDS-PAGE. Expression of WT1(17AA+) and WT1(17AA-) presents protein bands at 59 and 53 kDa respectively. GAPDH was used as an internal control. (+/+), (+/-), (-/+), and (-/-) represented as WT1(+/+), WT1(+/-), WT1(-/+), WT1(-/-) respectively. The molecular weight was referred from MajicMark™ XP Western Protein Standard protein marker (Invitrogen, USA).

Next experiment, stable WT1s overexpressing MCF-7 cells that expressed WT1(+/+), WT1(+/-), WT1(-/+), and WT1(17AA-/-) isoforms were knocked down using $siRNA_{WT1}$. The effect of WT1 isoforms on cell morphology was investigated under phase contrast microscopy. After transfection, the stable WT1s overexpressing MCF-7 cells exhibited growth faster than control ($MCF\text{-}7_{Empty}$) cells. Interestingly, WT1(+/+) and WT1(+/-) were able to induce cell growth that was observed in $MCF\text{-}7_{WT1(+/+)}$ and $MCF\text{-}7_{WT1(+/-)}$ cells while $MCF\text{-}7_{WT1(-/-)}$ cells presented slow growth. The characteristic of $MCF\text{-}7_{WT1+/+}$, $MCF\text{-}7_{WT1+/-}$, $MCF\text{-}7_{WT1-/+}$, and $MCF\text{-}7_{WT1-/-}$ cells was changed to an abnormal growth and morphology.

After decreasing of WT1 using $siRNA_{WT1}$, $MCF\text{-}7_{Empty}$ cells were detached and the cell number was decreased comparing with control cells ($MCF\text{-}7_{Empty}$). The stable WT1(+/+), WT1(+/-), and WT1(-/+) MCF-7 cells affect the decrease of cell number whereas the stable WT1(-/-) MCF-7 cells slowly grow.

To confirm the hypothesis that WT1 isoforms correlated with up-regulation of ER-α and HER2, the protein levels of WT1, ER-α, and HER2 were determined in the stable WT1s overexpressing MCF-7 cells using siRNA and Western blot analysis. In $siRNA_{neg}$ group (Figure 29A, 29B), high expression of WT1 correlated with the expression of ER-α and HER2, especially, WT1(+/+) and WT1(+/-) (Figure 29A). Protein expression level of WT1, ER-α, and HER2 was quantified by normalization with GAPDH protein band intensity. The ER-α expression in WT1(+/+), WT1(+/-), WT1(-/+), and WT1(-/-) cells was significantly expressed for 1.164, 1.091, 1.011, and 0.504 fold respectively comparing to control $MCF\text{-}7_{empty}$ cells (Figure 29B). In addition, the HER2 highly expressed in WT1(+/+) and WT1(+/-) cells (Figure 29A), at 1.773 and 1.599 fold respectively comparing to $MCF\text{-}7_{empty}$ cells (0.635 fold) (Figure 29B). In $siRNA_{WT1}$ group, the expression of WT1 was completely abolished and no expression of ER-α in all WT1 isoforms, comparing to $siRNA_{neg}$ group. Moreover, the expression of HER2 was also significantly decreased in all WT1s isoform (Figure 29A, 29B).

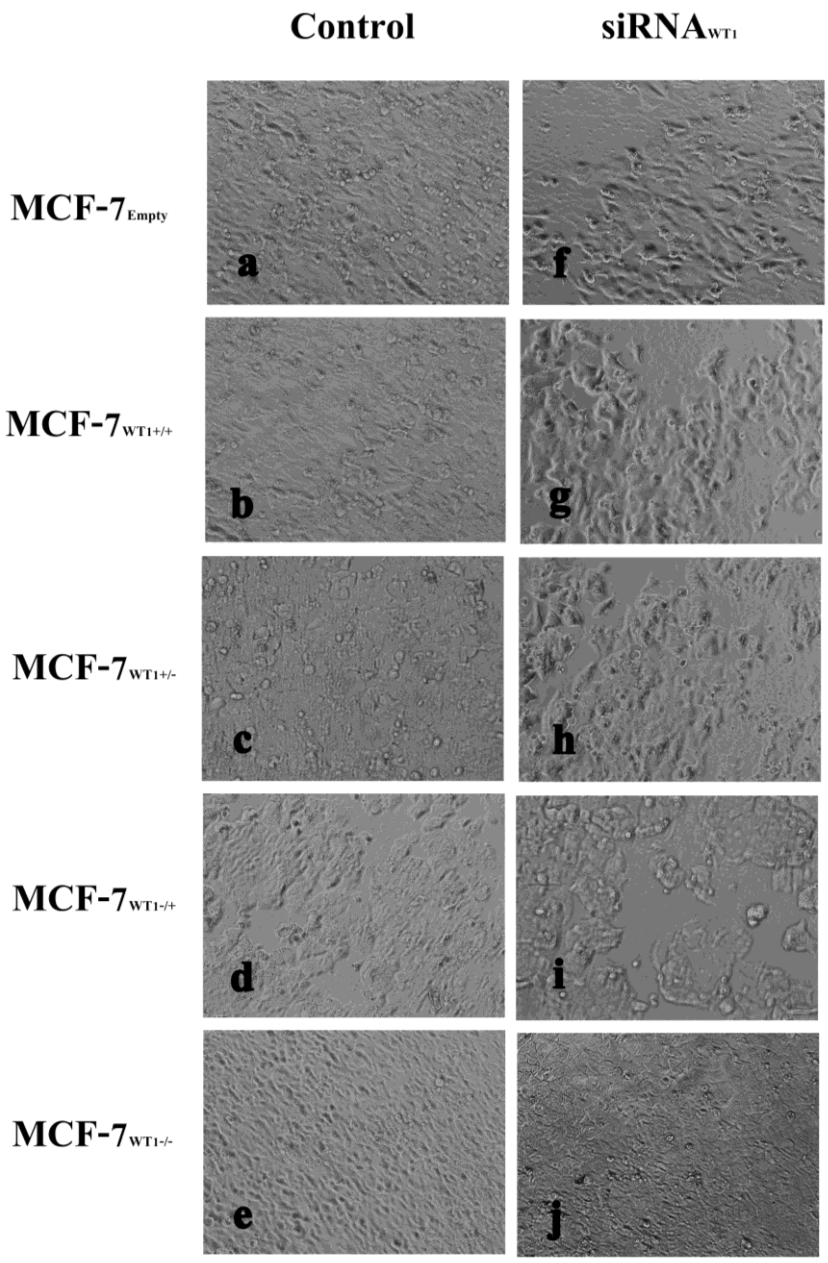


Figure 28. The morphology of stable WT1s overexpressing MCF-7 cells was observed under phase contrast microscope. Photos are shown 100x microscope magnification of two groups. Control group represented MCF-7 cells harboring (a) empty expression vector, (b) WT(+/+), (c) WT1(+/-), (d) WT1(-/+), and (e) WT1(-/-) isoforms that were challenged with 100 nM siRNA$_{neg}$. siRNA$_{WT1}$ group represented MCF-7 cells harboring (f) empty expression vector, (g) WT1(+/+), (h) WT1(+/-), (i) WT1(-/+), and (j) WT1(-/-) isoforms that were challenged with 100 nM siRNA$_{WT1}$.

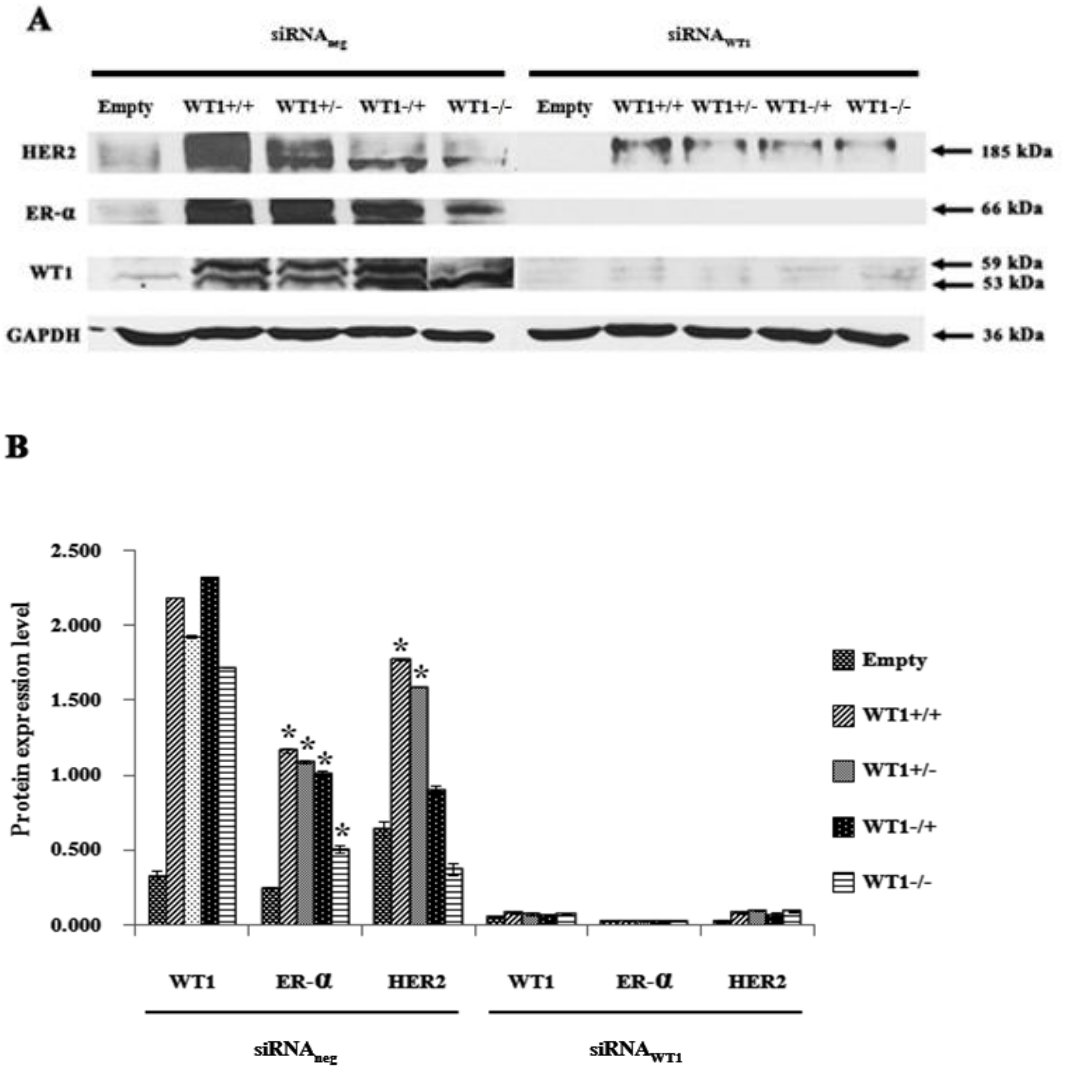


Figure 29. Western blot analysis of WT ER-α and HER2 expression in WT1 overexpressing MCF-7 cells. (A) The expressions of WT1, ER-α, and HER2 in MCF-7 cells treated with 100 nM siRNA$_{WT1}$. After 96 hrs of siRNA transfection, cells were harvested and performed Western blotting. GAPDH was used as a loading control. The molecular weight was referred form MajicMark^{TM} XP Western Protein Standard protein marker (Invitrogen, USA). (B) The intensity of WT1, ER-α, and HER2 protein bands were quantified using Scion Image software. siRNA$_{neg}$ group was used as a control that challenged with 100 nM siRNA$_{neg}$. siRNA$_{WT1}$ group was challenged with 100 nM siRNA$_{WT1}$. Results were normalized with the intensity of GAPDH protein levels. Results represent the average of three independent experiments and are mean ± S.D. (*$p<0.05$).

CHAPTER 4

DISCUSSIONS

4.1 Study of WT1 mRNA in breast cancer tissues

Previous researches investigates the growth inhibitory effect of small interference RNA silencing of WT1 gene in MCF-7. The results showed that siRNA against WT1 gene is decrease the level of WT1 protein in MCF-7 in dose dependent manner and decreasing the number of cells in time dependence. These observation suggested that small interference RNA silencing of WT1 gene plays inhibitory proliferation (Navakanit et al., 2007). As observed previously, WT1(+/+) and WT1(+/-) isoforms play anti-apoptotic function in MCF-7 cells. Since WT1(+/+) and WT1(+/-) isoforms play inhibition of apoptosis in doxorubicin treated MCF-7 cells (Graidist et al., 2010). In addition, WT1 gene is overexpressed in wide variety of solid tumors (Oji et al., 1999). Also, Loeb et al. (2001) have demonstrated that high expression of WT1 mRNA and protein was found over 90% in breast cancer, but rarely detected in adjacent normal breast tissues. Although WT1 is expressed in various solid tumors and its expression level serving as a significant prognostic factor, but WT1 expression has rarely been studied in breast cancers. This study underlined to explore the expression of WT1 isoform and its function. The results for study of WT1 expression consisted of two parts, WT1(17AA) and WT1(KTS) expression in adjacent normal breast and breast cancer tissues.

WT1(17AA) expression was detected in both adjacent normal breast and breast cancer tissues. These finding showed that the dominant expression of WT1(17AA-) mRNA was found in adjacent normal breast. Whereas breast carcinoma grade 1, 2 and 3 tissues dominantly presented mixed of WT1(17AA+) and WT1(17AA-) mRNA. Also, the expression of WT1 isoforms in protein level found that mixed of WT1(17AA+) and WT1(17AA-) isoform was highly similar expression in breast carcinoma tissuses. While, the expression of WT1(17AA-) isoform high presented in adjacent normal breast cancer tissues. The results suggested that the expression of mixed WT1(17AA+) and WT1(17AA-) isoforms in breast carcinoma plays a crucial function in cancer progression and development. Especially, WT1(17AA+) may be the partner molecule of WT1(17AA-) for inducement or inhibition multi-molecular mechanisms.

Previous supported reports found that the expression of WT1(+/+) isoform in cancer cells plays an important tumor suppressor effects because this isoform enhanced the inhibition of cell growth by WT1 antisense oligomer (Oji et al., 2004; Yang et al., 2007). In addition, WT1(+/+) and WT1(+/-) isoform also play anti-apoptotic functions through the apoptotic intrinsic pathway since WT1(17AA+) isoform was knocked down by using shRNA targeting exon 5. These evidence leaded to stabilize of mitochondrial membrane permeability. In addition, WT1(17AA+) activated pro-apoptotic Bax protein that acts as up-stream molecules for activation of various apoptotic signals pathways (Ito et al., 2006; Tatsu mi et al., 2008).

Recently, there is accumulating evidence that WT1(KTS+) activates carcinogenesis in cancer. Especially WT1(+/+) plays an oncogene in breast cancer cells and no effect in normal mammary epithelial cells (Nakatsuka el al., 2006), and dominantly expressed of WT1(+/+) contribute breast progression by activation of cell proliferation and by failure of cell differentiation (Caldon et al., 2008). Preliminary study of WT1(KTS) expression found that WT1(KTS-) dominantly expressed in adjacent normal breast tissues, and WT1(-/-) also significantly expressed in adjacent normal breast tissues. There is previous supported report to indicate that WT1(KTS-) plays a tumor suppressor in carcinogenesis (Loeb and Sukumar, 2002). Moreover, mammary epithelial cells that high expressed of WT1(-/-) isoform decreased cell growth (Burwell et al, 2007). While WT1(KTS+) expressed in both adjacent normal breast and breast cancer tissues. The mixed WT1(KTS+) and WT1(KTS-) was high exhibited in both cell types. Since KTS region is protein interaction domain, therefore WT1 presented KTS that it acts as both transcriptional repression and activational function depending on protein contexts (Reynolds et al., 2003). These result indicated that WT1(KTS+) and mixed WT1(KTS+) and WT1(KTS-) might be oncogene and tumor suppressor gene during breast tumorgenesis while WT1(KTS-) plays tumor suppressor function. Since there were the low case of breast cancer patients, therefore the investigation of WT1(KTS) should be explored in large scases of breast cancer patients.

4.2 Study of WT1, ER-α and HER2 proteins in breast cancer tissues

The steroid hormone, estradiol, plays an important role in the progression of breast cancer (Roy et al., 2011). It binds to one of the structurally and functionally distinct ERs , ER-α and ER-β, leading to the complex interaction between ERs and growth factor receptor signaling (Arpino et al., 2008; Roy et al., 2011). There are two different ER proteins, ER-α and ER-β, which are produced by distinct genes. ER-α plays the crucial role in breast malignancies (Hall et al., 1999; Speirs et al., 2002). Whereas the role of ER-β in breast cancer is still controversial (Osborn et al., 2001; Speirs et al., 2002; Arpino et al., 2008). Nontheless, studies indicates that ER-β can antagonize ER-α activity (Hall and DP, 1999) and suggest that reduced level of ER-β protein are associated with resistance to tamoxifen therapy (Hopp et al., 2004). ER signaling pathway consist of two distinct types, genomic and non-genomic ER pathway. In the genomic pathway, ERs genomic nuclear activity knows as nuclear-initiated steroid signaling (NISS) (Nemere et al., 2003) and its function as a ligand-dependent transcription factor and promotes expression of IGFR-I receptor (IGFR), the cell cycle regulator cyclin D1, the antiapoptotic factor Bcl-2 (Lee and Cui, 2001; Kling, 2001; Sanchez et al., 2002), and proangiogenic vascular endothelial growth factor (Klinge et al., 2001; Schiff et al., 2004) In addition, nuclear ER also induces the expression of TGFα and amphiregulin (Saeki et al., 1991). The non-genomic rapid ER activity, endogenous membrane ER directly or indirectly activates EGFR, HER2 and IGFR1 (Lee et al., 2000). These activation enhances the activation of activates multi-down stream molecule cascades, the cellular tyrosine kinase Src (Pedram et al., 2000), matrix metalloproteinases (MMPs) 2 and 9, and the release of EGFR ligand hairpin binding epidermal growth factor-like growth factor (HB-EGF), which, in turn, activates EGFR, down stream kinase cascades (i.e., Ras/Mek/MAPK and PI3K/AKT) (Razandi et al., 2003; Razandi et al., 2003). The crosstalk between ER and HER2 tyrosine kinase receptor family presumably promotes breast cancer cell proliferation, survival and tumor progression. Recently, Wang et al. (2010) investigated the function of WT1 in malignant progression of breast cancer via swith from estrogen-dependent to estrogen-independent growth in MCF-7 cells. Their finding showed that high passage MCF-7 cells ($MCF\text{-}7^{H}$, > 75 passages) presents high level of HER2 and EGFR expression comparing to low passage MCF-7 cells ($MCF\text{-}7^{L}$, < 35 passages). These results

indicated that MCF-7^{H} that is an estrogen-independent growth property, gained expression of two members of EGFR family, EGFR and HER2. In MCF-7^{H} cells, the levels of ER-α phosphorylation at Ser118 are also increased while the expression levels of ER-α not significantly change, compared to MCF-7^{L} cells. The expression levels of WT1 are greatly increased in $MCF7^{H}$ cells compared to $MCF7^{L}$ cells (Wang et al., 2010). These finding support the hypothesis that WT1 plays an oncogenic role on ERα and HER2 proteins regulation. Here, our data showed that the protein level of ER-α and HER2 were strongly expressed in breast carcinoma tissues but slightly expressed in adjacent normal breast tissues. The results of WT1(17AA+) mRNA was confirming explored using Western blotting. These observation showed that the high expression of ER-α and HER2 correlated with high expression of mixed WT1(17AA+) and WT1(17AA-) in breast carcinoma tissues. These finding presented in the experiment opening the possibility that WT1(17AA+) may be the crucial molecule in regulating ER-α and HER2 proteins.

4.3 Study of the correlation between WT1, ER-α and HER2 in breast cancer cell lines

Since the study of WT1 isoform in step 4.2 provided the evidence that the mixed of WT1(17AA+) and WT1(17AA-) mRNA and protein are markedly expressed in breast cancer tissues. Moreover the mixed WT1(17AA+) and WT1(17AA-) isoform positively correlated with ER-α and HER2 expression. These exploration leaded to study the oncogenic function of the mixed WT1(17AA+) and WT1(17AA-) isoform. The comparison between the protein levels in WT1s overexpressing MCF-7 cells and WT1 knocked down MCF-7 cells was examined to confirm WT1 function. The study exhibited that WT1(+/+) and WT1(+/-) associated with expression of ER-α and HER2. These results presents strong evidence to oncogenic roles of WT(17AA+) in up-regulating ER-α and HER2 expression resulting in breast cancer progression. In previous report, WT1(+/+) plays the oncogenic functions to contribute E-cadherin redistribution from the cell membrane to the cytoplasm leading to metastasis (Burwell et al., 2007). WT1(+/+) also expressed in malignant melanoma and it co-expressed with two proliferative nuclear antigen (Nestin and Zyxin), resulting in melanoma cell proliferation (Burwell et al., 2007; Caldon et al., 2008). Taking this into account with data showing different

WT1 isoforms effect on cell proliferation, MCF-7 presented WT1(17AA+), MCF-$7_{WT1(+/+)}$ and MCF-$7_{WT1(+/-)}$, exhibited growth faster than MCF-7 empty. In addition, the decreasing of cell number was found in $siRNA_{WT1}$treated MCF-$7_{WT1(+/+)}$, MCF-$7_{WT1(+/-)}$ and MCF-$7_{WT1(-/+)}$ cells. There are current finding to support the hypothesize that WT1(17AA+) regulate ER-α and HER2 expression leading to cell growth. The phenotype of mammary epithelial cells was promoted by WT1(+/+) isoform, and high expression of WT1(KTS+) isoform effects to enhance proliferative evidences in breast cancer cells (Burwell et al., 2007; Caldon et al., 2008). These supported evidence is quite possible that WT1(17AA+) and WT1(KTS+) plays an oncogenic property in breast cancer cells. In contrast, these observation demonstrated that the slow growth of cells was found in MCF-7 overexpressing WT1(-/-). After $siRNA_{WT1}$ transfection, the number of MCF-$7_{WT1-/-}$ cells was slight increase. In addition, overexpression of WT1(-/-) exhibited lower expression of ER-α and HER2 than WT1(+/+) and WT1(+/-). These results supported the results which represented the tumor suppression effects of WT1(-/-) isoform in mammary epithelial cells. Moreover, the up-regulation of p21 involved in cell proliferation and G2 cell cycle arrest (Reizner et al., 2005; Caldon et al., 2008). WT1(-/-) isoform also induced cytoskeletal changes in gastric cancer, esophageal cancer, breast cancer and fibrosarcoma cell lines, and it also promoted such as ovarian cancer cells (Jomgeow etal., 2006). This study has specifically show that WT1(17AA+) isoform might be a key molecule in cancer progression and its function requires more depth inverstigation.

CHAPTER 5

CONCLUSIONS

5.1 Study of WT1 mRNA in breast cancer tissues

5.1.1 WT1(17AA) mRNA in adjacent normal breast and breast cancer tissues

- The expression of WT1 mRNA was found 100% (15 of 15 samples), 100% (8 of 8 samples) and 83% (25 of 30 samples) in breast carcinoma tissues grade 1 and 2, breast carcinoma tissues grade 3, and adjacent normal breast tissues that expressed.

- Expression of WT1(17AA-) mRNA was dominantly presented in adjacent normal breast tissues (53%, 16 of 30 samples) whereas it was low expressed in breast carcinoma tissues grade 1 and 2 (13%, 2 of 15 samples) and grade 3 (14%, 1 of 8 samples).

- The mixture of WT1(17AA+) and WT1(17AA-) mRNA was highly expressed in breast carcinoma tissues grade 1 and 2 (73%, 11 of 15 samples) and grade 3 (88%, 7 of 8 samples) while it was low exhibited in adjacent normal breast tissues (13%, 4 of 30 samples).

5.1.2 WT1(KTS) mRNA in adjacent normal breast and breast cancer tissues

- The expression of WT1(KTS-) mRNA was significantly found in adjacent normal breast tissues (17%, 2 of 12 cases), but it was not found in breast carcinoma tissues grade 1 and 2 (0 of 5 cases) and grade 3 (0 of 6 cases).

- The expression of mixed WT1(KTS+) and WT1(KTS-) mRNA was both expressed in adjacent normal breast tissues (58%, 7 of 12 cases) and strongly expressed in breast cancer tissues grade 1 and 2 (80%, 4 of 5 cases).

5.2 Study of WT1, ER-α and HER2 proteins in breast cancer tissues

- WT1(17AA-) protein was found in adjacent normal breast tissues (88%, 15 of 17 cases), but it rarely expressed in breast carcinoma tissues grade 1 and grade 2 (0 of 10 cases), and grade 3 (0 of 7 cases).

- Protein expression of mixed WT1(17AA+) and WT1(17AA-) was highly presented in breast carcinoma tissues grade 1 and grade 2 at 100% (10 of 10 cases) and grade 3 at 100% (7 of 7 cases). The mixture of WT1(17AA+) and WT1(17AA-) expression was rarely found in adjacent normal breast tissues at 12% (2 of 17 cases)

- These results were similar to WT1 mRNA expression.

- The mixed WT1(17AA+) and WT1(17AA-) isoforms positively correlated with ER-α and HER2 expression.

- WT1(17AA+), WT1(17AA-), and the mixture of WT1(17AA+) and WT1(17AA-) positively correlated to express in breast carcinoma grade1, 2 and grade 3.

5.3 Study of the correlation between WT1, ER-α and HER2 in breast cancer cell lines

- WT1(+/+), WT1(+/-), WT1(-/+), and WT1(-/-) isoforms affected morphological changes, leading and cell proliferation. All isoforms except WT1(-/-) also correlated the up-relulation of ER-α and HER2.

- WT1(+/+), WT1(+/-), and WT1(-/+) isoforms correlated with the decrease of ER-α and HER2 in knocked down WT1 MCF-7 cells.

- WT1(+/+), WT1(+/-), and WT1(-/+) might play an oncogenic role in breast cancer by correlating with ER-α and HER2 expression, resulting in cell proliferation. However, functions of WT1 isoforms should be further explored to intensely attain its distinct mechanism.

- Further dissection of the mixed WT1(17AA-) and WT1(17AA+) with ER-α and HER2 expression should be necessary explored in depth for its mechanism. The new finding of these correlations may be useful for diagnosis and treatment in breast cancer patients.

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APPENDIX A

I. Reagents for cell culture

Complete RPMI 1640 medium

RPMI 1640 medium	10.4	g
$NaHCO_3$	2.0	g

Adjust pH with 1 M NaOH and 1M HCl to give final pH 7.3, add distilled water to final volume of 1 L

Supplemented with :

- Fetal Bovine Serum (FBS)	100	ml
- Penicillin/Streptomycin (10,000U/ml of Penicillin, 10,000 μg/ml Streptomycin)	10	ml
- L-glutamine (200 mM, 100X)	10	ml

Complete RPMI 1640 medium was filtered through 0.22 micron Millipore filter membrane. Store at $4^{o}C$.

Complete DMEM medium

Dulbecco's modified eagle medium	13.5	g
$NaHCO_3$	3.2	g

Adjust pH with 1 M NaOH and 1M HCl to give final pH 7.3, add distilled water to final volume of 1 L

Supplemented with :

- Fetal Bovine Serum (final conc. 10% of Fetal Bovine Serum)	100	ml
- Penicillin/Streptomycin (100U/ml of Penicillin, 100 μg/ml Streptomycin)	10	ml
- L-glutamine (final conc. 2 mM)	10	ml

Complete RPMI 1640 medium was filtered through 0.22 micron Millipore filter membrane. Store at $4^{o}C$

Ham's F12 medium

Ham's F12	10.7	g
$NaHCO_3$	1.2	g

Adjust pH with 1 M 1M HCl to give final pH 7.3, add distilled water to give final volume of 1 L and filter through 0.22 micron Millipore filter membrane.

Store at 4°C.

MCF-12A medium

Dulbecco's modified Eagle's medium	250.00	ml
Ham's F12	250.00	ml
Cholera toxin (final conconcentration 100 ng/ml)	0.05	ml
Bovin insulin (final conconcentration 0.01 mg/ml)	0.50	ml
Hydrocortisone (final conconcentration 500 ng/ml)	0.05	ml
Human epidermal growth factor (final conconcentration 20 ng/ml)	0.25	ml
Horse serum (final conconcentration 5% of Horse serum)	2.50	ml

Filter through 0.22 micron Millipore filter membrane.

Store at 4°C.

Trypsin-EDTA (trypsin 0.25% in 1 mM EDTA)

Store at 4°C.

Cholera toxin (stock concentration 1 mg/ml)

Store at -20°C.

Bovin insulin (stock concentration 10 mg/ml)

Store at -20°C.

Human epidermal growth factor (stock concentration 40 µg/ml)

Store at -20°C.

Hydrocortisone (stock concentration 5 mg/ml)

Hydrocortisone	0.005	g

Dissolve the ingredients in distilled water and bring up volume 1000 ml

Filter through 0.22 micron Millipore filter membrane. Store at -20^{o}C.

10X Phosphate Buffer Saline (PBS) pH 7.4

NaCl	80.0	g
KCl	2.0	g
KH_2PO_4	2.4	g
Na_2HPO_4	14.4	g

Adjust pH with 1 M NaOH and 1M HCl to give final pH 7.4, add distilled water to give final volume of 1 L. Sterilize by autoclaving at 121^{o}C, 15 pounds per square inch for 20 min and stored at 4^{o}C. The 1X of working solution was used in experiments. Store at 4^{o}C.

HCl (1 M)

37% w/w or 12 M of HCl	16.6	ml
Distilled water	183.4	ml

Carefully add the solution of HCl into Distilled water. Store at room temperature.

NaOH (1 M)

NaOH	8	g

Dissolve the ingredients in distilled water and bring up volume 200 ml

Sterilization is not necessary. Store the solution in a plastic container at room temperature.

II. Regents for gel electrophoresis

5X TBE buffer pH 8.0

Tris-base	54.0	g
Boric acid	27.5	g
0.5 M EDTA pH 8.0	20.0	ml

Add distilled water to volume 1000 ml. Stored at 4°C. The 1X of working solution was used in experiments.

EDTA (0.5 M) pH 8.0

EDTA	18.6	g

Dissolve the ingredient in 80 ml of distilled water and stir vigorously by magnetic stirrer. While stirring vigously on magnetic stirrer add NaOH pellet approximately 20 g or 10 N of NaOH to adjust the solution pH 8.0 and adjust the volume to 100 ml with deionized water. Sterilize by autoclaving at 121°C, 15 pounds per square inch for 20 min. Store solution at 15-25°C.

Gel loading buffer

Glycerol (final conc. 25% (V/V))	2.500	ml
EDTA (final conc. 60 mM)	7.500	ml
Bromophenol Blue (0.25% (W/V))	0.025	g

Mix EDTA and bromophenol blue then add the glycerol. Store at 4°C. Sterilization is not necessary.

EDTA (60 mM) pH 8.0

EDTA	2.2	g

Dissolve the ingredient in 80 ml of distilled water and stir vigorously by magnetic stirrer. While stirring vigously on magnetic stirrer add NaOH pellet or 10 N of NaOH to adjust the solution pH 8.0 and adjust the volume to 100 ml with deionized water. Sterilize by autoclaving at 121^{o}C, 15 pounds per square inch for 20 min. Store solution at 15-25°C.

Silver Nitrate for Silver staining (55% (V/V)) 900 ml

0.15% (W/V) $AgNO_3$	500	ml
0.1% formaldehyde	400	ml

The mixture should be freshly prepared only and the mixture have to kept in 4 °C until use. Store at room temperature.

AgNO3 (0.15% (W/V))

$AgNO_3$	1.5	g

Dissolve the ingredients in distilled water and bring up volume 100 ml

Formaldehyde (0.1% (V/V)) 100 ml

Formaldehyde	100	µl
Distilled water	100	ml

Store at room temperature.

Developing solution for Silver staining 800 ml

Na_2CO_3	10	g
Distilled water	400	ml
Formaldehyde	400	µl
Sodium Thiosulfate	400	ml

Na_2CO_3 was mixed firstly with 400 ml of distill water and stir by magnetic stirrer until solution completely dissolve. Next, formaldehyde and sodium thiosulfate were added to give final volume 800 ml following by formula. Store solution at 4 °C until use.

Sodium carbonate (2.5% (w/v))

Na_2CO_3	2.5	g

Dissolve the ingredients in distilled water and bring up volume 100 ml.

The mixture should be freshly prepared only and the mixture have to kept in 4 °C until use.

Store at room temperature.

Glycerol (10% (v/v)) for Silver staining

Glycerol	20	ml
Distilled water	180	ml

Store at room temperature. Sterilization is not necessary.

Acetic acid (10% (v/v)) for Silver staining

Distilled water	190	ml
99% Acetic acid	10	ml

Preparation let start to take distilled water to beaker first, and acetic acid should be then carfully take to distilled water. The mixture was mixed by spatula and stored at room temperature.

Denaturing buffer for Silver staining

1% Xylen cyanol	625.00	µl
Formamide	11.87	ml

Store at 4 °C. Dissolve the ingredients in formamide and bring up volume 12.495 ml.

Xylene cyanol (1% (w/v))

Xylen cyanol	0.1	g

Dissolve the ingredients in distilled water and bring up volume 10 ml

Store at 4 °C.

III. Reagents for RNA extraction

Trizol® reagent

Chloroform

Isopropanol

75% Ethanol

Deionized water treated with DEPC (Diethylpyrocarbonate)

Note: 100 ml of deionized water was mixed with 1 ml DEPC for 24 hours and then sterilize by autoclaving at 121°C, 15 pounds per square inch for 20 min. Store solution at room temperature.

IV. Reagents for cloning

1 M $CaCl_2$

$CaCl_2$	22.2	g

Dissolve the ingredients in distilled water and bring up volume 200 ml.

Sterilize by autoclaving at 121°C, 15 pounds per square inch for 20 min and store at at 4°C.

0.1 M $CaCl_2$

1 M $CaCl_2$	100	ml
Distilled water	1000	ml.

Sterilize by autoclaving at 121^{o}C, 15 pounds per square inch for 20 min and store at at 4^{o}C.

LB (Luria-Bertani) broth

Yeast extract	5.0	g
Peptone	10.0	g
Sodium chloride	5.0	g

Dissolve the ingredients in distilled water and bring up volume 1000 ml.

Sterilize by autoclaving at 121^{o}C, 15 pounds per square inch for 20 min and store at at 4^{o}C.

LB (Luria-Bertani) agar

Yeast extract	10.0	g
Peptone	10.0	g
Sodium chloride	5.0	g
Agar	15.0	g

Dissolve the ingredients in distilled water and bring up volume 100 ml.

Sterilize by autoclaving at 121^{o}C, 15 pounds per square inch for 20 min and store at at 4^{o}C.

LB agar (supplement with 10 μg/ml tetracyclin)

Yeast extract	10.0	g
Peptone	10.0	g
Sodium chloride	5.0	g
Agar	15.0	g

Dissolve the ingredients in distilled water and bring up volume 1000 ml.

Sterile by autoclaving at 121^{o}C, 15 pounds per square inch for 20 min. Add 1 ml of tetracycline (10 mg/ml) into medium (50 ^{o}C). The medium was poured into sterilized glass plate.

LB agar (supplement with 100 µg/ml amplicillin)

Yeast extract	10.0	g
Peptone	10.0	g
Sodium chloride	5.0	g
Agar	15.0	g

Dissolve the ingredients in distilled water and bring up volume 1000 ml.

Adjust the volume of the solution to 1000 ml with distilled water and sterile by autoclaving at 121^{o}C, 15 pounds per square inch for 20 min. Add 1 ml of ampicillin (100 mg/ml) into medium (50 ^{o}C). The medium was poured into sterilized glass plate.

Ampicillin sodium salt (100 mg/ml)

Amplicillin	0.1	g
Sterile distilled water	1.0	ml

Store at 4^{o}C.

10 µg/ml Tetracyclin

Tetracyclin	0.001	mg
Absolute ethanol	1.00	ml

STET buffer

Glucose	80.0	g
Triton X-100	50.0	ml
EDTA	18.6	g
Tris-base	12.1	g

Adjust the pH to 8.8 by adding HCl. Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving. Store at room temperature.

Ethidium bromide (EtBr) (1% W/V)

Ethidium bromide	0.05	g
Distilled water	5.00	ml

Mix by vortex to ensure that the dye has dissolved. Wrap the container in aluminium foil and store the solution in a dark box at room temperature.

RNAse (10 mg/ml)

RNase A	0.01	g
Sterile distilled water	1.00	ml

Dissolve the RNase A with the sterile distilled water and boil in water for 5 min.

store at -20 ^{o}C.

V. Reagents for reverse transcription

Enzyme mix (Reverse transcriptase and Taq polymerase)

5X Reverse transcription buffer

6 μM (17AA) WT1 Forward primer

6 μM (17AA) WT1 Reverse primer

dNTP mix 10 mM

Reverase transcriptase 200 U

Deionized water treated with DEPC

Diethyl pyrocarbonate water (DEPC water)

DEPC	1	ml
Deionized water	1000	ml

Let the solution incubate for 24 hours at room temperature. Autoclave to remove any trace of DEPC. Store at room temperature.

VI. Reagents for Western blotting

Tris-HCl (1.5 M)

Tris-base	18.1	g

Dissolve the ingredients in distilled water and bring up volume 100 ml.

Adjust the pH to 8.8 by adding concentrated HCl. Sterilize by autoclaving.

Tris-HCl (1 M)

Tris-base	12.1	g

Dissolve the ingredients in distilled water and bring up volume 100 ml.

Adjust the pH to 6.8 by adding concentrated HCl. Sterilize by autoclaving.

SDS (10%W/V)

Sodium dodecyl sulfate	10.0	g

Dissolve the ingredients in distilled water and bring up volume 100 ml.

Heat to 68 $^{\circ}$C and stir with a magnetic stirrer to assist dissolution. Sterilization is not necessary.

APS (10%W/V)

Ammonium persulfate	1.0	g
Distilled water	10.0	ml

Sterilization is not necessary. Store at 4 $^{\circ}$C.

Ponceau S (0.25% W/V)

Ponceau S	0.3	g
Acetic acid (final conc. 5%V/V)	5.0	ml
Distilled water	95.0	ml

Mix Ponceau S powder in Distilled water to ensure that the Ponceau S was dissolved. Add carefully the acetic acid into Ponceau S solution. Sterilization is not necessary. Store at room temperature.

BSA (10 mg/ml)

Bovine Serum Albumin	0.05	g
Sterile distilled water	5.00	ml

Dissolve BSA in sterile water and filter the solution through 0.22 micron Millipore filter membrane.

4X SDS Gel loading buffer

Table 27 The ingredients for SDS Gel loading buffer preparation.

Reagents	Volume/Weight	Final concentration
1M Tris HCl pH 6.8	10 ml	200 mM
SDS	4.0 g	412.8 mM
DTT	3.1 g	400 mM
Bromophenol blue	0.2 g	0.4% w/v
2-β-mercaptoethanol	3.58 ml	8% v/v
100% Glycerol	20 ml	59.5% v/v

Note: Adjust the volume of the solution to 50 ml with distilled water and gently mix by rock machine at 4 ^{o}C overnight. Aliqout solution to sterile microtube and store at -80^{o}C.

5X TTBS buffer

Tris-Base	5.80	g
NaCl	45.00	g
Tris-HCl	31.75	g
Tween 20	5.00	ml

Dissolve the ingradients in distilled water and bring up volume 1000 ml distilled water. The 1X of working solution was used in experiments.

10X Running buffer (Tris-glycine buffer)

Tris-base	15.1	g
Glycine	94.0	g
20% SDS	25.0	ml

Dissolve the ingradients in distilled water and bring up volume 1000 ml distilled water. The 1X of working solution was used in experiments.

20% SDS

Sodium dodecyl sulfate	20.0	g

Dissolve the ingredients in distilled water and bring up volume 100 ml

1X Electroblotting buffer

Glycine	7.9	g
Tris-base	5.8	g
Methanol	200.0	ml

Dissolve the ingredients in distilled water and bring up volume 1000 ml distilled water.

Blocking buffer

Low fat dry milk	5.0	g
1X TTBS	100.0	ml

Washing buffer

Low fat dry milk	10.0	g
1X TTBS	1000.0	ml

Comassie blue staining

Comassie Brillant Blue R250	2.5	g
Methanol	450.0	ml
Acetic acid	100.0	ml
Distilled water to	450.0	ml

Mix the ingredients in distilled water and store at room temperature.

Destain solution

Methanol	500	ml
Acetic acid	100	ml
Distilled water	100	ml

Mix the ingredients in distilled water and store at room temperature.

APPENDIX B

There are three different breast cancer cell lines that were used in this study containing MCF-7, MDA-MB-231 and MDA-MB-468 cells, and one normal breast cancer cell lines is MCF-12A cells. Each cells present the differences of biological properties, indicating in Table 16, 17 and 18.

Table 28 Properties of MCF-7 cells

Category	Biological properties
Organism	Homo sapiens (Human)
ATCC No.	HTB-22 ™
Source	mammary gland; breast: pleural effusion, Disease: adenocarcinoma
Culture properties	adherent
Cell types	epithelial
Marker	estrogen receptor
Genes expression	insulin-like growth factor binding proteins (IGFBP) BP-2; BP-4; BP-5
Comments	The MCF7 line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. The cells express the WNT7B oncogene. Growth of MCF7 cells is inhibited by tumor necrosis factor alpha (TNF alpha). Secretion of IGFBP's can be modulated by treatment with anti-estrogens (Huguet EL, et al., 1994).
Complete growth medium and culture condition	RPMI-1640, supplement with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml of Penicillin, 100 µg/ml of Streptomycin. Cell grow at 95% humidity, 5% carbon dioxide (CO_2), 37 °C.

Table 28 Properties of MCF-7 cells (continued)

Category	Biological properties
Subculture	Remove medium, and rinse the cell layer with 0.25% trypsin, 0.53 mM EDTA then placed the cell culture plate at 37 °C until the cell detach. Add the complete growth medium and aspirate cells by gently pipetting. Remove the supernatant to collect the cell pellet and add fresh complete growth mediumfor subculture the cells ata subcultivation ratio of 1: 3, 1:6. After propagation, the medium could renew for 2 or 3 times per week.
Cryopreservation	Complete growth medium supplemented with 5% (v/v) DMSO or fetal bovine serum in 5% (v/v) DMSO. Freeze cells are stored at liquid nitrogen vapor phase.

Table 29 Properties of MDA-MB-231 cells

Category	Biological properties
Organism	Homo sapiens (Human)
ATCC No.	HTB-26 ™
Source	Organ: mammary gland; breast, Disease: adenocarcinoma
Culture properties	adherent
Cell types	epithelial
Marker	epidermal growth factor (EGF), transforming growth factor alpha (TGF alpha)
Genes expression	-
Comments	The cells express the WNT7B oncogene (Huguet EL, et al., 1994)
Complete growth medium and culture condition	DMEM, supplement with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml of Penicillin, 100 µg/ml of Streptomycin. Cell grow at 95% humidity, 5% carbon dioxide (CO_2), 37 °C.
Subculture	Remove medium, and rinse the cell layer with 0.25% trypsin, 0.53 mM EDTA then placed the cell culture plate at 37 °C until the cell detach. Add the complete growth medium and aspirate cells by gently pipetting. Remove the supernatant to collect the cell pellet and add fresh complete growth medium for subculture the cells ata subcultivation ratio of 1: 2, 1:4 After propagation, the medium could renew for 2 or 3 times per week.
Cryopreservation	Complete growth medium supplemented with 5% (v/v) DMSO or fetal bovine serum in 5% (v/v) DMSO. Freeze cells are stored at liquid nitrogen vapor phase.

Table 30 Properties of MDA-MB-468 cells

Category	Biological properties
Organism	Homo sapiens (Human)
ATCC No.	HTB-132 ™
Source	mammary gland; breast: pleural effusion, Disease: adenocarcinoma
Culture properties	adherent
Cell types	epithelial
Marker	epidermal growth factor (EGF), transforming growth factor alpha (TGF alpha)
Genes expression	Blood Type AB; HLA Aw23, Aw30, B27, Bw35, Cw2, Cw4 (patient)
Comments	Although the tissue donor was heterozygous for the G6PD alleles, the cell line consistently showed only the G6PD A phenotype. There is a G -> A mutation in codon 273 of the p53 gene resulting in an Arg -> His substitution. EGF receptor is present at 1 X 10(6) per cell.
Complete growth medium and culture condition	DMEM, supplement with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml of Penicillin, 100 µg/ml of Streptomycin. Cell grow at 95% humidity, 5% carbon dioxide (CO_2), 37 °C.
Subculture	Remove medium, and rinse the cell layer with 0.25% trypsin, 0.53 mM EDTA then placed the cell culture plate at 37 °C until the cell detach. Add the complete growth medium and aspirate cells by gently pipetting.
Cryopreservation	

Table 30 Properties of MDA-MB-468 cells (continued)

Category	Biological properties
Cryopreservation	Complete growth medium supplemented with 5% (v/v) DMSO or fetal bovine serum in 5% (v/v) DMSO. Freeze cells are stored at liquid nitrogen vapor phase.

Table 31 Properties of MCF-12A cells

Category	Biological properties
Organism	Homo sapiens (Human)
ATCC No.	CRL-10782 ™
Source	mammary gland; breast
Culture properties	adherent
Cell types	epithelial
Marker	positive for epithelial cytokeratins 8, 14 and 18, and negative for cytokeratin 19
Genes expression	epithelial mucin, milk fat globule membrane antigen, sialomucin
Comments	No tumogenetic cells. MCF-12A cells exhibit typical luminal epithelial morphology, three dimensional growth in collagen, and form domes in confluent cultures. The cells are positive for epithelial cytokeratins 8, 14 and 18, and negative for cytokeratin 19.
Complete growth medium and culture condition	1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, 20 ng/ml Human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin and 500 ng/ml hydrocortisone, and 5% horse serum
Subculture	Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and add an additional 1 to 2 mL of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37°C) until the cells detach. Centrifuge the cell suspension at 1000 rpm for 10 minutes, resuspend the pellet in fresh medium, aspirate and dispense into new flasks. Inoculate new flasks at 1 X 10^4 cells per cm^2. After propagation, the medium could renew for 2 or 3 times per week.

Table 31 Properties of MCF-12A cells (continued)

Category	Biological properties
Cryopreservation	Complete growth medium supplemented with 5% (v/v) DMSO or fetal bovine serum in 5% (v/v) DMSO. Freeze cells are stored at liquid nitrogen vapor phase.

VITAE

Name Miss Tapanawan Nasomyon

Student ID 5210330005

Educational Attainment

Degree	Name of Institution	Year of Graduation
B.Sc. (Microbiology)	Khon Kaen University	2004
M.Sc. (Medical Microbiology)	Khon Kaen University	2008

Scholarship Awards during Enrolment

Research Grant, Faculty of Medicine, Prince of Songkla University

Graduate School Prince of Songkla University scholarship

List of Publication and Proceeding

Nasomyon T, Samphao S, Sangkhathat S, Mahattanobon S and Graidist P. Correlation of Wilms' Tumor 1 isoforms with HER2 and ER-α and its oncogenic role in breast cancer. Anticancer Res 2014;34:1333-42.

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