การศึกษาการเกิด LINE-1 ไฮเปอร์เมทิลเลชันในเนื้อเยื่อเซลล์สโตรมาของมะเร็งเต้านม

นายเจริญชัย พุฒิปัญญาเลิศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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LINE-1 HYPERMETHYLATION IN BREAST CANCER STROMAL CELLS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	LINE-1 HYPERMETHYLATION IN BREAST CANCER STROMAL CELLS
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เจริญชัย พุฒิปัญญาเลิศ : การศึกษาการเกิด LINE-1 ไฮเปอร์เมทิลเลชันในเนื้อเยื่อเซลล์สโตรมาของมะเร็งเต้านม (LINE-1 HYPERMETHYLATION IN BREAST CANCER STROMAL CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ. ดร.อภิวัฒน์ มุทิรางกูร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.นครินทร์ กิตกำธร, หน้า.

การเปลี่ยนแปลงระดับดีเอ็นเอเมทิลเลชันในยีนที่มีไลน์-1 นั้นจะสามารถเปลี่ยนแปลงการแสดงออกของยีนได้ ในงานวิจัยนี้ ได้ทำการทดลองเพื่อแสดงให้เห็นถึงการเหนี่ยวนำการเปลี่ยนแปลงดีเอ็นเอเมทิลเลชันในเซลล์ข้างเคียงเซลล์มะเร็งเต้านม อันเป็นผลจากสารพาราไคลน์ที่หลั่งออกมาจากเซลล์มะเร็ง โดยขั้นแรกได้ทำการพิสูจน์การเปลี่ยนแปลงระดับดีเอ็นเอเมทิลเลชันที่เพิ่มขึ้นของไลน์-1 ในเนื้อเยื่อต่อมน้ำเหลืองของผู้ป่วยมะเร็งเต้านมระยะแพร่กระจาย ใช้การแยกบริเวณเซลล์มะเร็งและเซลล์รอบๆเซลล์มะเร็งออกจากกันด้วยวิธี laser-capture microdissected ขั้นต่อมา เพื่อแสดงให้เห็นถึงการกระตุ้นเซลล์โดยรอบเซลล์มะเร็งด้วยพาราไคลน์ จึงได้ทำการทดลองในห้องปฏิบัติการด้วยวิธีเพาะเลี้ยงเนื้อเยื่อร่วมกับเซลล์มะเร็งหรือ co-culture ซึ่งได้ทำการเพาะเลี้ยงเซลล์มะเร็งเต้านม, มะเร็งโพรงจมูก หรือ มะเร็งปากมดลูก ร่วมกับเซลล์เม็ดเลือดขาวชนิด PBMCs ทั้งจากผู้บริจาคเพศหญิงและชาย และยังได้ทำการเพาะเลี้ยงเซลล์มะเร็งร่วมกับเซลล์เนื้อเยื่อเกี่ยวพัน fibroblast ด้วย ผลการทดลองที่ได้พบว่าระดับเมทิลเลชันของไลน์-1มีค่าเพิ่มสูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเพาะเลี้ยงเซลล์มะเร็งเต้านม ร่วมกับเซลล์เม็ดเลือดขาว PBMCs หรือเซลล์เนื้อเยื่อเกี่ยวพัน Fibroblast ที่เวลา 4 และ 8 ชม. ซึ่งการเปลี่ยนแปลงนี้ไม่สามารถตรวจพบเมื่อทำการเพาะเลี้ยงเซลล์มะเร็งเต้านมร่วมกับเซลล์เม็ดเลือดขาว PBMCs จากเพศชาย หลังจากนั้นจึงได้ทำการวิเคราะห์ยีนที่มีไลน์-1 และมีการเปลี่ยนแปลงระดับเมทิลเลชันของไลน์-1 ด้วย bioinformatics program ในเนื้อเยื่อรอบๆเซลล์มะเร็งเต้านม และนำไปใช้ย้อมสี immunohostochemistry เพื่อตรวจหาต่อมน้ำเหลืองที่มีมะเร็งเต้านมระยะแพร่กระจาย ซึ่งจากผลการทดสอบพบว่า ยีน MUC-1 สามารถใช้ตรวจหาต่อมน้ำเหลืองที่มีการแพร่กระจายของเซลล์มะเร็งเต้านมได้ด้วยความไวและความจำเพาะที่สูง โดยสรุปจากงานวิจัยนี้สามารถกล่าวได้ว่าเซลล์มะเร็งเต้านมสามารถกระตุ้นการเปลี่ยนแปลงระดับเมทิลเลชันของเซลล์ข้างเคียงได้ด้วยการใช้พาราไคลน์ และยีนที่มีไลน์-1 และมีการแสดงออกเปลี่ยนแปลงไปนั้นสามารถนำมาประยุกต์ใช้เพื่อตรวจหาเซลล์มะเร็งเต้านมระยะแพร่กระจายได้

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5487853120 : MAJOR BIOMEDICAL SCIENCES
KEYWORDS: BREAST CANCER / LINE-1 HYPERMETHYLATION / TUMOR SUPPORTING TISSUE

CHAROENCHAI PUTTIPANYALEARS: LINE-1 HYPERMETHYLATION IN BREAST CANCER STROMAL CELLS. ADVISOR: PROF. APIWAT MUTIRANGURA, Ph.D., CO-ADVISOR: ASSOC. PROF. NAKARIN KITKUMTHORN, Ph.D., pp.

Change in methylation level of genes containing Long Interspersed Element-1 (LINE-1) alters host genes regulation. Here we demonstrated paracrine signalling of breast cancer influents the epigenetic regulation of stromal cells. First, we proved by an increased level of genome-wide LINE-1 methylation of laser-captured lymphocytes of metastatic breast cancer lymph nodes. To demonstrate paracrine signaling, in vitro co-cultures between breast cancer cell lines and male or female peripheral blood mononuclear cells (PBMCs) of normal individuals, female fibroblasts or the cervical cancer cell line (HeLa) were set up. Interestingly, LINE-1 hypermethylation occurred exclusively in female PBMCs (P values = 0.0044) or fibroblasts (P values = 0.0288) after co-culture at 4-8 h. No LINE-1 methylation change was observed in co-cultured male PBMCs or HeLa cells. Next, genes containing LINE-1 of breast cancer stromal cells were upregulated. Finally, one of the genes, MUC-1, was validated to have expression in plasma cells from lymph nodes of patients with lymph node metastasis or micrometastasis. In conclusion, breast cancer sends a paracrine signal in stroma cells causing LINE-1 epigenetic regulation. Moreover, the regulated genes in stroma cells are potential biomarkers for detecting breast cancer micrometastasis.

Field of Study: Biomedical Sciences
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

LINE-1	Long INterspersed Element-1
CU-DREAM-extra	Connection Up- or Down- Regulation Expression Analysis of Microarrays Extra
MRI	Magnetic Resonance Imaging
ER	Estrogen Receptor
PR	Progesterone Receptor
HER-2	Human Epidermal Growth Factor Receptor 2
CAF	Cancer-associated Fibroblasts
DNMT	DNA methylatransferase
CpG	CpG oligodeoxynucleotides
LCM	Laser Captured Microdissected
LN	Lymph node
PBMCs	Peripheral Mononuclear Cells
FBS	Fetal Bovine Serum
HPLC	High Performance Liquid Chromatography

CHAPTER I

INTRODUCTION

Gene regulation by intragenic Long Interspersed Element (LINE-1), one type of interspersed repeat sequences, is involved in several biological processes such as inflammation, immune function and hormonal responses (Wanichnopparat W et al. 2013). In cancer, hundreds of genes containing LINE-1s are down regulated due to LINE-1 hypomethylation (Aporntewan C et al. 2011, Kitkumthorn N and Mutirangura A 2011). Reduction of LINE-1 methylation promotes intragenic LINE-1 transcription and produces antisense RNAs to pre mRNA (Figure 1). The LINE-1 RNA-pre-mRNA complex will bind to AGO2, which leads to an RNA degradation process. Then, genes containing LINE-1s will be down-regulated. In cancer, LINE-1s are demethylated by a generalized process such as oxidative stress (Kaer K and Speek M 2013, Baba Y et al. 2014). Therefore, the degree of gene repression is correlated with methylation level of not only the particular intragenic but also genome wide LINE-1s (Aporntewan C et al. 2011).

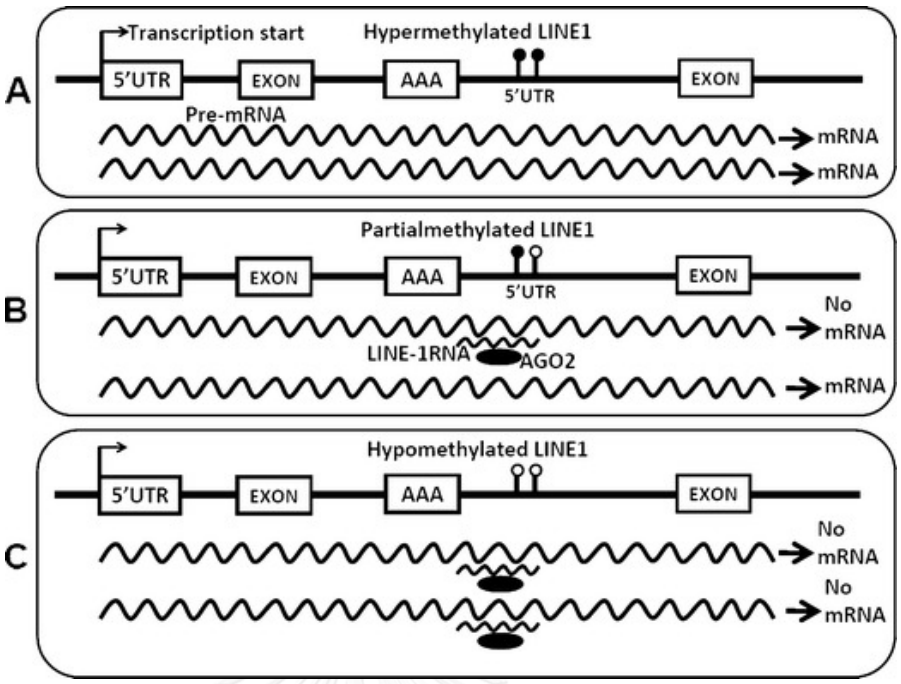


Figure 1 Intragenic LINE-1 can regulate gene expression. The LINE-1 RNA-Pre-mRNA complex was bound to AGO2, which led to an RNA degradation process and mRNA production not occurring. a) Up-regulated gene expression occurred by hypermethylation of intragenic LINE-1. b) Partial methylation of intragenic LINE-1 could transcript LINE-1 RNA, which was complementary to Pre-mRNA. c) LINE-1 RNA had increased hypomethylation of intragenic LINE-1, which led to down-regulated gene expression. (Aporntewan C et al. 2011, Kitkumthorn N and Mutirangura A 2011)

While researchers studied the regulation of genes containing LINE-1s in several disease conditions using CU-DREAM-extra program (Khowutthitham S et al. 2012), genes containing LINE-1 expression changes in breast cancer stromal cells were discovered. Therefore, the hypothesis was hypothesized that breast cancer sends paracrine signalling to regulate LINE-1 methylation levels of stromal cells and

consequently, to genes containing LINE-1 expression. Molecular biology of cancer is a complex process involving multiple factors from both tumor cells and host stromal cells. Many studies have shown that tumor stromal cells perform important functions in tumor initiation, progression and metastasis (Phokaew C et al. 2008). Stromal cells such as T lymphocytes (infiltrating lymphocytes) and macrophages (tumor-associated macrophages) are involved in breast cancer progression (Patchsung M et al. 2012). Many studies have suggested that the activation of fibroblasts is induced by many cancer secretion factors, such as tumor growth factor (TGF)-β, CXCL12/SDF-1, PDGF-α/β, basic fibroblast growth factor (b-FGF) or IL-6 (Man YG and Sang QX 2004, Kalluri R and Zeisberg M 2006, Kojima Y et al 2010, Aporntewan C and Mutirangura A 2011). Some studies have found that human adipose tissue-derived stem cells can differentiate into CAF-like myofibroblasts by the induction of TGF-β from the culture medium of breast cancer cell lines such as MCF-7 and MDA-MB-231 (Shao ZM et al. 2000). CAFs not only play a role in tumor progression but also induce therapeutic resistance (Giannoni E et al. 2010).

During cancerous tissue development, system interaction as a microenvironment between cancer cells and surrounding stromal cells occurs. Recent studies revealed promoter methylation in some specific genes of breast cancer stromal cells, including PGR, HSD1734, and CDH 13, and suggested that the epigenetic changes in stromal cells may be affected by the tumour (Hugo HJ et al.

2012). However, the epigenomic changes particularly at interspersed repetitive sequences in surrounding tissue induced by cancer cells have not been verified. Here in this study we proved that paracrine signaling from breast cancer cells can increase LINE-1 methylation level of stromal cells. Consequently, gene expression and cellular phenotypes were altered. Moreover, because a few breast cancer cells can upregulate proteins in a large number of stromal cells, this study demonstrated that these proteins are sensitive tumor markers for micrometastatic detection.

CHAPTER II

LITERATURE REVIEWS

1. Breast cancer

Breast cancer is a one type of cancer locates at breast tissue. There are 2 types of breast cancer divided by origin of cancer cell including ductal and lobular carcinoma (Sariego J 2010). This cancer majority occur in female but male breast cancer also found (about 0.01 percent). Breast cancer is the most common invasive cancer in women worldwide (16 percent of all female cancers) (World cancer report, 2008) and incidence rate is high in developed countries. The noticeable symptom of breast cancer is typically a lump that feels different from the rest of breast tissue. More than 80% of breast cancer cases were discovered when the patient feel a lump (Watson M 2008). However, the earliest breast cancer can detect by using mammogram and lump found in lymph node located in the armpit can also indicate the breast cancer. Moreover, indication of breast cancer others than lump are including thickening different from the other breast tissue, one breast become larger or lower size, a nipple change position, skin puckering, a rash around nipple, discharge from nipple, constant pain and swelling around the collar bone. Inflammatory breast cancer is a particular type of breast cancer which may has

symptoms resemble as breast inflammation including itching, pain, swelling, nipple inversion and redness. Occasionally, breast cancer presents as metastatic disease which can spread beyond the original organ. The common sites of breast cancer metastasis are including bone, liver, lung and brain (Sariego J 2010, Lacroix M 2006). Then, the patient might has non-specific symptom from metastasis breast cancer such as fever, chill, joint pain and jaundice. Anyway, most symptoms of breast disorders do not turn into the breast cancer because less than 20 percent of lump can change to breast cancer. Those breast disorders might turn into benign breast disease such as mastitis (breast tissue inflammation from some bacteria; *S. aureus*, *S. epidermidis* and streptococci), fibroadenoma (lump compose of fibrous and grandular tissue) (Reeder JG and Vogel VG 2008).

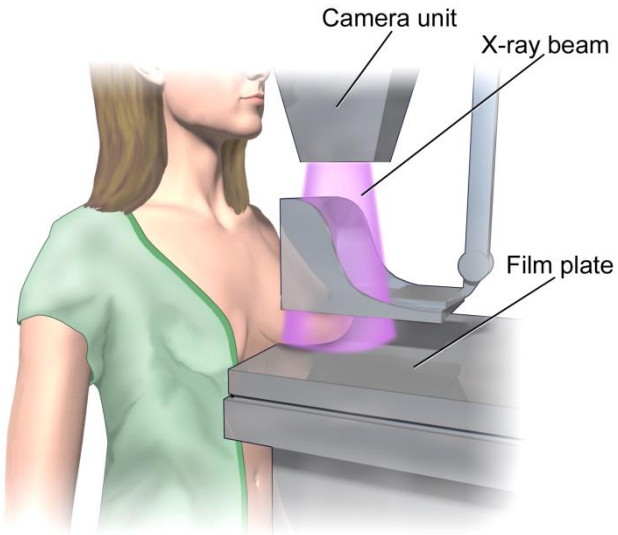


Figure 2. The process of mammography used to examine the human breast and screening tool for breast cancer

1.1 Diagnosis

Most types of breast cancer are easy to diagnose and evaluate the affected area of the breast tissue by microscopic examination or biopsy. But some cases of breast cancer are need to performed special examination such as ultrasound or magnetic resonance imaging (MRI) to diagnose the breast cancer. There are two common screening methods for breast cancer including physical examination and mammography (Saslow D et al. 2004). Physical examination can observe the lump or cyst in breast tissue. Core needle biopsy and fine needle aspiration are the technique to collect tissue sample or some discharge fluid for microscopic examination and histopathology (Yu YH et al. 2010). However, biopsy tissue can be collected with other ways including surgical, incisional and excisional biopsy. Next, mammography is the method to observe breast cancer by using X-ray (Biesheuvel C 2011). This technique might be useful for early detection for breast abnormality. Nowadays, combine methods among breast physical examination, mammography and tissue biopsy can be used to diagnose breast cancer with a good degree of accuracy. Although therapeutic approaches, such as surgery, chemotherapy, radiation, endocrine therapy, and targeted therapy, have reduced cancer mortality, there still are many therapeutic failures which result in cancer recurrence, metastasis and death (Tara LH and Rache MS 2005, Mohammad J 2007).

1.2 Risk factors

There are many risk factors to promote breast cancer carcinogenesis and still in ongoing research (Reeder JG and Vogel VG 2008, Yager JD 2006, Yang L and Jacobsen KH 2008). At this situation, the known risk factors were shown the information below:

1.2.1 Age and race

Although breast cancer can occur early in life, more than 65 percent of breast cancer patient are found in older female with menopause status (> 65 years old) (Reeder JG and Vogel VG 2008). The occurrence of breast cancer has increase with advancing age. About race, some epidemiological study found that breast cancer is the most frequently diagnosed malignancy for women in the western world especially for American women (Parkin DM et al. 2005). This incidence might be involved with other factors such as lifestyle and environment.

1.2.2 Lifestyle

Smoking behavior appears to increase the risk 35 to 50 percent of breast cancer (Johnson KC et al. 2011, Hamajima N et al. 2002). The higher risk depends on the amount smoked and the earlier in life that smoking began. The association between breast feeding and breast cancer has been identified that it can lower breast cancer risk especially for breast feeding longer than 1 year (Yang L and Jacobsen KH 2008, Awatef M et al. 2010). Moreover, there are some studies suggest

that the risk of breast cancer also increase in women who receiving radiation therapy (Boyd NF et al. 2002, Willett W et al. (2004). For ionizing radiation, the risk of breast cancer increases with higher doses (dose–effect relationship) but commonly performed medical procedure, such as chest radiography and mammography, has very low dose radiation exposure (mean doses to breast tissue around 0.002–0.0002 Gray) which relate to very small risk for breast cancer.

1.2.3 Hormone

Breast cancer cells also have hormone receptor on their surface, cytoplasm and nucleus. There are 3 important receptors including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). The breast cancer cells, which have estrogen receptors called ER+, can be treated with drug to block estrogen effects (such as tamoxifen). HER+ breast cancers are generally more aggressive than HER- breast cancers but HER+ also response to drug such as trastuzumab (Sotiriou C and Pusztai L 2009, Romond EH et al. 2005). Cancer cells that do not have 3 receptor types are called triple negative, however they might be express other receptors such as androgen receptor and prolactin receptor.

At this time, several steroid hormones are clearly related to risk of breast cancer. One of them is estrogen. In animals, estrogens have been shown to increase mammary tumor development and promote cell proliferation with existing mutation. For premenopausal women, the major group of estrogen is estradiol produced by

the ovaries. When the ovaries stop working in postmenopausal women, the main estrogen is estrone, which came from androgens produced in adipose tissue. Then, obese postmenopausal women have both higher levels of estrogens and a higher risk of breast cancer (Watson M 2008). The other hormone which relate to breast cancer risk is androgen. Although androgens, such as testosterone, are typically considered as male hormones, they are also found in women, secrete from the ovaries and adrenal glands. They might increase breast cancer risk either by increasing cell proliferation, or convert to estrogens (Hankinson SE and Eliassen AH 2007). Moreover, there are 2 female hormones which have studies related to breast cancer risk including progesterone and prolactin (Willett W et al. 2004). Before menopause, progesterone is produced by the corpus luteum in the ovaries but progesterone levels are extremely low after menopause. Prolactin is secreted by the pituitary gland, and prolactin levels are much lower after menopause. As with estrogens, both progesterone and prolactin promote mammary tumour development in animals. In addition, breast cell proliferation is highest during the luteal phase of the menstrual cycle, when progesterone levels also peak. As we known, circulating level of hormones are associated with breast cancer risk. Then, exogenous sources of hormones, usually taken in the form of medications, also may influence breast cancer risk (Santow G 1991, Hamajima N et al. 2002). However, there was a small increase in risk while women were using combined oral contraceptive pills and in the 10 years after stopping. It found that the risk of women who taking the pills, was

increased by an average of 24% compared with women who never used the pills (Kumle M et al. 2002, Marchbanks PA et al. 2002, Hannaford PC et al. 2007, Boyd NF et al. 2003).

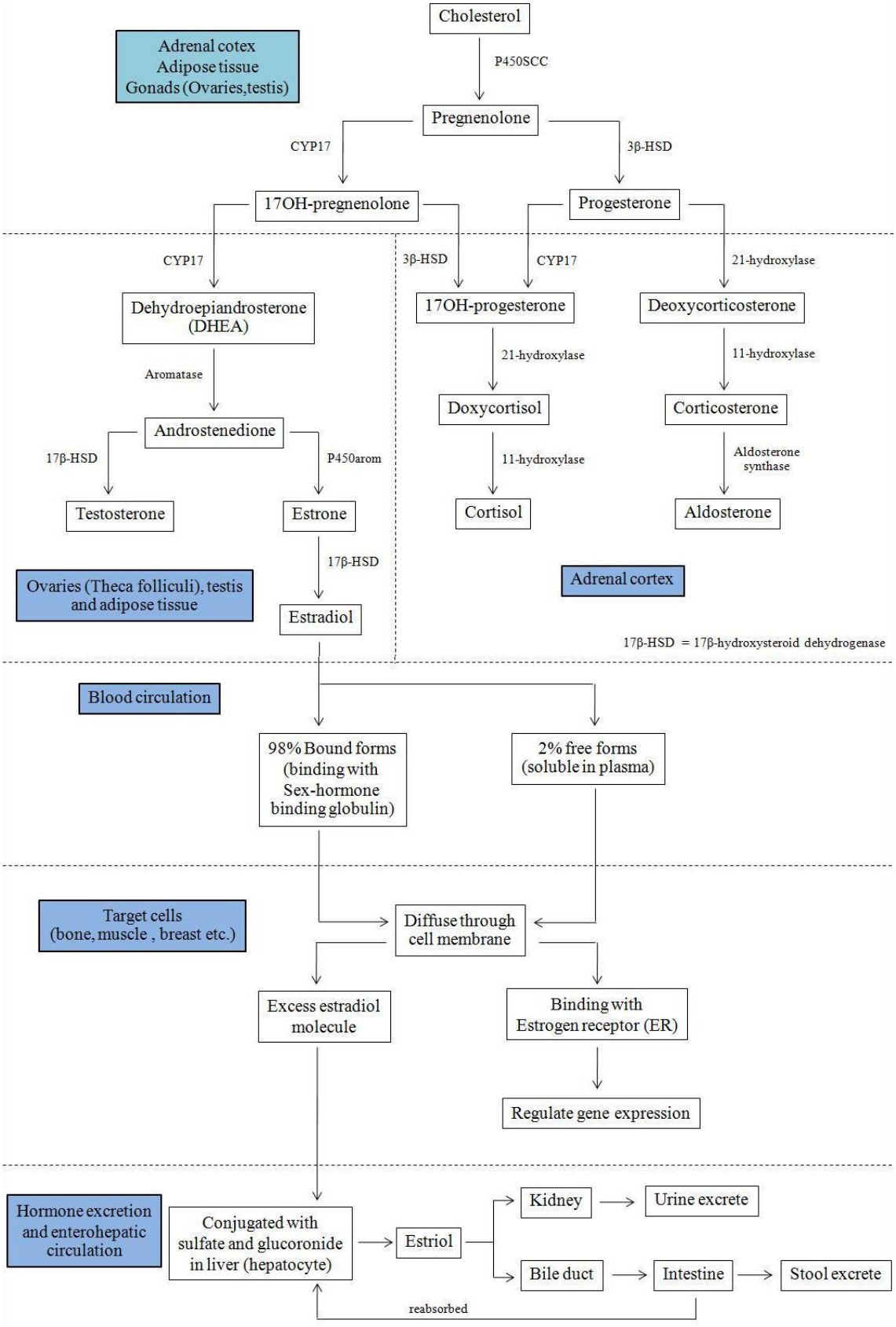

Cholesterol
P450SCC
Adrenal cotex
Adipose tissue
Gonads (Ovaries,testis)
Pregnenolone
CYP17
3β-HSD
17OH-pregnenolone
Progesterone
CYP17
3β-HSD
CYP17
21-hydroxylase
Dehydroepiandrosterone (DHEA)
17OH-progesterone
Deoxycorticosterone
Aromatase
21-hydroxylase
11-hydroxylase
Androstenedione
Doxycortisol
Corticosterone
17β-HSD
P450arom
11-hydroxylase
Aldosterone synthase
Testosterone
Estrone
Cortisol
Aldosterone
17β-HSD
Ovaries (Theca folliculi), testis and adipose tissue
Estradiol
Adrenal cortex
17β-HSD = 17β-hydroxysteroid dehydrogenase
Blood circulation
98% Bound forms (binding with Sex-hormone binding globulin)
2% free forms (soluble in plasma)
Target cells (bone, muscle , breast etc.)
Diffuse through cell membrane
Excess estradiol molecule
Binding with Estrogen receptor (ER)
Regulate gene expression
Hormone excretion and enterohepatic circulation
Conjugated with sulfate and glucoronide in liver (hepatocyte)
Estriol
Kidney
Urine excrete
Bile duct
Intestine
Stool excrete
reabsorbed

Figure 3. The metabolism and synthesis pathway of estrogen which related to breast cancer risk factor

1.2.4 Diet

There are some correlation between breast cancer and high fat diet consumption especially for animal fat and red meat. It has been suggested that meat consumption might increase the risk of breast cancer induced from some components of red meat including heterocyclic amines, iron and exogenous hormone residuals (Boyd NF et al. 2003). Some studies suggest that consumption of meat cooked by methods that promote formation of carcinogens (meat cooked at high temperature) might increase postmenopausal breast cancer risk (Gerber B et al. (2003). For alcohol consumption, there was little promotion in risk of breast cancer for alcohol consumption compared with women who drinking no alcohol (Hamajima N et al. 2002)]. Anyway, there are some studies shows small to moderate reduction of breast cancer risk with increasing consumption vegetables and fruits (Smith-Warner SA et al. 2001, Riboli E and Norat T 2003).

1.2.5 Genetics

Genetic mutation can increase risk of breast cancer for those women who inherit them especially for some high risk gene BRCA1 and BRCA2 . Both of them have been identified that show strongest association with breast cancer development. They have biological function to repair DNA damage. For both genes,

the risk of breast cancer for carriers of specific mutation form is substantially higher (10–30 times) than for women who not inheriting those genetic variants (Antoniou A et al. 2003). Despite the large increase in breast cancer risk associated with BRCA1 and BRCA2 mutation, they can cause for only about 5% of all breast cancers (hereditary breast cancer) because only 1 in 1000 women has inherited one of them (Scott CL et al. 2003, Nkondjock A and Ghadiria P 2004). Therefore, all women who inherit a BRCA1 or BRCA2 mutation do not necessary to develop breast cancer, it also needs other factors (environmental or genetic) to influence the risk of disease (Matros E et al. 2005). There are some other significant mutations which relate to breast carcinogenesis including p53 (Li–Fraumeni syndrome), PTEN (Cowden syndrome), and STK11 (Peutz–Jeghers syndrome) (Gage M et al. 2012).

1.3 Microenvironment

Generally, cancer is a systemic disease composes of multiple components from both tumor cells and host stromal cells. Recently, many supportive researches notify that tumor stromal cells play important roles in tumor initiation, progression, and metastasis (Paget S 1989, Kalluri R and Zeisberg M 2006). Fibroblasts, which are the most abundant cells in connective tissues, were normally found to be activated in wound healing and fibrosis. Tumors are similar to a chronic and non-healing wound, so fibroblasts have been found to be activated in cancer. Cancer-associated fibroblasts (CAFs) are the name of activated fibroblast which majority found in the

component of tumor stromal cells, especially for breast tissue (Kalluri R and Zeisberg M 2006, Ostman A et al. 2009)]. There are evidences suggesting that the activation of fibroblasts is induced by many cancer secretion factors, such as TGF-β, CXCL12/SDF-1, PDGF-α/β, basic fibroblast growth factor (b-FGF), or interleukin (IL)-6 (Kojima Y et al. 2010, Bronzert DA et al. 1987, Shao ZM et al. 2000, Strutz F et al. 2000, Giannoni E et al. 2010, Hugo HJ et al. 2012). TGF-β from the tissue culture medium of MCF7 and MDA-MB-231 (both of them are breast cancer cell line) can promote the differentiation of human adipose tissue-derived stem cells into a CAF-like myofibroblastic phenotype (Jotzu C et al. 2010). CAFs can promote tumor development and progression in different ways by secreting many types of factors including HGF, TGF-β, SDF-1, VEGF, IL-6, and matrix metalloproteinases (MMPs) (Hasebe T et al. 2001, Orimo A et al. 2005, Saito RA et al. 2010). Moreover, CAFs not only promote tumor progression but also induce therapeutic resistance (Martinez-Outschoorn UE et al. 2010). Then, targeting CAFs can provide a novel way to control tumors with therapeutic resistance. There are other stromal cells which can promote breast cancer progression such as T lymphocyte (infiltrating lymphocyte) and macrophage (tumor-associated macrophage) (Ryan L et al. 2013). Nowadays, there is technique to observe breast cancer microenvironment by culture multiple cell lines in the same condition called co-culture. This technique can be used to reveal cell adhesion, cell migration or cell interaction (Thierry T et al. 2000). The tumor

microenvironment is an interesting area for the development of novel therapies with the potential of augmenting existing treatment and prevention options [53]. Actually, some new related therapeutic targets have been developed and are under preclinical evaluation and clinical trials (Loeffler M et al. 2006, Jotzu C et al. 2010, Man YG and Sang QX 2004).

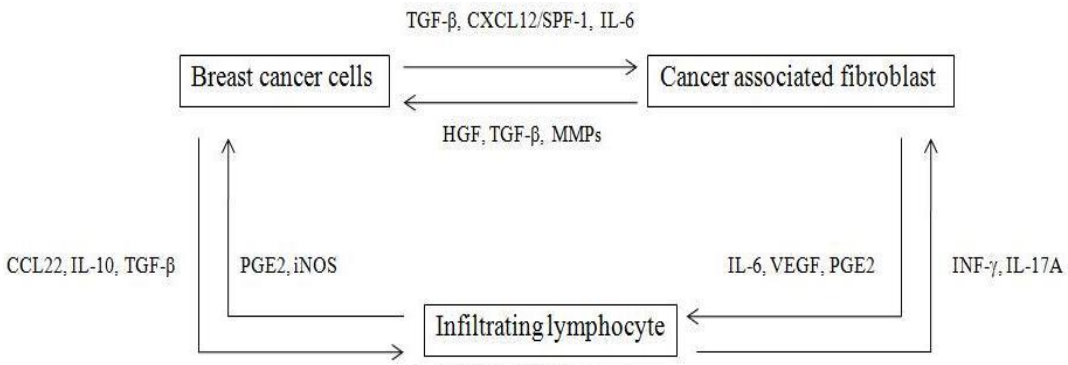


Figure 4. There are interaction molecules between breast cancer and surrounding tissue which can regulate cell development.

2. DNA methylation

The biochemical processes that involve with the addition reaction of methyl group to the nucleotide of DNA called DNA methylation. This vital process can be found in many organisms such as bacteria, fungi, insect, plant and mammal. In mammal, this epigenetic mechanism is a post-transcriptional process and majority occurred at cytosine of CpG dinucleotide sequence. CpG dinucleotides are found in clusters called CpG islands and mostly presented at 5' regulatory region of the gene.

At least 70 percent of CpG islands in whole genome are methylated in normal condition (Jaenisch R et al. 2003). During cell development and differentiation, DNA methylation at CpG islands can change to alter gene expression pattern and level in many key processes such as genomic imprinting, X chromosome inactivation and carcinogenesis (Iqbal K et al. 2011, Ehrlich M et al. 1982). From present studies, the methylation of DNA have 3 mainly purposes composed of 1) maintain gene expression pattern during cell division 2) methylation at promoter to regulate specific gene expression and 3) directly form the basis structure of chromatin (Wossidlo M et al. 2011, Rudolf J et al. 2003). By changing gene regulation expression and losing stability of genome structure, the DNA methylation mechanism can relate to many types of cancer carcinogenesis such as colon cancer, breast cancer, leukemia and also prostate cancer (Rudolf J et al. 2003, Ehrlich M et al. 1982). In cancer cell, the abnormal DNA methylation pattern can be observed in both hypermethylation and hypomethylation when compared to normal. Promoter CpG hypomethylation can up regulated gene expression and change to down regulated gene expression when its hypermethylation. According to present study, global genome hypomethylation has been discovered as a motivation mechanism of cancer progression through different pathways (Hon GC et al. 2012). Then, breast cancer is a one type of cancers which reveal global genome hypomethylation (Yu Y et al. 2003, Monk M 1990).

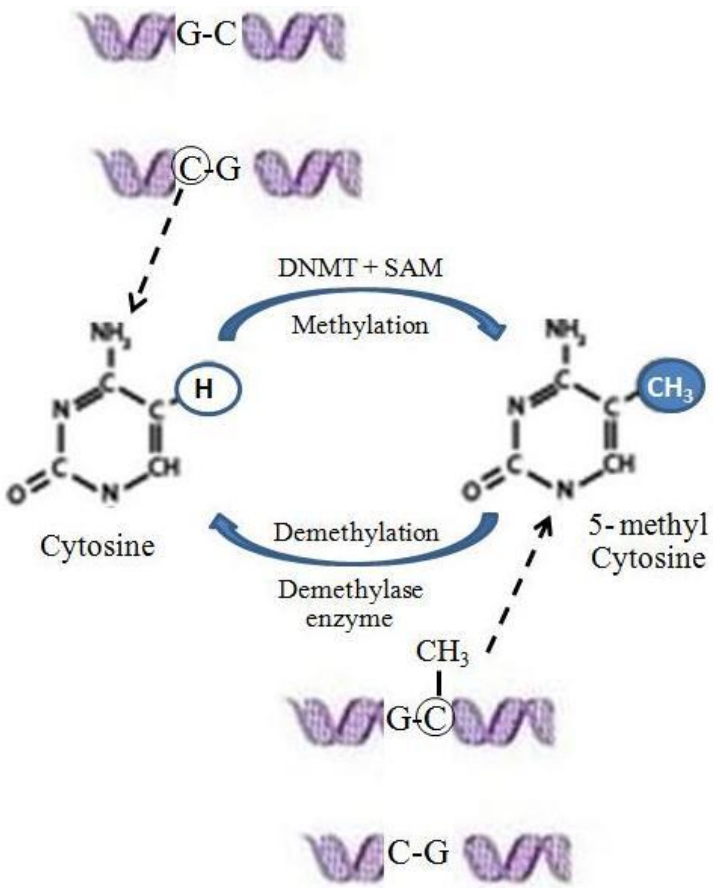


Figure 5. DNA methylation (at CpG), SAM = S-adenosyl-L-methionine

In this time, there are many currently techniques to detect DNA methylation including methylation specific PCR (MSP), whole genome bisulfate sequencing and pyrosequencing of bisulfate treated DNA. Most of them take the benefit from bisulfate reaction to convert unmethylated cytosine to uracil and methylated cytosine to unmethylated cytosine. Then, the amount of methylated and unmethylated can be calculated.

2.1 Type of DNA methylation

There are two types of DNA methylation in eukaryotic cell including:

2.1.1 De novo methylation

This methylation type involve with the methylation rearrangement pattern during embryogenesis or cell differentiation process in adult cell (Jones P et al. 1990). For embryogenesis, *de novo* methylation can occurred after zygote implantation until gastrulation stage. Somehow, it seems to occur rarely after post-gastrulation. Recently, *de novo* methylation can frequently found in cell lines (in vitro) and cancer cells (Kawai J et al. 1994, Leonhardt H et al. 1992, Robertson KD et al. 1999).

2.1.2 Maintainance methylation

The maintainance methylation is responsible for maintaining methylation pattern after it was established. This type of methylation is so vital important to keep the similar DNA methylation pattern after cell division in cell developmental and differentiation process (Bestor T et al. 1988). This process occurs during DNA replication in cell division.

2.2 DNA methyltransferase (DNMT)

The construction of methylation mechanism and pattern during cell development or cancer carcinogenesis came from the function of DNA methylatransferase (DNMT) enzyme. There is various type of DNMT as describe below:

2.2.1 DNA methyltransferase 1 (DNMT-1)

This enzyme plays a major role to maintainance DNA methylation process. In the first, the researcher found DNMT-1 in mouse genome (Yen RW et al. 1992). After that, the homologous gene (located on short arm of chromosome 19) in same function was found in human (Li E et al. 1992). During DNA replication, DNMT-1 as the one part of replication complex can recognized CpG methylation pattern in parental strand and catalyzed the additional reaction of methyl group into corresponding CpG site in daughter strand. DNMT-1 is vital gene for cell developmental process as observe in DNMT-1 knockout mice which die in embryogenesis (Goll MG et al. 2006).

2.2.2 DNA methyltransferase 2 (DNMT-2)

DNMT-2 (located on chromosome 10p15.1) is not methylate to DNA structure but it has function to methylate cytosine-38 residue in the anti-codon loop of aspartic acid tRNA (Robertson KD et al. 1999). Then, it has another name refer to its function called tRNA aspartic acid methyltransferase.

2.2.3 DNA methyltransferase 3 (DNMT-3a and DNMT-3b)

Both DNMT-3a (located on chromosome 2p23) and DNMT-3b (located on chromosome 20q11.2) play an important role in *de novo* methylation of embryo developmental process. Thus, they have no effect in maintainance methylation mechanism (Xie S et al. 1999, Laird PW 1997).

According to alteration of methylation pattern, there might be mechanism to remove methyl group from methylated CpG dinucleotide. Nowadays, there are two pathways of demethylation process that already knows including passive and active demethylation. Passive pathway occurs immediately when DNMT-1 fail to maintain existing methylated CpG site (Bhattacharya SK et al. 1999). Active pathway might be occurred by demethylase enzyme which have been previous describe (Nemanja R et. al 2013).

3. Repetitive sequences

The sequence of DNA that found in multiple copies spread in all genome called repetitive sequences. Repetitive sequence can make up nearly 50 percent of human genome (Jeffrey AJ 2005). There are composed of three major types as follow:

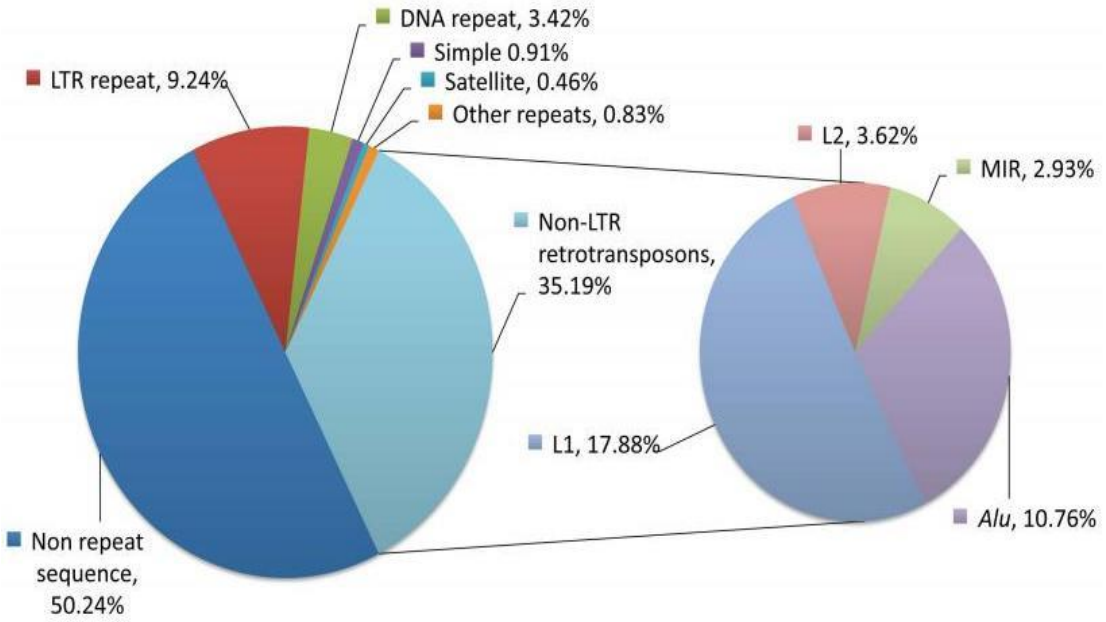


Figure 6. Amount of repetitive sequence in human genome [89]

3.1 Terminal repeats

Another name of this repeat is Long Terminal Repeats (LTRs). This repeats occur from insertion of retroviral nucleic acid into host genome (Jeffrey AJ 2005, Singer MF 1982). Human immunodeficiency virus (HIV) is the one of retroviral which perform this process. LTRs are comprised of three domains including U3 (Unique 3), R (Redundant) and U5 (Unique 5). Both U3 and U5 have transcription start site for Poly A which play an important part after reverse transcription to integrate into host genome.

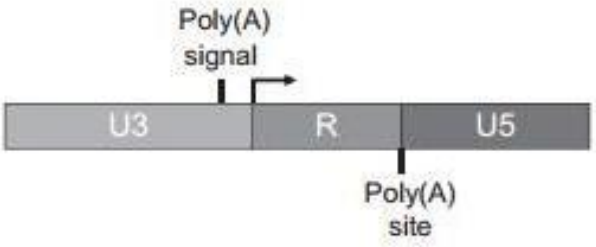


Figure 7. Three domains of retroviral LTRs (Singer MF 1982)

3.2 Tandem repeats

This repeat component found in DNA sequence with more than two nucleotides is repeated. There are subtypes in this repeats divided by number of repeated nucleotides. When it has only two nucleotides repeated, it can called dinucleotide repeat. In the same way, if three nucleotides repeated, it can called trinucleotide repeat. Somehow, the repeated sequence between 4 to 9 nucleotides

can called microsatellite or Short Tandem Repeats (STRs). The repeatitive sequence in larger scale between 10 to 100 nucleotides can called minisatellite. This type of repeated sequence can be used to determine an individual's inherited trait (Eva-Maria Schrom et al. 2013).

3.3 Interspersed nuclear repeats (IRS)

Interspersed repeat or interspersed nuclear element is located mainly in all eukaryotic genome and distributed more than tandem repeats (Lander ES et al. 2001). These elements, that pose ability to amplify themselves by DNA or RNA intermediate and spread around genome, can call mobile element or transposon. DNA transposons can mobile with cut-and-past mechanism which found in non-protein coding sequence. In the other way, RNA transposons or retrotransposons can mobile and amplify with copy-and-paste mechanism (Jeffrey AJ 2005, Lander ES et al. 2001). There are compose of two types of IRS in eukaryotic cell which are LINE (majority is LINE-1) and SINE (majority is Alu).

3.3.1 Long interspersed nuclear element (LINEs)

There are many types of LINEs but the main component in this group is LINE-1. LINE-1 is a retrotransposon which contain more than 500,000 copies in human genome (about 17 percent). These elements are longer than 4.5 kb and compose of three parts including a 5' untranslated region (UTR), two open reading frames (ORF) and a 3' UTR which provide polyadenylation signal for amplification mechanism

(Aporntewan C et al. 2011). In the past, LINE-1 was also known as a junk or parasitic DNA. However, the recent study express that LINE-1 can regulate gene expression which controlled by 5' UTR methylation status (Shengyan X et al. 2010). In eukaryotic cell, LINE-1 has three steps in its life cycle. The first step, LINE-1 can transcript to RNA which has 2 ORF regions by RNA polymerase II. In the second step, ORF1 can translate to RNA binding protein and ORF2 can translate to a protein which has reverse transcriptase and endonuclease activity. The third step, RNA binding protein can form ribonucleoprotein complex with LINE-1 transcript and move into nucleus. Reverse transcriptase perform its function to construct LINE-1 DNA (almost start from consensus sequence; 5' TTTTAA 3') and insert into genome with endonuclease property (Penzkofer T et al. 2005). The other path way of LINE-1 to control gene expression is interference RNA. The mechanism occurred from complementary LINE-1 transcript can reverse to bind with LINE-1 element in the genome.

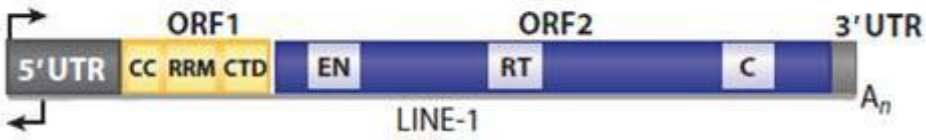


Figure 8. Genetic structure of LINE-1 which contain both ORF1 and ORF2 (Aporntewan C et al. 2011).

3.3.2 Short interspersed nuclear element (SINEs)

SINEs are a short sequence of DNA (less than 500 bp). The majority in these compartments is Alu element. These elements found more than 10% of human

genome which also can represent genome methylation (Penzkofer T et al. 2005, Pedraza V et al. 2010, Finak G et al. 2008). Full-length Alu elements are about 350 bp which commonly found in introns, 3′ untranslated regions of genes and intergenic genomic regions. There are evidence indicated that these mobile elements were present in the human genome with high copy number (about 500,000 copies) (Casey T et al. 2009). Alu transcripts are not encoding the protein but they can regulate expression, affect recombination, influence nucleosome formation and genome evolution. Demethylation of Alu elements is a marker of lower genome stability, which effect for gene recombination and chromosome translocation (Penzkofer T et al. 2005, Pedraza V et al. 2010).

According to throughout spreading of interspersed nuclear elements especially for LINE-1and Alu, the methylation status of this element can represent the whole genome methylation. Recently, researchers observed global methylation via LINE-1 or Alu methylation in both normal situation and various cancers (Penzkofer T et al. 2005, Moses H et al. 1990). Nowadays, The DNA methylation levels of LINE-1 and Alu at 5’ UTRs in cancer have been extensively evaluated for potential use as an epigenomic marker for cancer (Kitkumthorn N and Mutirangura A 2011, Shengyan X et al. 2010, Pedraza V et al. 2010).

CHAPTER III

RESEARCH CONCEPT

1. Research Question

"How the breast cancer cells can induce LINE-1 methylation change in normal surrounding tissue?"

2. Hypothesis

Breast cancer cells can regulate LINE-1 hypermethylation in breast stromal cells

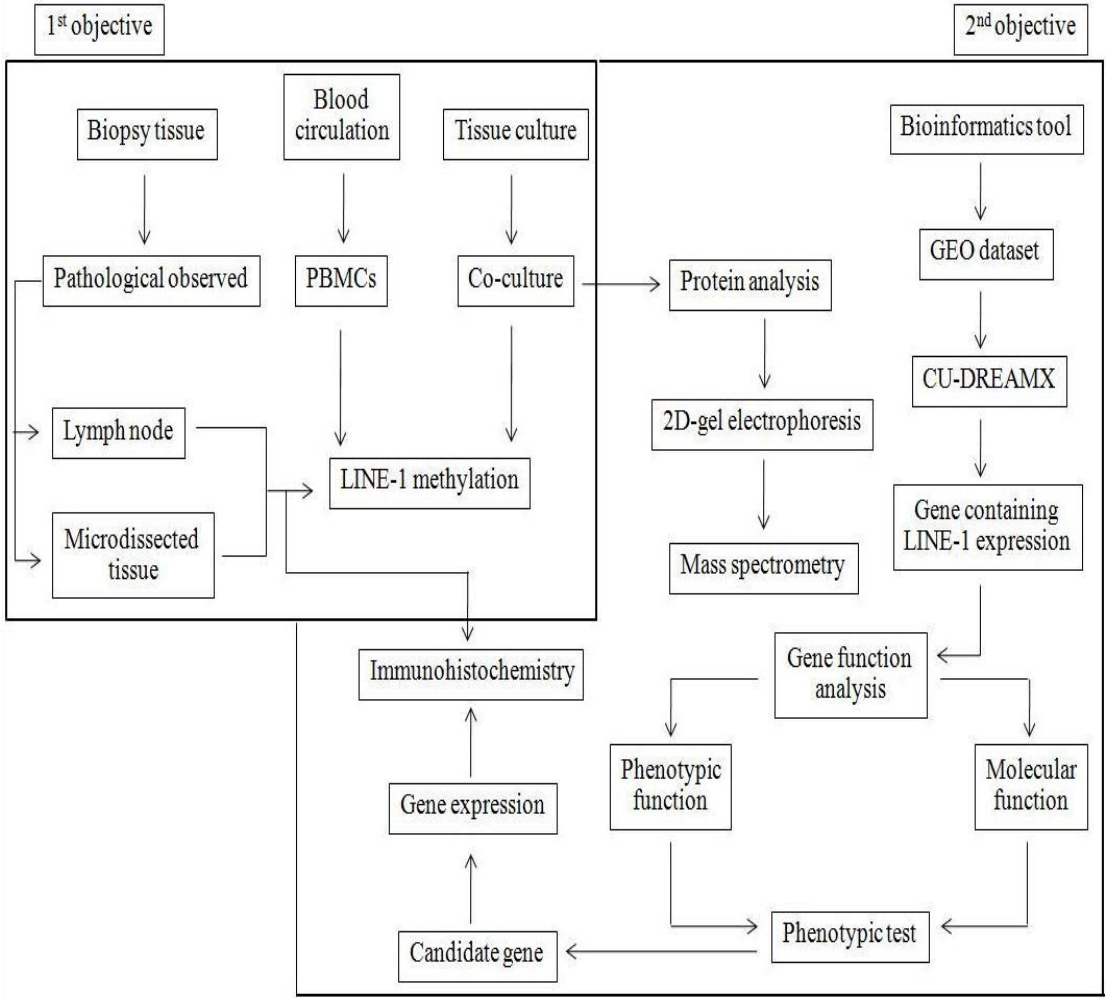
3. Objectives

There are 2 main objectives in this research including;

1. To investigate LINE-1 methylation change in breast cancer adjacent tissue

2. To identify possible mechanism that the breast cancer cells can promote methylation change in breast adjacent tissue

4. Conceptual framework



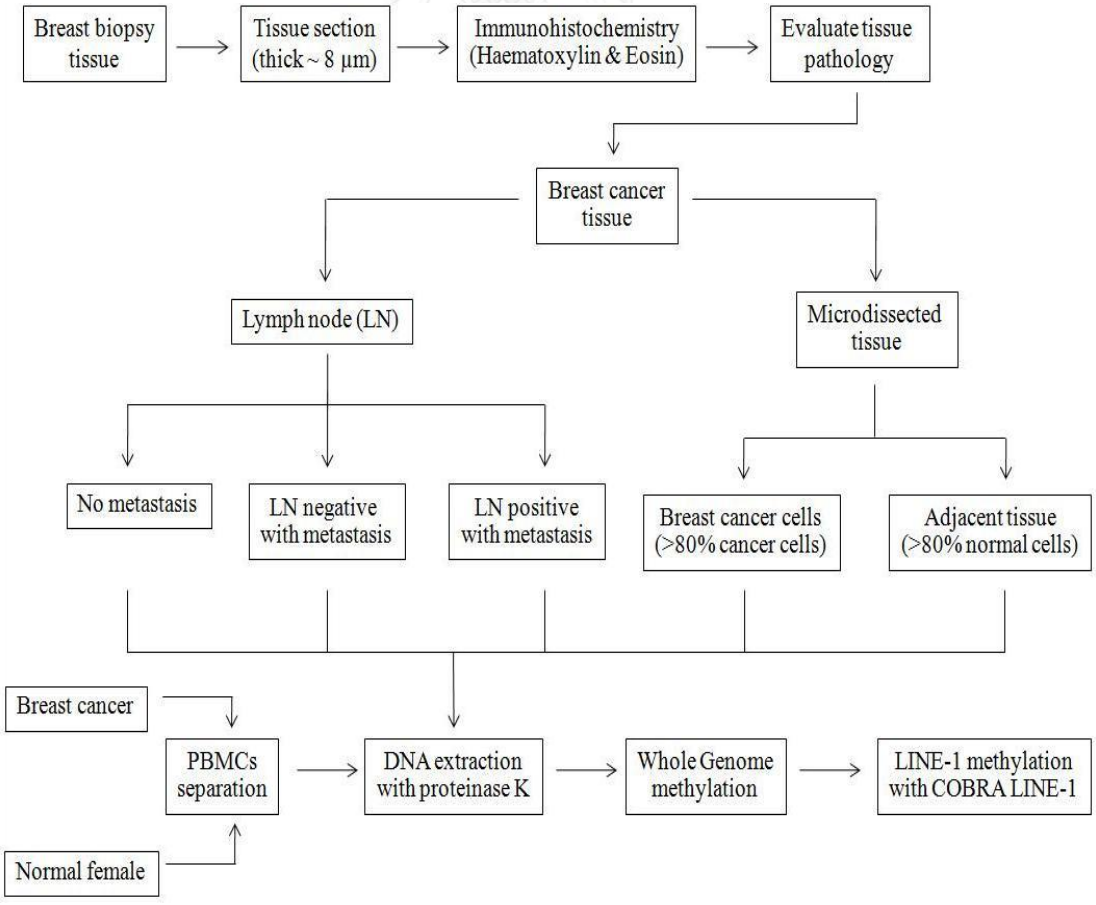
CHAPTER IV

MATERIALS AND METHODS

1. Experimental design

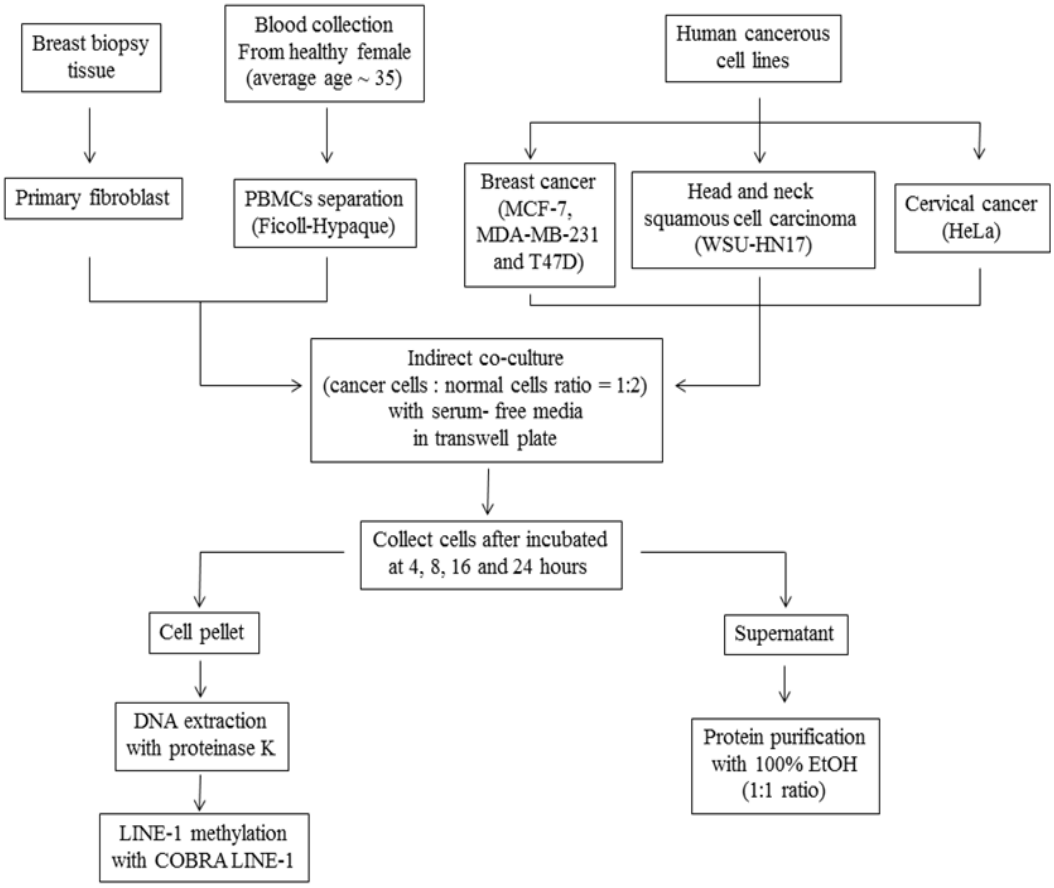
Experiment 1: The study to observe the effect of breast cancer cell to breast cancer adjacent tissue

Purpose: To verify LINE-1 methylation change in breast cancer adjacent tissue



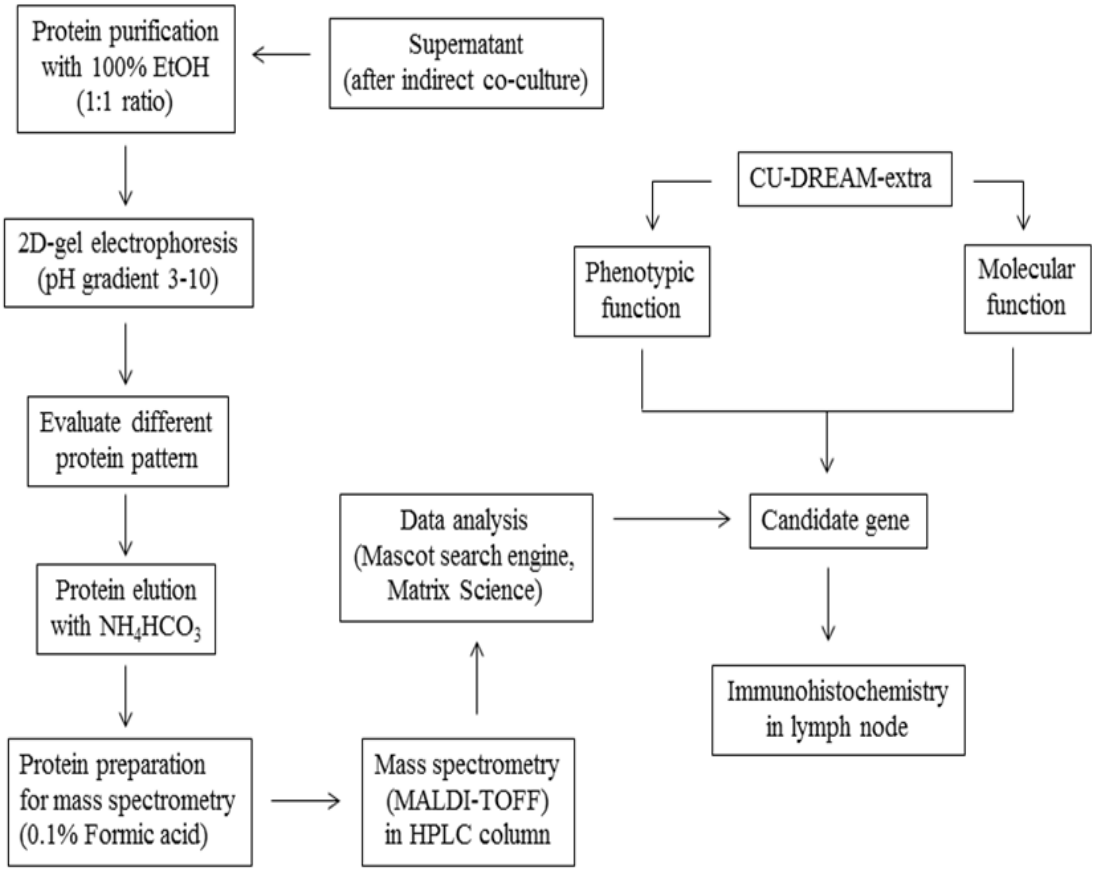
Experiment 2: The study to observe the epigenetic change in PBMCs and primary fibroblast

Purpose: To verify LINE-1 methylation change in PBMCs and primary fibroblast



Experiment 3: Searching for candidate which found epigenetic change only in breast cancer adjacent tissue

Purpose: To verify the possible mechanism that breast cancer can promote LINE-1 methylation change in breast surrounding tissue



2. Research methodology

In this study, samples were collected from 3 groups including lymph node biopsy tissue, peripheral mononuclear cells and cell cultures.

2.1 Tissue culture

The established human carcinoma cell lines which obtain from American Type Culture Collection (Rockville, MD) are including 3 mammary carcinoma (MCF-7; ATCC® HTB-22, MDA-MB-231; ATCC® HTB-26, T47D; ATCC® HTB-133), head and neck squamous cell carcinoma (WSU-HN17) and cervical carcinoma (HeLa, ATCC® CCL-2). Normal human primary fibroblasts were isolated from periodontal ligament in the absence of tumoral lesion and retained a fibroblastic morphology more than 10 passages. All of them were grown up with DMEM medium supplemented with 10% FBS (Gibco® BRL, Life technologies) at 37°C in humidified atmosphere (95% air and 5% CO_2). The cell lines were passaged twice a week, and the medium were changed every other day. All cell lines will be performed mycoplasma-free test with the Boehringer Mannheim BM-Cyclin test (F. Hoffmann-La Roche Ltd.). The cells, grown in culture flasks (CytoOne T225 flask, USA Scientific®.Inc), were harvested at 65-70% confluence using 0.05% Trypsin, 0.5 mM EDTA, and washed in a phosphate buffer saline (PBS).

Indirect co-culture experiments associating cancer cells with PBMCs or fibroblast were performed in Transwell® culture plates (Costar, Dutscher, Brumath, France) allowing paracrine exchanges between both cell types. The cancer cells were

seeded in 24-well culture plates ($5x10^4$ cells/well) and allowed to attach overnight in DMEM serum-free medium, whereas PBMCs or fibroblasts were plated in permanent membrane culture inserts ($1x10^5$ cells/well) which is 6.5-mm diameter; tissue culture treated polycarbonate membranes, 0.4-mm pore size ($1x10^5$ cells/well). Culture inserts containing PBMCs or fibroblasts were placed in the wells containing cancer cells. Both PBMCs and fibroblasts were co-cultivated for 4, 8, 16 and 24 hours to harvest and determine DNA methylation.

After obtain the preliminary result, sample size were calculated with following formula;

$$n = [(Z_{\alpha/2} + Z_{\beta})^2 \sigma^2_d] / X_d^2$$

n = Sample size

d = Different of value in each group

X_d = Different of mean in each group

σ^2_d = Different of variance in each group

Z = Standard value from normal distribution

2.2 Sample collection

2.2.1 PBMCs preparation

Blood samples were derived from 21 healthy females and 7 healthy males without immune disorder and chronic disease. PBMCs were purified using standard Ficoll-Hypaque gradient centrifugation according to the instructions of the manufacturer (Amersham Pharmacia®, Uppsala, Sweden). Briefly, 4 ml of Ficoll-

HyPaque gradient were pipetted into two 15-ml centrifuge tubes. The heparinized blood will be diluted 1:1 in phosphate-buffered saline (PBS) and carefully layered over the Ficoll-Hypaque gradient (9 to 10 ml/tube). The tubes were centrifuged for 20 min at 1,200 g. The cell interface layer will be harvested carefully, and the cells will be washed twice in PBS (for 10 min at 640 g followed by 10 min at 470 g) and resuspended in RPMI 1640 medium with Glutamax supplemented with penicillin (50 U/ml)-streptomycin (50 g/ml) and 10 mM HEPES (complete RPMI medium) before counting.

2.2.2 Lymph node and microdissected tissue

Lymph nodes samples from thirty-eight breast cancer patients (12 cases of no metastasis and 26 cases of metastasis) were collected during January 2013-December 2014 from the Faculty of Medicine, Chulalongkorn University. Patients who had prior chemotherapy or radiotherapy were excluded. All lymph nodes will be immediately cut from the resected breast tissue, embedded in Tissue Tek® OCT medium (Sakura™, Tokyo, Japan), and frozen in liquid nitrogen. All patients will had no anti-inflammatory treatment, chemical, hormonal or radical therapy. The frozen tissues will be sectioned by a cryostat (Carl Zeiss Microimaging™, New York, USA) at 8 μm, mounted on glass slides and covered with PEN foil (2.5 μm thick; Carl Zeiss Microimaging™). The slice samples will be quickly fixed using a mixture of absolute ethanol and acetic anhydride (19:1), and stored at -80°C until use. Slides will be

stained with hematoxylin and eosin (H&E) at room temperature for investigation to confirm histopathologically component by a pathologist and dehydrated for 5 sec each with 70, 80, 95 and then 100% ethanol. The breast cancer sections which included in this study will be consisted of at least 80 percent of tumor cells. Then, sixty-four lymph node samples were classified into 3 sample groups including 1) lymph nodes of non-metastatic breast cancer (No metastasis, n=12), 2) lymph nodes negative of metastatic breast cancer (Metastasis LNs-, n=26), 3) matched lymph nodes positive of metastatic breast cancer (Metastasis LN+, n=26). After being air-dried, the sections will be microdissected using the LMD system with a 337-nm nitrogen ultraviolet (UV) laser (Zeiss PALM Microbeam LCM System, Carl Zeiss Microsystems™). The target cells dissected from a section will be dropped immediately into a microcentrifuge tube cap filled with 30 μl of RLT lysis buffer (Qiagen®, Hiden, Germany). The samples of noncancerous tissues will be selected from at least 5 cm away from cancer tissue, and pathologically composed of >80% normal breast tissue without cancerous and dysplastic cells. Laser capture microdissection will be used to collect approximately 1,500 cells for further experiment.

2.3 DNA extraction

Cells from the tissue culture (PBMCs and fibroblasts) and section breast biopsy tissue will be centrifuged at 4 $^{\circ}$C 2,500 rpm, 15 minutes. Cell pellet will be

collected and discarded supernatant. Wash cell pellet in sterile phosphate buffer saline solution and centrifuged at 4 °C 2,500 rpm, 15 minutes. Cell pellet will be added with 1 ml of extraction buffer with 10% SDS and proteinase K 0.5 mg/ml. The mixture will be incubated at 50 °C, 72 hours. Phenol-chloroform solution will be added into the digest cell pellets or tissues mixture. After that, the mixtures will be centrifuged at 4 °C 14,000 g for 15 minutes. Collect the upper phase and add 10 M ammonium acetate, absolute ethanol for DNA precipitation. The air-dry DNA will be suspended in distill water and applied for COBRA to measure the LINE-1 and Alu methylation level.

2.4 Quantitative Combine Bisulfite Restriction Analysis for LINE-1 (COBRA LINE-1)

All DNA samples will be converted by bisulfite reaction using sodium bisulfate as previously described (Xiong Z and Laird PW 1997). Unmethylated cytosine at CpG island will be changed into uracil while methylated cytosine will not be changed. A total of 1 µg DNA samples will be denatured in 0.22 M NaOH at 37°C, 10 minutes and added 10 mM hydroquinone (Sigma-Aldrich®, Singapore) with 3 M sodium bisulfited (pH 5.0) incubated at 50°C, 16-20 hours. After that, DNA will be recovered using the Wizard DNA Clean-Up Kit (Promega, Madison, WI) follow by the manufacturer's protocol. DNA samples will be eluted by warm water and precipitated with sodium acetate in ethanol. Then, Bisulfited DNA pellets will be resuspended in distills water. One microliter of bisulfited DNA will be subjected into 45 cycles of PCR process using

LINE-1 forward primer (5′-CCGTAAGGGGTTAGGGAGTTTTT-3′) and LINE-1 reverse primer (5′-RTAAAACCCTCCRAACCAAATATAAA-3) with annealing temperature 50°C. The amplified products will be digested with 2 U of *TaqI* and *TasI* restriction endonuclease in *TaqI* buffer (MBI Fermentas®, Glen Burnie, MD) at 65 °C, overnight. The digested products will be identified by 8% non-denaturing polyacrylamide gel electrophoresis and stained with SYBR green (SYBR® Green JumpStart™ Taq ReadyMix™, Sigma-Aldrich® .Co.LLC). All samples will be process in duplicate.

According to LINE-1 analysis and calculation, the amplified products will be classified into 3 types depended on the methylation pattern of the 2 CpG dinucleotides. There are hypermethylated loci ($^{m}C^{m}C$), partial methylated loci ($^{u}C^{m}C$ and $^{m}C^{u}C$) and hypomethylated loci ($^{u}C^{u}C$). After enzyme digestion, four sizes of products will be detected for COBRA LINE-1 (160 bp, 98 bp, 80 bp and 62 bp). Then, the band intensities will be measured by a phosphoimager using ImageQuant Software (Molecular Dynamics, GE Healthcare®, Slough, UK).

Next, the percentage of LINE-1 methylation pattern will be calculated as follow; First, the intensity of each band will be divided by bp of DNA length; %160/160 = A, %98/94 = B, %80/78 = C, and %62/62 = D. After that, the percentage of methylation will be calculated as following formula; ^{m}C = 100×(C+A)/(C+A+A+B+D), $^{m}C^{u}C$ = 100×(A)/(((C–D +B)/2)+A+D), $^{u}C^{m}C$ = 100×(D-B)/(((C–D+B)/2)+A+D), $^{u}C^{u}C$ = 100×B/(((C–D+B)/2)+ A+D) and $^{m}C^{m}C$ = 100×((C–D+B)/2)/(((C–

D+B)/2)+D+A). The same DNA preparations from HeLa (Cervical cancer), DauDi (B-cell lymphoma) and JurKat (T-cell lymphoma) cell lines will be used as a positive control in the experiment and for inter-assay adjustments.

2.5 Quantitative Combine Bisulfite Restriction Analysis for Alu (qCOBRA Alu)

This process is similar to qCOBRA LINE-1 but using different set of primers (Kitkumthorn N et al. 2012) with the following sequences; Alu forward primer (5′-GGCGCGGTGGTTTACGTTTGTAA-3′) and Alu reverse primer (5′-TTAATAAAAACGAAATTTCACCATATTAACCAAAC-3′) at annealing temperature 53 °C. Three product sizes (117bp, 75 bp and 43 bp) were found after *Taq*I digestion. The methylation pattern can be calculated using the following; the intensity of each band is separated by bp of DNA length; %117/117=A, %75(74)/74.5=B, %42(43)/42.5=D and C (represent of mCmC)=D-B. Next, the percentage of methylation was calculated as following formula; mC=100×(2D)/(2A+2D), mCmC=100×C/(A+B+C), uCmC+mCuC=100×B/(A+B+C) and uCuC=100×A/(A+B+C).

2.6 Protein purification

Protein will be extracted from cell pellet and supernatant after co-culture procedure. Then, 100 µl of isoelectric focusing lysis solution which composed of 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, and protease inhibitor mixture, will be applied directly to the cells pellet and vortexed vigorously for 5 min until all cells were completely lysed. The protein from cell lysates will be precipitated in 10%

trichloroacetic acid/80% acetone. The protein in supernatant will be added with 100% EtOH (1:1 ratio) for protein precipitation. The precipitated proteins will be dissolved in 20 mM Tris-Cl (pH 8.5), 8 M urea, 4% CHAPS, 5 mM magnesium acetate, according to the manufacture's protocol. The labeled samples were combined and mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 1% Pharmalyte (pH 3–10 NL)) before being applied to an 18-cm immobilized pH gradient strip (pH 3–10 NL) for overnight rehydration.

2.7 2D-gel electrophoresis

First-dimension isoelectric focusing will be carried out on an Amersham Biosciences®, Inc. The system process essentially as described by the manufacturer. Pre-cast immobilized pH gradient strips (18 cm; pH 3–10) will be used for the first-dimensional separation for a total focusing time of 25 kV. The strips will be equilibrated with a solution containing 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris (pH 8.8) (Sigma-Aldrish®, Singapore) reduced with 100 mM Dithiothreitol (DTT, Fisher Scientific®, Inc.) and directly applied to a 8% SDS-PAGE for second dimension gel electrophoresis overnight at 60 mA constant current. The resulting gel will be visualized by Oriole™ fluorescent gel staining (Bio-Rad Laboratories®, Inc.). The Oriole™ fluorescent gel stained image will be scanned at an excitation wavelength of 400 nm and an emission wavelength of 630 nm. All the images will be collected on a 2D 2920 Master Imager (Amersham Biosciences®, Inc.). Statistics and quantitation of

protein expression will be carried out in Decyder-DIA software (Amersham Biosciences®, Inc.). Only those spots with over 3 fold changes in volume after normalization between the two populations will be defined as altered.

2.8 Mass spectrometry

The separated proteins in SDS-PAGE gels will be visualized by Oriole™ fluorescent gel staining. The spots of interest will be excised, in-gel digested, and extracted as described previously [90]. The dried peptide sample will be dissolved in 6 µl of HPLC Buffer A solution (water:acetonitrile:acetic acid, 97.5:2:0.5 (v/v/v)) with 0.1% formic acid for mass analysis. HPLC-MS/MS analysis will be performed in an LCQ (ThermoFinnigan®, San Jose, CA) coupled on-line with an HPLC system (SMART System; Amersham Biosciences®, Inc.). 2 µl of protein digests obtained above will be loaded on the HPLC connected with an in-house packed C18 column (~5–10 cm length; 75 µm inner diameter). To reduce the dead volume, a high voltage (1.2 to 1.4 kV) will be applied to the HPLC side of the column through a zero dead volume three-way Y connector (Valco® Instruments Co., Inc., Houston, TX). The peptides will be sequentially eluted from the HPLC column with a gradient of 5 to 90% of Buffer B (acetonitrile:water:acetic acid, 90:9.5:0.5) in Buffer A (acetonitrile:water:acetic acid, 2:97.5:0.5) at a flow rate of ~ 0.7 µl/min. The eluted peptides will be sprayed directly from the tip of the capillary column to the LCQ mass spectrometer for mass spectrometry analysis. The LCQ will be operated in a data-dependent mode where

the machine measured intensity of all peptide ions in the mass range of 400 to 1400 m/s (mass-to-charge ratios) and isolated the peptide peak with the highest intensity for collision-induced dissociation. In this way, masses of both the parent peptide and its daughter ions will be detected. The accurately measured masses of the tryptic peptide and its fragments will be used to search for protein candidates in the protein sequence data base with the program Mascot Search engine (Matrix Science® LLC., London, UK).

2.9 Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue specimens were randomized from No metastasis (5 nodes), Metastasis LNs- (5 nodes) and Meatstasis LNs+ (5 nodes). Three-micrometer-thick sections of FFPE were deparaffinized and dehydrated. After antigen retrieval by heating in an autoclave for 10 min at 120 °C, the sections were incubated with 2% normal bovine serum to block any non-specific reactions. Then, the sections were incubated with an antihuman monoclonal antibody against MUC-1 (dilution 1:100, Novocastra®, Newcastle upon Tyne, England) or ALK-1 (dilution 1:70, Dako®, Denmark) or prolactin (dilution 1:100, Abcam®, USA) or Collagen type IV (dilution 1:150, Abcam®, USA) or Ubiquitin (dilution 1:250, Biomol GmbH®, Germany) or $CD138^{+}$ (dilution 1:40, Serotec®, Raleigh, USA) or pan-cytokeratin (dilution 1:200, Abcam®, USA) at 4 °C overnight followed by incubation with a biotinylated secondary antibody (anti-mouse IgG, 1:200; Vector® Laboratories,

Burlingame, CA) and Vectastain Elite ABC reagent (Vector® Laboratories) at room temperature for 30 min each. The sections were treated with 3,3′-diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin. Tissue specimens that were detected as having positive immunoreactivity were used as a positive control for staining including breast cancer cells for MUC-1, lymphoma for ALK-1, kidney tissue for prolactin, keratinocyte for Collagen type IV, pancreas for ubiquitin, bone marrow for $CD138^{+}$ and human hepatocyte for pan-cytokeratin. Then, positive cells from immunohistochemistry staining were counted and calculated as the percentage of positive cells per lymph node.

2.10 Bioinformatics analysis

The GSE datasets will be collected from GEO databases including GSE10797, GSE9014, GSE5364 and GSE10810. CU-DREAM-extra program (Connection Up- or Down- Regulation Expression Analysis of Microarrays Extra, website; http://pioneer.netserv.chula.ac.th/~achatcha/cu-dream) will be used to analyze the expression of gene-containing LINE-1 in all GSE datasets. Then, the group of upregulated genes containing LINE-1 will be observed molecular and phenotypic function. There are two bioinformatics tool will be used to evaluate the gene function including Genecard® (Lifemap Science, USA) and DAVID bioinformatics resource version 6.70 (National institute of allergy and infectious diseases, NIH). The upregulated genes with high significant *p*-value will be selected into candidate genes.

Moreover, the results from mass spectrometry will be analyzed with the program Mascot Search engine (Matrix Science® LLC., London, UK) which is one part of bioinformatics search. This program will be analyzed by using protein sequence and charge ratio from mass spectrometry compared to protein database.

2.11 Statistical analysis

Statistical analyses will be determined by using SPSS (Statistical Package for the Social Sciences) software for Windows version 17.0.1 (SPSS Inc., Chicago, IL). The data will be expressed in mean ± SD and independent sample *t*-test will be performed to calculate significant differences among metastasis lymph nodes, microdissected tissues and co-culture cells. The LINE-1 methylation percentage will be compared between control and co-culture group. Fisher exact test were performed to calcaulate significant different in the percentage of MUC-1 positive cells. All *p*-values will be obtained by two sided and *p*-values less than 0.05 will be observed to statistical significant.

3. Ethical approval

This research project needs to observe and use sample specimen from breast cancer patients. The benefit will be helped scientist to understand breast carcinogenesis and useful for finding new therapeutic way. Before sample collection process performed, all participants will be signed inform consent and given a self-administered questionnaire to collect medical history and demographic information.

After completing the questionnaire then the clinical examination will be recorded and confirmed pathology by physician. Next, tissue samples will be obtained under a protocol approved by Ethical Research Committee, Faculty of medicine, Chulalongkorn University (IRB No. 409/57).

CHAPTER V

RESULTS

1. Bioinformatics analysis

First, The GEO datasets, which compared between breast cancer cells and stromal associated cancer cells, were extracted from NCBI database including GSE10797, GSE9014, GSE5364 and GSE10810. CU-DREAM-extra is a program to categorize genes, containing intragenic LINE-1 that gives different expression. The expression informations of gene containing LINE-1 in each GSE were shown in table 1. The analysis showed that genes, containing intragenic LINE-1 in cancer cells were repressed. Surprisingly, in contrast to cancer cells, genes, containing LINE-1 of adjacent stromal cells were up regulated. Some research proved that intragenic LINE-1s in cancer cells repress gene expression because of LINE-1 hypomethylation (Yen RW et al. 1992, Xie S et al. 1999). This data supported the hypothesis that intragenic LINE-1s of breast cancer adjacent cells were hypermethylated.

GEO dataset	Case	Control	Down reglated		Upregulated	
			Odd ratio	*P* -value	Odd ratio	*P* -value
GSE10797 (93)	Stromal cells from invasive breast cancer	Stromal cells from healthy normal	0.52	8.36E-03	1.68	3.08E-08
GSE9014 (94)	Invasive breast cancer, stromal cells	Normal adjacent stromal cells	0.72	1.52E-06	1.39	1.34E-08
GSE5364 (95)	Breast cancer sample	Adjacent normal breast tissue	1.51	1.11E-07	0.60	6.24E-09
GSE10810 (96)	Invasive breast cancer	Adjacent normal breast tissue	1.64	8.09E-10	0.66	3.17E-06

Table 1. The results from CU-DREAM-extra program. Genes containing intragenic LINE-1 expression were significantly up-regulated in stromal cells from invasive breast cancer and significantly down-regulated in breast cancer cells.

The results show genes containing LINE-1s of breast cancer cells were down-regulated (GSE5364; P values = 6.24×10^{-9}, OR = 1.51, Upper 95% CI = 1.40, Lower 95% CI = 1.85 and GSE10810; P values = 3.17×10^{-6}, OR = 1.64, Upper 95% CI = 1.43, Lower 95% CI = 1.98). However, in contrast, genes containing LINE-1s of breast cancer associated stromal cells were up-regulated (GSE10797; P values = 3.08×10^{-8}, OR = 1.68, Upper 95% CI = 1.80, Lower 95% CI = 2.13 and GSE9014; P values = 1.34×10^{-8}, OR = 1.39, Upper 95% CI = 1.19, Lower 95% CI = 1.51) (Figure 9a).All 709 up-regulated genes containing LINE-1 from the GSE9014 breast cancer stromal cell microarray library were analyzed and classified (Figure 9b). Interestingly, a number of genes were associated with angiogenesis, inflammation and immunological processes

A

GSE9014	Up (0.05)	Not up			
L1	709	488	1,197	P-value	1.34E-08
No L1	9,524	8,054	17,578	Odd Ratio	1.39
	10,233	8,542	18,775	Upper 95% CI	1.19
				Lower 95% CI	1.51

B

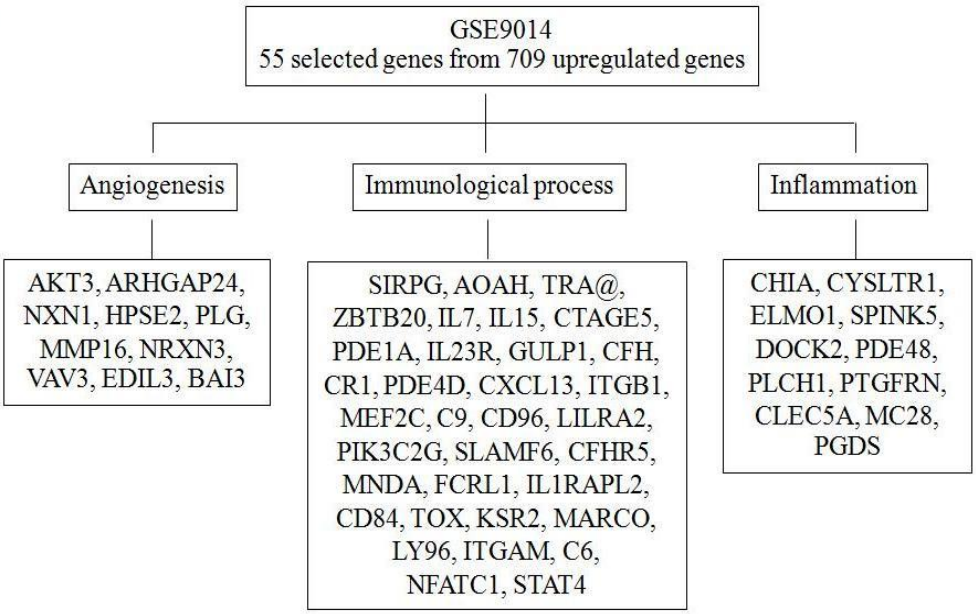


Figure 9. The analysis results from the CU-DREAM-extra program. a) Intersection analysis results from GSE 9014 which show 709 up-regulated genes from 1,197 genes containing LINE-1. b) All the up-regulated genes containing LINE-1 were classified into 3 major groups including angiogenesis, inflammation and immunological processes.

2. LINE-1 methylation in lymph node

Because up-regulated genes contain LINE-1s in breast-cancer-associated stromal cells, we hypothesized that LINE-1s in breast cancer stromal cells are hypermethylated. We evaluated the LINE-1 methylation status in breast cancer lymph nodes. Three types of LN were evaluated (Figure 10a-c). Group I or No metastasis were LNs from breast cancer patients without LN metastasis. Group II or Metastasis LNs- were LNs from breast cancer patients with LN metastasis, but the LN was without an identifiable breast cancer cell under the microscope. Group III or Metastasis LNs+ were LNs containing breast cancer cells. The LINE-1 hypermethylation was observed in group III or Metastasis LNs+ (P values No metastasis vs Metastasis LNs+ = 0.0020, Metastasis LNs- vs Metastasis LNs+ = 0.0080).

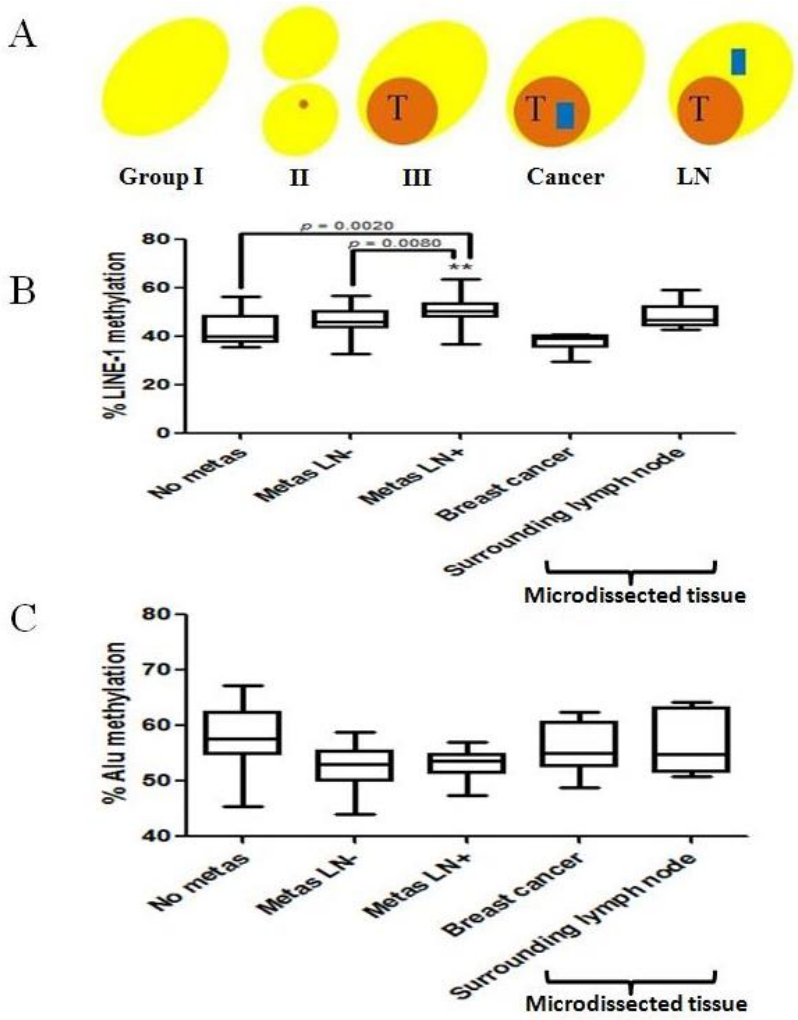


Figure 10. The results of LINE-1 methylation in lymph nodes. a) Demonstrates figures for each group. b) The percentage of LINE-1 methylation in LNs from breast cancer patients showed increasing levels from no metas (N=12), metas LNs- (N=26) and metas LNs+ (N=26). Moreover, LINE-1 hypermethylation still occurred in microdissected samples when comparing breast cancer (N=6) and surrounding LNs (N=6). c) The percentage of Alu methylation in the same group of LNs, which were

not significantly different. The levels of LINE-1 and Alu methylation are shown as box plots, with the boxes representing the interquartile ranges (25^{th} to 75^{th} percentile) and the median lines representing the 50^{th} percentile. The whiskers represent the minimum and the maximum values (**p<0.01).

3. LINE-1 mythylation in PBMCs and fibroblast cell co-culture

First, direct co-culture was performed to observe the LINE-1 methylation change. Because there are an immune response between peripheral blood mono nuclear cells (PBMCs) and cancer cells, PBMCs were clumping around cancer cells within 4 hours (Figure 11b). LINE-1 hypomethylation occur in PBMCs co-culture with MCF-7, WSU-HN17 and HeLa (Figure 11a). This evidence occurs from cancer DNA contamination in the sample. Then, we applied an indirect co-culture with transwell plate technique into this study (Figure 12).

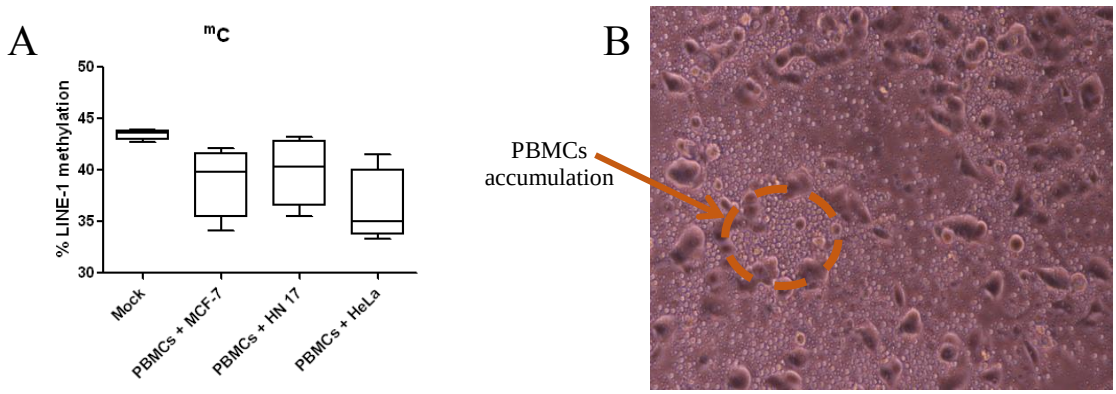


Figure 11. LINE-1 methylation in direct co-culture (4 hours). A) LINE-1 hypomethylation show in PBMCs sample after co-culture with 3 cancer cell lines. B) In the MCF-7 and PBMCs direct co-culture, PBMCs were immunized and accumulated around cancer cells which cause cancer cell death.

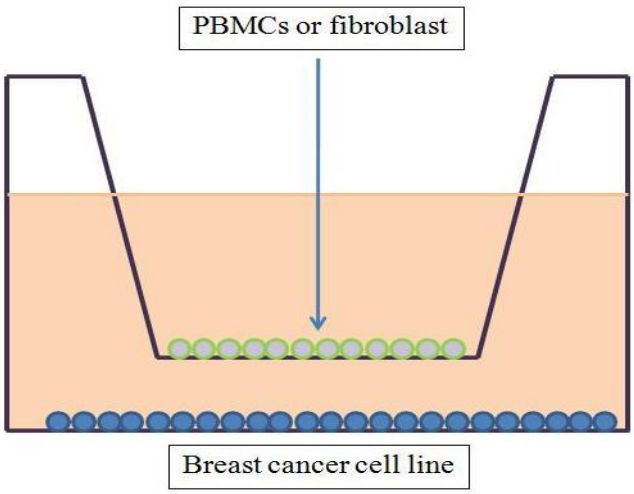


Figure 12. Schematic of indirect co-culture composed of the lower layer, which was breast cancer cell lines, and the upper layer, which was PBMCs or fibroblasts.

To test if breast cancer cells send paracrine signalling to methylated LINE-1s, we set up indirect co-cultures among three cancer cell lines including breast cancer (MCF-7, MDA-MB-231 and T47D), head and neck squamous cell carcinoma (WSU-HN17) and cervical cancer (Hela) with PBMCs. Indirect co-cultures were performed in 4, 8, 16 and 24 hours (Figure 13 a-d). LINE-1 hypermethylation occur in 4 and 8 hours of indirect co-culture. Then, we focus on 4 and 8 hours of indirect co-culture

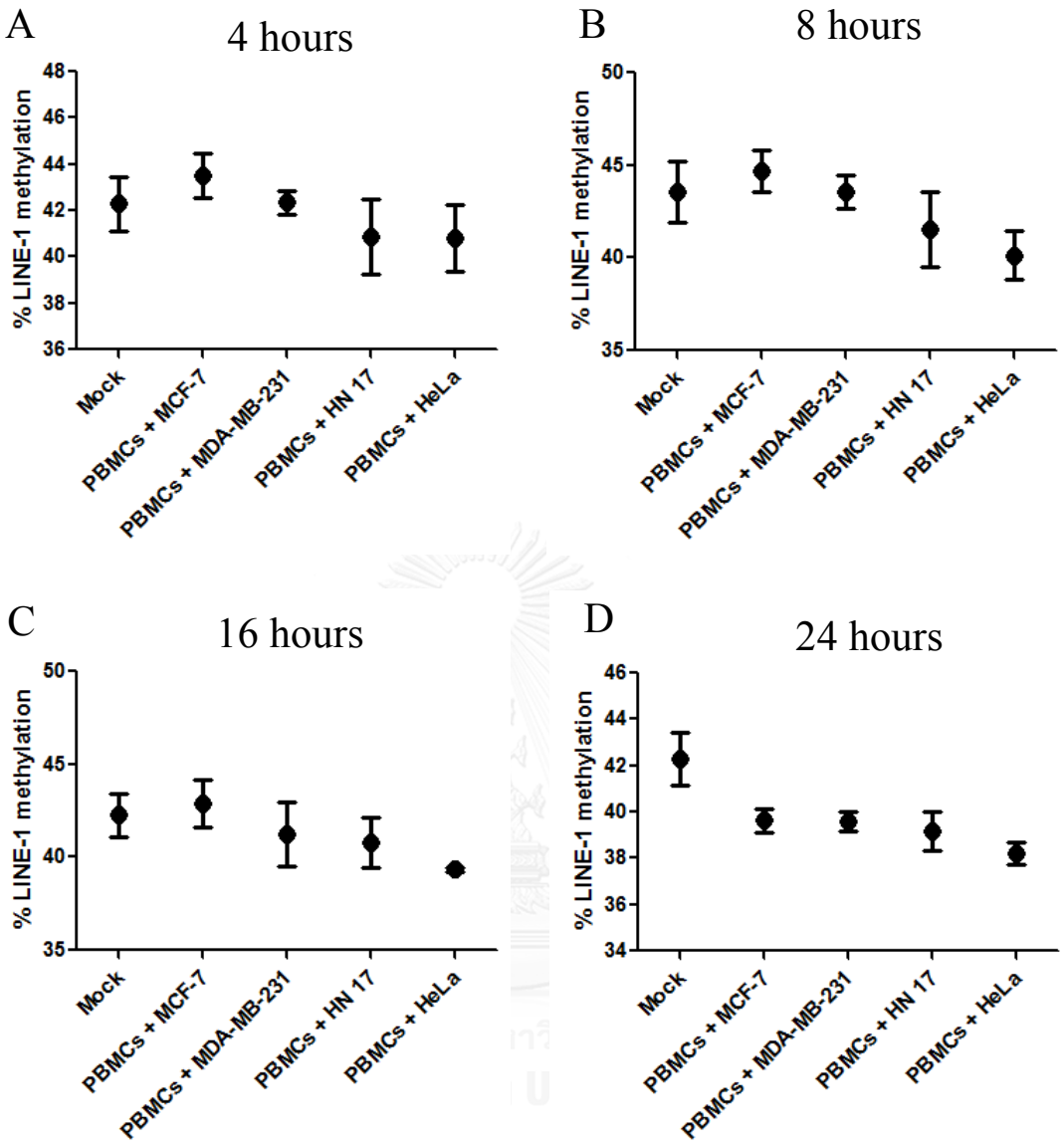


Figure 13. Percentage of LINE-1 methylation in various time of indirect co-culture.

LINE-1 hypermethylation were observed in 4 and 8 hours.

When the sample size increase, the results showed significant LINE-1 hypermethylation in female PBMCs (P values =0.0044; Figure 14). This

hypermethylation evidence was also found in the indirect co-culture between MCF-7 and female primary fibroblasts (P values =0.0288; Figure 15b). No LINE-1 methylation change was observed in co-cultured male PBMCs and HeLa cells. We then evaluated whether the epigenetic changes were specific to LINE-1 by testing Alu methylation status. Alu methylation in female PBMCs showed no significant changes (Figure 15a).

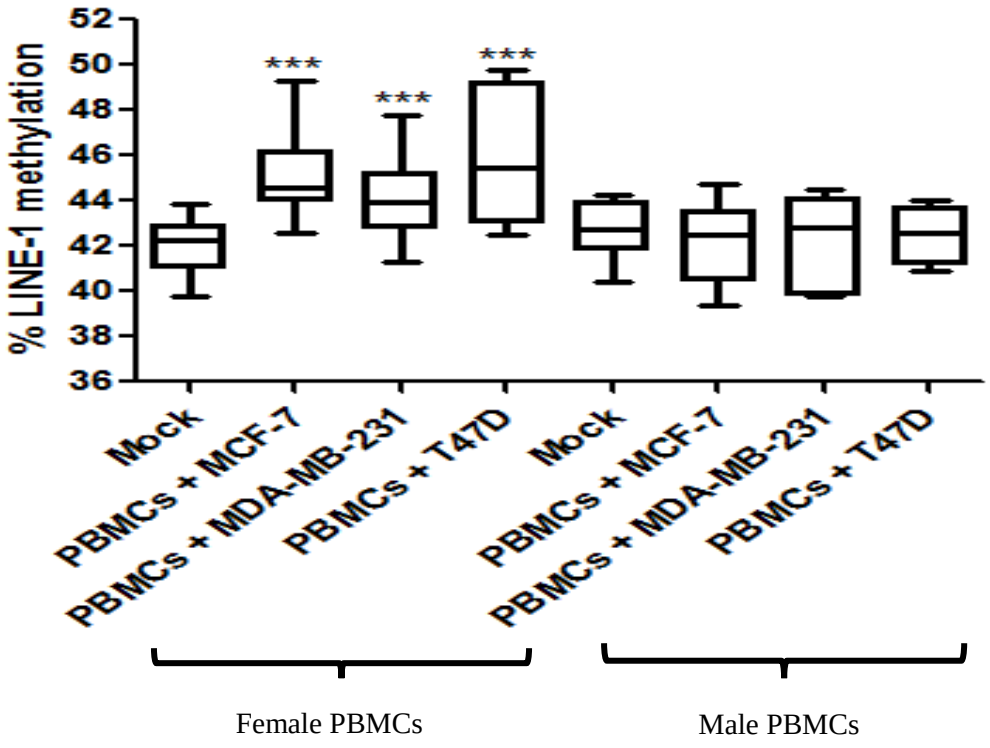


Figure 14. The results of LINE-1 methylation from 4 hours co-cultured PBMCs (N=21) found LINE-1 hypermethylation only in female PBMCs co-cultured with 3 types of breast cancer cell lines (MCF-7, MDA-MB-231 and T47D).

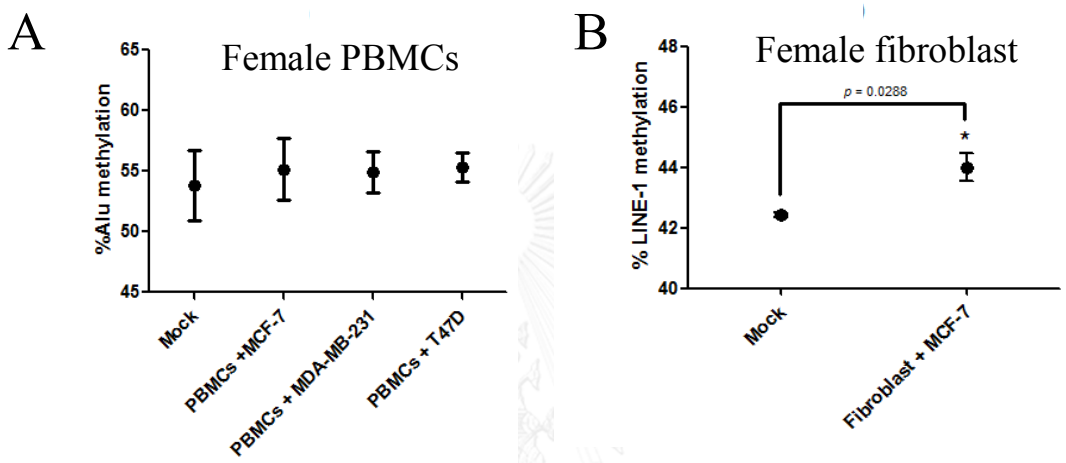


Figure 15. The percentage of LINE-1 and Alu methylation including A)Alu methylation of co-cultured female PBMCs (N=3) with the breast cancer cell line, which was not significant. B) LINE-1 hypermethylation also occurred in fibroblasts (N=3) when co-cultured with the breast cancer cell line.

LINE-1 and Alu methylation data

Sample		N	LINE-1 methylation (%± S.D.)				Alu methylation (%± S.D.)			
Breast cancer, lymph node										
	Group 1……No metastasis……	12	42.65±6.63				58.24±6.03			
Match	Group 2……Metastasis LN-……..	26	46.36±5.18				52.72±3.63			
	Group 3……Metastasis LN+……..	26	50.55±5.08				53.12±2.50			
Match	Microdissected breast cancer	6	37.81±4.19				55.91±4.81			
	Microdissected surrounding lymph node	6	48.55±5.74				56.62±5.66			
Cell Co-culture		N	LINE-1 methylation (%± S.D.)				Alu methylation (%± S.D.)			
			4hr	8hr	16hr	24hr	4hr	8hr	16hr	24hr
Female PBMCs control		21	42.02±1.23	41.69±3.44	42.57±1.26*	42.63±1.31*	55.15±4.45*	55.33±2.78*	54.01±1.87*	54.29±3.38*
Female PBMC:MCF-7		21	45.16±1.98	45.74±4.13	43.41±2.72*	40.12±0.99*	54.92±3.03*	54.99±3.33*	53.02±3.17*	54.84±3.73*
Female PBMC:MDA-MB-231		12	44.12±1.96	44.43±1.99	41.78±3.93*	39.72±0.50*	55.34±2.09*	55.39±3.12*	53.85±4.11*	54.11±2.74*
Female PBMC:T47D		9	46.23±3.02	44.34±0.98	40.55±1.41*	39.63±1.15*	53.83±4.96*	55.07±3.98*	53.41±3.44*	53.07±2.88*
Male PBMCs control		7	42.68±1.31	42.10±1.74	42.14±2.11*	40.51±095*	N/A	N/A	N/A	N/A
Male PBMC:MCF-7		7	42.27±1.84	42.39±1.44	42.33±198*	39.88±1.11*	N/A	N/A	N/A	N/A
Male PBMC:MDA-MB-231		7	42.16±1.93	42.39±1.66	42.65±1.12*	39.12±088*	N/A	N/A	N/A	N/A
Male PBMC:T47D		4	42.49±1.28	43.02±0.42	43.01±1.02*	39.66±1.31*	N/A	N/A	N/A	N/A
Fibroblast Control		3	42.43±0.14	42.14±0.85	N/A	N/A	N/A	N/A	N/A	N/A
Fibroblast:MCF-7		3	44.00±0.80	43.67±1.02	N/A	N/A	N/A	N/A	N/A	N/A
HeLa:MCF-7		3	25.29±2.34	N/A	N/A	N/A	N/A	N/A	N/A	N/A
HelLa control		3	27.33±1.66	N/A	N/A	N/A	N/A	N/A	N/A	N/A

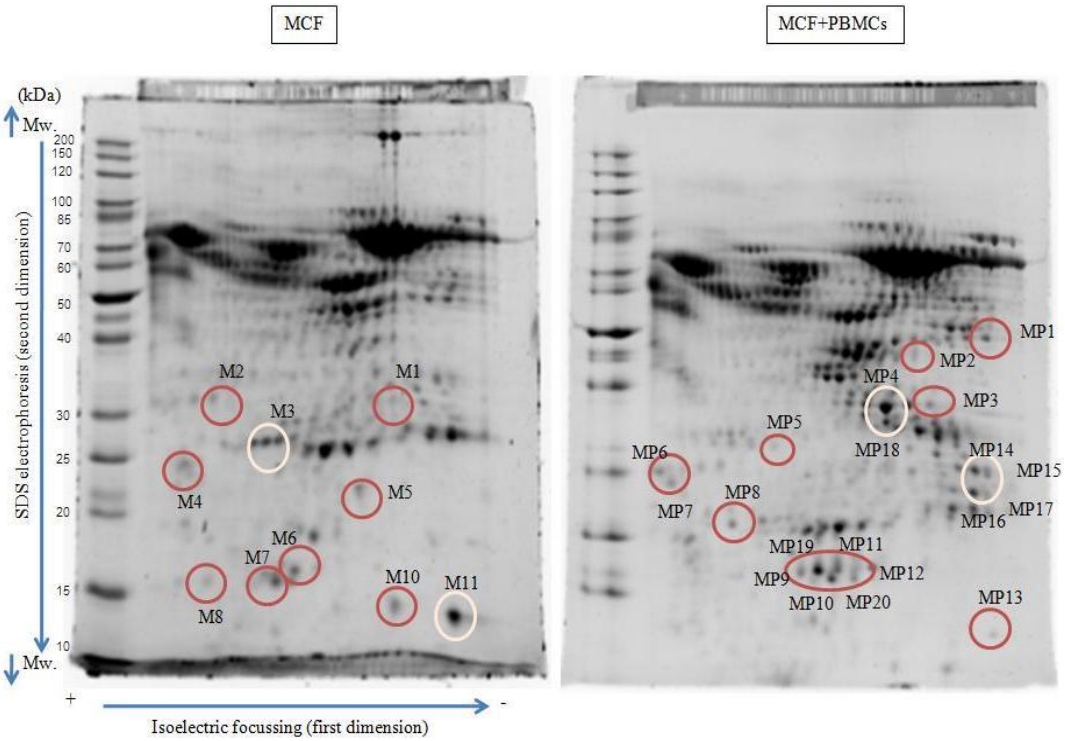
*N/A = not determine

Table 2. LINE-1 and Alu methylation data in lymph nodes and co-culture cells represent in average ±SD.

4. 2D gel electrophoresis and Mass spectrometry

The releasing proteins in co-culture supernatant were analyzed by using 2D-gel electrophoresis. The proteins from co-culture supernatant were compared between MCF-7 group and MCF-7+PBMCs co-culture group (Figure 16). The samples were performed in triplicate and combine to analyze with 2D-gel analysis program.

All protein spot which have significantly change expression level were extracted from SDS-PAGE and prepared for Mass spectrometry.



Protein spot	MCF-7 (supernatant)
M1	Albumin fragment
M2	F box, LRR repeat protein
M3	Actin filament
M4	Keratin typ II, cytoskeleton
M5	Keratin typ I, cytoskeleton
M6	Albumin fragment
M7	Transmembrane protein like 7
M8	Keratin typ II, cytoskeleton
M9	Actin filament
M10	Keratin type I, cytoskeleton
M11	Dynein heavy chain I

Protein spot	MCF-7 + PBMCs (supernatant)
MP1	Keratin typ II, cytoskeleton
MP2	peroxisome biogenesis factor I
MP3	peroxisome biogenesis factor I
MP4	Golgi apparatus
MP5	Na+,K+ transmemebrane
MP6	Albumin fragment
MP7	Albumin fragment
MP8	Albumin fragment
MP9	Actin filament
MP10	Keratin typ I, cytoskeleton
MP11	Keratin typ II, cytoskeleton
MP12	Na+,K+ transmemebrane
MP13	Keratin typ II, cytoskeleton
MP14	Keratin typ II, cytoskeleton
MP15	Fibroblast growth factor receptor II
MP16	Actin filament
MP17	Actin filament
MP18	Trypsin
MP19	Statuim domain containing protein
MP20	Albumin fragment

Figure 16. Shows 2D-gel electrophoresis and protein spot that have significantly changed in supernatant co-culture medium. Most of protein spots are structural proteins.

For mass spectrometry analysis, most of significant different proteins are structure protein including actin filament, keratin, and membrane bound protein.

5. Immunohistochemistry staining in lymph node with micrometastic cancer

Due to paracrine action, secretion of one cancer cell should regulate multiple stromal cells. Therefore, upregulated proteins in breast cancer stromal cells should be highly sensitive tumour markers. We selected candidate genes containing LINE-1s for immunohistochemistry staining including ALK-1, MUC-1, prolactin, collagen type IV and ubiquitin. However, only MUC-1 staining demonstrated clear potential in micrometastatic detection (Figure 17). Figure 5.9 shows the results for No metastasis, Metastasis LNs- and Metastasis LNs+. Both No metastasis and Metastasis LNs- groups showed no pan-cytokeratin stained cells, suggesting limited presentation of breast

cancer cells. CD138+ was used as a marker for plasma cells. Cells that were MUC-1 positive and CD138+ positive were MUC-1 positive plasma cells. Whereas MUC-1 positive plasma cells were found in Metastasis LNs- and Metastasis LNs+, very limited numbers of positive cells were found in the No metastasis group.

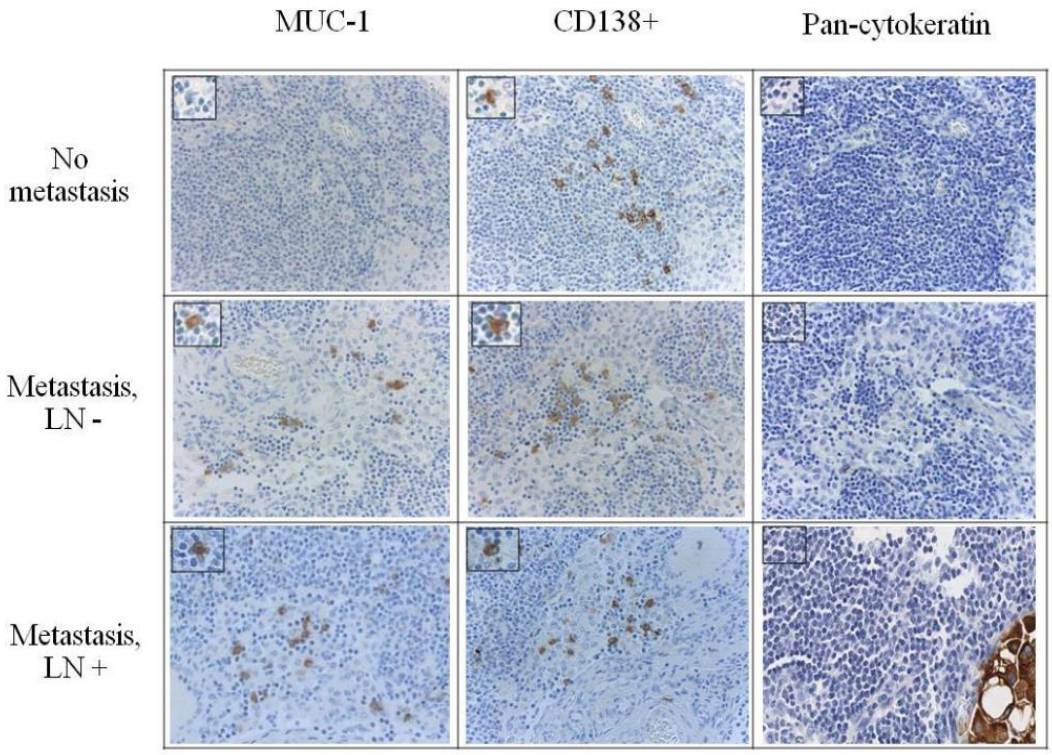


Figure 17. Immunohistochemistry of No metastasis, Metastasis LNs- and Metastasis LNs+. MUC-1 positive cells were found in metastasis LNs- and Metastasis LNs+. CD138+ is a maker for plasma cells, which were found in all groups, and some of them were MUC-1 positive cells. Pan-cytokeratin is a marker for breast cancer, which

was found in Meats LNs+ and negative in both No metastasis and Metastasis LNs- groups.

When comparing the 3 groups; i.e., No metastasis, Metastasis LNs- and Metastasis LNs+, the percentages of MUC-1 positive plasma cells were 5.74+/-3.01, 80.74+/-6.15 and 77.41+/-9.56 (average +/-SD), respectively (Table 2). (P values No metastasis vs Metastasis LNs- = 3.15E-25, No Metastasis vs Metastasis LNs+ = 3.92E-24) (Figure 19).

	CD138+	CD138+, MUC-1+	% of MUC-1+	Average±SD	P value*
No metastasis (5 nodes)	43	2	4.65	5.74±3.01	-
	56	6	10.71		
	35	2	5.71		
	40	2	5.00		
	38	1	2.63		
Metastasis LN- (5 nodes)	54	41	75.93	80.74±6.15	3.15E-25
	48	40	83.33		
	71	63	88.73		
	60	44	73.33		
	51	42	82.35		
Metastasis LN+ (5 nodes)	45	31	68.89	77.41±9.56	3.92E-24
	66	49	74.24		
	48	33	68.75		
	39	34	87.18		
	75	66	88.00		

Table 3. Positive cells from immunohistochemistry staining were counted and calculated as the percentage of positive cells per lymph node. MUC-1 positive plasma cells had high potential to discriminate between the No metastasis and Metastasis LN- groups. P values* were calculated by Fisher exact test.

No metastasis Vs Metastasis LNs-

	Metastasis LNs-	No metastasis	
MUC-1+	230	13	243
CD138+	284	212	496
	514	225	739

P value = 3.15E-25

No metastasis Vs Metastasis

	Metastasis LN+	No metastasis	
MUC-1+	213	13	226
CD138+	273	212	485
	486	225	711

P value = 3.92E-24

Figure 18. Show the 2x2 table for Fisher exact test statistic analysis to calculated significant different in the MUC-1 positive cells including No metastasis vs Metastasis LNs- and No metastasis vs Metastasis LNs+.

CHAPTER VI

DISCUSSION

The experiments in this study demonstrated that breast cancer cells release paracrine signaling to increase LINE-1 methylation and upregulation of genes containing LINE-1s of breast cancer stromal cells. This paracrine signal can present in different metastatic potential breast cancers. Therefore, breast cancer commonly releases the paracrine and this paracrine action may not involve in metastatic process. Previously reports founded that cancer decreased LINE-1 methylation levels by generalized mechanisms (Baba Y et al. 2014, Kaer K and Speek M 2013) causing genome wide hypomethylation of LINE-1s. This decrement in LINE-1 methylation represses gene containing LINE-1 expression (Aporntewan C et al 2011). Therefore, in stromal cells, it is possible that breast cancer cells release paracrine to genome widely increase LINE-1 methylation levels of stromal cells and consequently genes containing LINE-1s were regulated in an opposite direction to cancer. There are many

molecules released from breast cancer cells such as CXCL2, TGF and MMPs (Pedraza V et al. 2010, Finak G et al. 2008). These chemokines can bind to receptors on the epithelial cells and enhance cancer cell proliferation and invasion. However, water-soluble molecules responsible for paracrine signalling in promoting LINE-1 hypermethylation in breast cancer stromal cells are still unknown. We found that the roles of some of the up-regulated genes containing LINE-1s functions were inflammation, angiogenesis and immunological processes. Therefore, we hypothesized that this epigenomic regulation process may promote tumour phenotypes.

An interesting finding of this study was LINE-1 hypermethylation induction in co-culture from breast cancer occurred exclusively in normal female cells but not in normal male cells and female cancer cells. The mechanisms underlining this finding are unknown. A few possible explanations worth considering are first, there are two functional binding sites (cis-element) for transcription factors of the SRY family, namely, SOX factors, were found in the LINE-1 promoter whereas *SRY* gene is male

specific (Casey T et al. 2009). In HeLa cell lines, hypomethylated LINE-1s possessed higher transcriptional activity (Aporntewan C et al. 2011, Allinen M et al. 2004). The LINE-1 transcription initiation complexes in HeLa cells might prevent paracrine signalling from breast cancer cells and inhibit LINE-1 hypermethylation induction.

There are few markers that can predict cancer metastasis (Moses H et al. 1990, Thierry T et al. 2000). Some metastatic cancer detections were observed by PET or CT scans using radioactive materials (Lee HS et al. 2015). Serum tumor markers (CA15-9) and a molecular technique (RT-PCR) were also applied to detect micrometastatic breast cancer, but both of them required the presence of cancer cells in the breast tissue (Rosen PP et al. 1989, Hellman S 1994). Nevertheless, there is no highly effective approach for detecting micrometastasis in breast cancer. Here, paracrine action of cancer cell should regulate stromal cells in amplification fashion. Because a limited number of cancer cells can promote MUC-1 protein in a large number of plasma cells, upregulated proteins in stromal cells by cancer paracrine signalling are promising tumour markers in identifying cancer LN metastasis.

Interestingly, the high proportion of plasma cells in metas LNs- expressed MUC-1 protein at similar levels to plasma cells in metas LNs+. The precise mechanisms for a high proportion of metas LNs- expressing MUC-1 proteins requires further study. However, MUC-1 protein was not positive in all stromal cells but present exclusively in plasma cells. Therefore, MUC-1 protein is promoted by not only LINE-1 hypermethylation but also some unknown tissue specific factors in plasma cells.

In summary, breast cancer has the potential to regulate LINE-1 hypermethylation in cancer stromal cells, which resulted in the up-regulation of genes containing LINE-1s. Without detectable cancer cells in an early stage of metastasis in LNs, MUC-1, one up-regulated gene, was still identified. MUC-1 can be developed as a metastasis marker that could be useful for breast cancer therapy.

CHAPTER VII

PUBLICATIONS

1. Puttipanyalears C, Subbalekha K, Mutirangura A, Kitkumthorn N. Alu hypomethylation in smoke-exposed epithelia and oral squamous carcinoma. *Asian pacific journal of cancer prevention* 2013(14): 5495-5501.
2. Tiwawech D, Srisuttee R, Rattanatanyong P, Puttipanyalears C, Kitkumthorn N, Mutirangura A. Alu methylation in serum from patients with nasopharyngeal carcinoma. *Asian pacific journal of cancer prevention* 2014(15): 9797-9800.
3. Puttipanyalears C, Kitkumthorn N, Buranapraditkun S, Keelawat S, Mutirangura A. Breast cancer up-regulating genes in stromal cells by LINE-1 hypermethylation and micrometastasis detection. *Epigenomics*. (accepted, in press)

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APPENDIX

1. Reagents

DMEM (GIBCO®, USA)

Fetal Bovine Serum (Bio Whittaker, Maryland, USA)

DMSO (Sigma®, UK)

Ficoll-HypaqueIsoprep (Amersham Pharmacia®, Uppsala, Sweden)

Phosphate Buffer Saline (Sigma®, UK)

Trypan Blue (Sigma®, UK)

2. Antibodies

MUC-1 (Novocastra®, Newcastle upon Tyne, UK)

ALK-1 (Dako®, Denmark)

Prolactin (Abcam®, USA)

Collagen type IV (Abcam®, USA)

Ubiquitin (Biomol GmbH®, Germany)

$CD138^{+}$ (Serotec®, Raleigh, USA)

Pan-cytokeratin (Abcam®, USA)

Biotinylated secondary antibody (anti-mouse IgG, Vector® Laboratories, Burlingame, CA)

Vectastain Elite ABC reagent (Vector® Laboratories)

3. Materials

24-wells flat plate (Costar®, USA)

Transwells permeable support (Costar®, USA)

EDTA tube (Becton-Dickinson®, USA)

Automatic pipette (Gilson®, France)

Conical tube 15 and 50 ml (Falcon®, USA)

Counting chamber

Cryotube (Sarstedt, Germany)

Disposal Serological pipette 2, 5, 10 and 25 ml (Costar®, USA)

Flask 50 and 250 cm^3 (Nunc®, Denmark)

Glove

Micro centrifuge tube (Eppendorf®, USA)

Pipette boy and pipette tip

4. Instruments

Centrifuge

Biological Safety Cabinet type II

CO_2 incubator (Forma Scientific®, USA)

Freezer -80°C

PCR machine (Thermo Scientific®, USA)

Refrigerator

Water bath (Shel lab, USA)

5. Programs

DAVID bioinformatics database (https://david.ncifcrf.gov/)

CU-DREAM-extra (http://pioneer.netserv.chula.ac.th/~achatcha/CU-DREAM/)

Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/)

Genecard® (http://www.genecards.org/, Lifemap Science, USA)

Mascot Search engine (http://www.matrixscience.com/search_form_select.html, Matrix Science® LLC., London, UK)

Oligoanalyzer 3.1 (http://sg.idtdna.com/calc/analyzer)

6. Certification of ethical approval

COA No. 785/2014

IRB No. 409/57

คณะกรรมการจริยธรรมการวิจัยในคน

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

1873 ถ.พระราม 4 เขตปทุมวัน กรุงเทพฯ 10330 โทร. 0-2256-4493 ต่อ 14, 15

เอกสารรับรองโครงการวิจัย

คณะกรรมการจริยธรรมการวิจัยในคน คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากลได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ : การศึกษาการเกิด LINE-1 ไฮเปอร์เมทิลเลชั่นในเนื้อเยื่อเซลล์สโตรมาของมะเร็งเต้านม

เลขที่โครงการวิจัย : -

ผู้วิจัยหลัก : นายเจริญชัย พุฒิปัญญาเลิศ

สังกัดหน่วยงาน : สาขาชีวเวชศาสตร์ บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

วิธีทบทวน : คณะกรรมการเต็มชุด

รายงานความก้าวหน้า : ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือส่งรายงานฉบับสมบูรณ์หากดำเนินโครงการเสร็จสิ้นก่อน 1 ปี

เอกสารรับรอง :

1. โครงร่างการวิจัย Version 1.0 Date 3 Sep 2014
2. โครงการวิจัยฉบับย่อ Version 1.1 Date 20 Oct 2014
3. เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย Version 1.2 Dated 4 Nov 2014
4. เอกสารแสดงความยินยอมเข้าร่วมในโครงการวิจัย Version 1.0 Date 2 Sep 2014
5. CASE REPORT FORM (For primary fibroblast) Version 1.1 Dated 20 Oct 2014
6. CASE REPORT FORM (For formalin fixative paraffin embedded tissue: FFPE) Version 1.1 Dated 20 Oct 2014

7. Research article

1. Puttipanyalears C, Subbalekha K, Mutirangura A, Kitkumthorn N. Alu hypomethylation in smoke-exposed epithelia and oral squamous carcinoma. *Asian pacific journal of cancer prevention* 2013(14): 5495-5501.

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Alu Hypomethylation in Smoke-exposed Epithelia and Oral Cancer

RESEARCH ARTICLE

Alu Hypomethylation in Smoke-Exposed Epithelia and Oral Squamous Carcinoma

Charoenchai Puttipanyalears[1], Keskanya Subbalekha[2*], Apiwat Mutirangura[1], Nakarin Kitkumthorn[3*]

Abstract

Background: Alu elements are one of the most common repetitive sequences that now constitute more than 10% of the human genome and potential targets for epigenetic alterations. Correspondingly, methylation of these elements can result in a genome-wide event that may have an impact in cancer. However, studies investigating the genome-wide status of Alu methylation in cancer remain limited. Objectives: Oral squamous cell carcinoma (OSCC) presents with high incidence in South-East Asia and thus the aim of this study was to evaluate the Alu methylation status in OSCCs and explore with the possibility of using this information for diagnostic screening. We evaluated Alu methylation status in a) normal oral mucosa compared to OSCC; b) peripheral blood mononuclear cells (PBMCs) of normal controls comparing to oral cancer patients; c) among oral epithelium of normal controls, smokers and oral cancer patients. Materials and Methods: Alu methylation was detected by combined bisulfite restriction analysis (COBRA) at 2 CpG sites. The amplified products were classified into three patterns; hypermethylation (%mCmC), partial methylation (%mCmC+%mCmC), and hypomethylation (%mCmC). Results: The results demonstrate that the %mCmC value is suitable for differentiating normal and cancer in oral tissues (p=0.0002), but is not significantly observe in PBMCs. In addition, a stepwise decrease in this value was observed in the oral epithelium from normal, light smoker, heavy smoker, low stage and high stage OSCC (p=0.0003). Furthermore, receiver operating characteristic (ROC) curve analyses demonstrated the potential of combined %mC or %mCmC values as markers for oral cancer detection with sensitivity and specificity of 86.7% and 56.7%, respectively. Conclusions: Alu hypomethylation is likely to be associated with multistep oral carcinogenesis, and might be developed as a screening tool for oral cancer detection.

Keywords: Alu element - hypomethylation - oral cancer - smoke-exposed epithelia

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Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent malignant neoplasm of the oral cavity which represents approximately 3% of all malignancies affecting humans (Yasusei et al., 2004; Song et al., 2011). OSCC accounts for more than five hundred thousand newly diagnosed cases every year worldwide (Massimo et al., 1995; Massimo et al., 2012). Generally, the highest incidence rates of oral cancer are found in South-East Asia, and Central and Eastern Europe for both males and females (Ahmedin et al., 2011). Because of its high mortality and low cure rate, OSCC represents a major global public health and socioeconomic problem (Massimo et al., 2012). At present, OSCC still lacks reliable diagnostic and prognostic molecular markers.

Cancers including OSCC are now known to develop and progress through a series of genetic and epigenetic alterations (Lingen et al., 2011; Saintigny et al., 2011). While on one hand genetic aberrations constitute irreversible changes (increased copy number) or mutations in the DNA coding sequences resulting in overexpression/ increased activity or inactivation, of key oncogenes and tumor suppressor genes, respectively (Lingen et al., 2011; Saintigny et al., 2011). On the other hand, promoter hypermethylation of tumor suppressor gene and genome-wide hypomethylation are the main features commonly associated epigenetics events (Chalitchagorn et al., 2004; Kitkumthorn and Mutirangura, 2011; Song et al., 2011). Of interest though, both types of alterations are now thought to occur in the transition of normal oral epithelium to premalignant lesion and to overt carcinomas (Diez-Perez et al., 2011; Lingen et al., 2011). Furthermore, with recent data suggesting that smoking related oral premalignant conditions might be associated with genome-wide hypomethylation (Demarini, 2004; Ian et al., 2007; Subbalekha et al., 2009) further investigation can likely afford the possibility of identifying novel molecular markers of OSCC.

Genome-wide hypomethylation can occur on

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interspersed repetitive sequences (IRS) and are dispersed throughout the genome. Long Interspersed Element 1 (LINE-1) and Alu accounting for the majority of IRS can likely represent key targets for genome-wide methylation that can lead to abnormal epigenetic events and consequently cancer. However, while the methylation status of LINE-1 is now reported to be widespread in many cancers, corresponding analysis of Alu methylation remains sparse and unclear (Debra et al., 2007; Moore et al., 2008; Hou et al., 2010; Wilhelm et al., 2010; Pobsook et al., 2011).

Fundamentally, Alu elements are Short Interspersed Elements (SINEs), widely dispersed with a notably high copy number (~500,000 copies) and accounting for ~10% of the human genome (Rubin et al., 1980). Thus, Alu elements can represent likely targets for genome-wide methylation (Xiang et al., 2010; Nakkuntod et al., 2011). In general though, Alu elements are normally methylated and transcriptionally inactive, but in certain stress-induced conditions, for example cellular heat shock, can lead to demethylation (hypomethylation) of CpG islands and activate Alu transcription (Peter et al., 2008). Although Alu transcripts are not protein encoding, nonetheless they can regulate associated gene expression, affecting processes such gene recombination, chromosome translocation, nucleosome formation and genome evolution that impacts genomic instability (Alexandros et al., 2008; Ana et al., 2009; Kristy et al., 2009).

While reduction in Alu methylation levels have been observed in several cancers (breast, colon, stomach, liver, lung, ovarian, urinary bladder, prostate gland) (Choi et al., 2007; 2009; Rodriguez et al., 2008; Watts et al., 2008; Yoo et al., 2008; Bollati et al., 2009; Daskalos et al., 2009; Lee et al., 2009; Park et al., 2009; Cho et al., 2010; Hehuang et al., 2010; Hou et al., 2010; Kwon et al., 2010; Xiang et al., 2010; Yoshida et al., 2011), those occurring in OSCC have not been reported. Here, we evaluated and compared levels and pattern of Alu methylation levels in formalin-fixed and paraffin-embedded (FFPE) specimens of normal and OSCC,and in peripheral blood mononuclear cells (PBMCs). Furthermore, we compared this emerging data with that of oral rinse samples from control patients and those with OSCC with known smoking habit that may provide new knowledge of Alu methylation in the pathogenesis of oral cancer.

Materials and Methods

Samples

In this study, samples were retrieved from 3 patient cohorts collected during January-December 2011. The demographics of these patient samples were collected from the available answer from questionnaires and records (Table 1). The patient cohorts that were used in this study include:

Cohort 1

FFPE archived cases (9 OSCC and 22 normal oral mucosa) were derived from the Faculty of Dentistry, Chulalongkorn University. From each retrieved case, 3-5 sections of approximately 5 μm-thickness, were prepared onto clean microscopic glass slides. One section underwent haematoxylin and eosin (H&E) staining, which than used for confirmatory histopathological evaluation by a pathologist (NK). All oral cancer samples consisting of at least 80% tumor cells were included for analysis.

Table 1. Demographic Data of All Sample Groups

Sample groups	N	Male:Female	Age (Average±SD)
Paraffin-embedded tissue			
Normal	22	6:16	47.59±13.87
Oral cancer	9	5:4	64.33±14.76
PBMCs			
Normal	31	14:17	48.28±11.78
Oral cancer	36	16:20	63.03±11.58
Oral rinse			
Normal (Non-smoker)	42	12:31	48.37±11.65
Light smoker	42	36:6	41.09±8.06
Heavy smoker	24	19:5	55.21±9.66
Oral cancer	43	21:22	60.40±12.95
Low stage (I+II)	14	5:9	63.79±11.68
High stage (III+IV)	29	16:13	58.76±13.41

Cohort 2

The PBMCs were derived from 36 patients with OSCC (36) and normal controls (31) and patients. The collection was carried out at three centers (Rajavithi Hospital, Bangkok; Buddhachinaraj Hospital, Bangkok; Faculty of Dentistry, Chulalongkorn University, Bangkok). Patients who had prior chemotherapy or radiotherapy were excluded. From each patient, six mL of blood was collected in heparinized tube, which than after underwent Ficoll-Hypaque centrifugation to separate the PBMCs which were used as source for DNA extraction.

Cohort 3

A total of 153 oral rinse samples were collected. Sample groups included normal, which was essentially sub-divided into non-smoker (42) and light to heavy smokers (66). The remaining group constituted samples from patients with histopathologically confirmed OSCC (43). Oral rinse from OSCC patients was collected prior to any treatment. All oral rises was done with 10 mL of sterile 0.9% normal saline solution and after gargling for 15 sec, solutions underwent centrifugation, and the cell pellet underwent DNA extraction within 24 hours of collection (see below). Total oral cancers were classified into 2 groups depended on patient pathological status including low and high stage oral cancer.

All participating subjects in cohorts 2 and 3 were given a self-administered questionnaire to collect medical history and information on smoking, prior to sample collection. Smoking consumption as number of years smoked, number of cigarettes smoked daily, age at which patient started smoking and the numbers of years since quitting, were carefully recorded. However, total smokers were divided into light and heavy smoker groups base on the average mean of pack/year value as previously described (Godtfredsen et al., 2004). After completing the questionnaire, patients underwent clinical examination by an oral surgeon (KS) and confirmation of patient histopathology by a pathologist (NK), prior to oral

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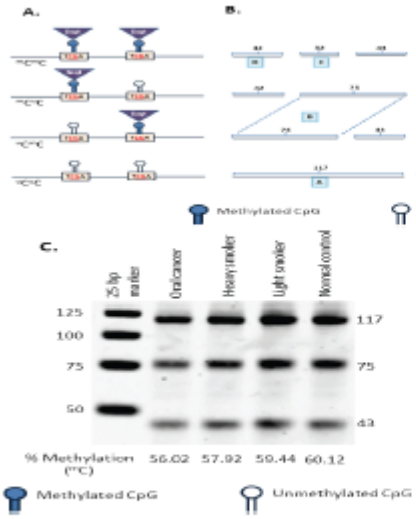

Figure 1. Alu Methylation Pattern in COBRA Alu Method. Amplified products digested with *TaqI* are represented in 3 patterns; hypermethylation (mCmC) 42/43 base pair (bp), partial methylation (mCC+mCmC) 74/75 bp and hypomethylation (CC) 117 bp. A) *TaqI* can digest only methylated CpG; B) different pattern of amplified product after *TaqI* digestion including A, B, C, and D which used calculate % methylation; and C) Gel electrophoresis showing 3 size of *TaqI* digested amplified product (117bp, 75 bp and 43 bp) in the indicated groups of oral rinse samples

rinse was collection. All samples were obtained under a protocol approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, Thailand (approval number: 7/2010) and signed informed consent.

DNA extraction

Cells from oral rinses and PBMCs were centrifuged at 4ºC at 2500 rpm for 15 min. Next, the supernatant from oral rinse samples was discarded and the resulting cell pellets resuspended in sterile PBS. For PBMCs, after standard Ficoll-Hypaque centrifugation, the layer corresponding to PBMCs was removed and placed in PBS for washing. Washed cells were centrifuged at 4ºC at 2,500 rpm for 15 min and the resulting pellet underwent DNA extraction with 1 mL of extraction buffer supplemented with 10% SDS and proteinase K 0.5 mg/mL. For FFPE tissue sections, these first underwent de-paraffination in xylene prior to lysing the tissue off the slide with 1 mL of extraction buffer and than transferring the extracts to a clean eppendorf tube. All the lysed extracts were first incubated at 50ºC for 72h, and then 0.5 mL of phenol-chloroform solution was added to each before mixing thoroughly. After, the mixtures were centrifuged at 4ºC at 14000g for 15 min and for each sample, the resulting clear upper phase was carefully removed and transferred to a clean eppendorff tube and the DNA precipitated by adding 10M ammonium acetate and absolute ethanol. The precipitated DNA was than centrifuged at 14000g, washed with 70% ethanol and after air drying, the pellet was re-suspended in distill water and used for COBRA Alu anaysis.

Combined bisulfite restriction analysis of Alu (COBRA Alu)

All DNA samples were converted to bisulfite DNA by using sodium bisulfate as previously described (Chalitchagorn et al., 2004). Briefly, a total of 1 μg of DNA of each sample first underwent denaturation in 0.22 M NaOH at 37ºC for 10 min and after the addition of 10 mM hydroquinone (Sigma-Aldrich, Singapore) and 3M sodium bisulfite (pH 5.0) samples underwent an additional incubation at 50ºC for 16-20h. After, DNA was recovered using the Wizard DNA Clean-Up Kit (Promega, Madison, WI) following the manufacturer's protocol. DNA samples were eluted from the columns by distilled water and precipitated with sodium acetate and 100% ethanol as indicated previously. Then, COBRA Alu was performed as previously described (Kitkumthorn et al., 2012; Sirivanichsunthorn et al., 2013). Briefly, the modified DNA pellets were resuspended in distilled water 1 μL of this was subject to 45 cycles of PCR using forward (GGCGCGGTGGTTTACGTTTGTAA) and reverse (TTA ATAAAAACGAAATTTCACCATATTAACCAAAC) primers with an annealing temperature of 53ºC. After, all amplified products were than digested with 2U of *TaqI* in *TaqI* buffer (MBI Fermentas, Glen Burnie, MD) overnight at 65ºC. The digested products were identified by 8% non-denaturing polyacrylamide gel electrophoresis and visualized with SYBR green.

Alu methylation analysis and calculation

The amplified products of DNA samples from the 3 patient cohorts were classified into 3 types depended on the methylation pattern of the 2 CpG dinucleotides. These are the hypermethylated (mCmC), partial methylated (mCmC and mCC) and hypomethylated loci (CC). After enzyme digestion, three product size (117bp, 75bp, 43bp) depending on the methylation status of the loci are generally detected as shown in Figure 1. Then, band intensities can be measured and quantitated by a phosphoimager using ImageQuant Software (Molecular Dynamics, GE Healthcare, Slough, UK). Next, the percentage of each methylation pattern can be calculated using the following; First, the intensity of each band is divided by bp of DNA length; %117/117=A, %75(74)/74.5=B, %42(43)/42.5=D and C (represent of mCmC)=D-B. After that, the percentage of methylation was calculated as following formula; mC=100×(2D)/(2A+2D), mCmC=100×C/(A+B+C), mCmC+mCmC=100×B/(A+B+C) and CC=100×A/(A+B+C).

DNA extracted from HeLa, DauDi and JurKat cell lines were used as positive controls in the experiments and for inter-assay adjustments.

Statistical analysis

Statistical analysis was performed using SPSS software for Windows version 17.0 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) and independent

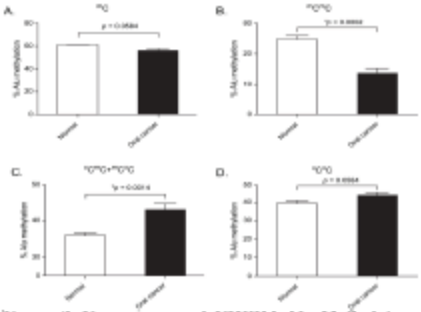

Figure 2. Comparisons of COBRA Alu Methylation Levels in FFPE Derived Normal Oral Mucosa and Oral Cancer Tissue

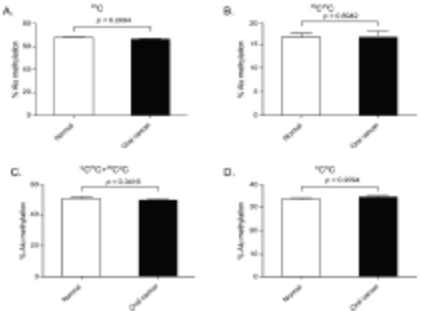

Figure 3. Comparison of Alu Methylation Levels in PBMCs from Normal and Oral Cancer Patients

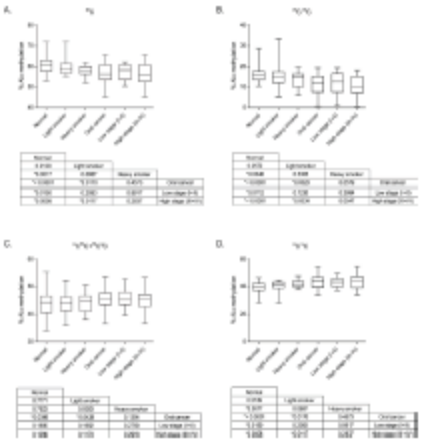

Figure 4. Comparisons of Alu Methylation Levels in Oral Rinses from Normal Controls, Light Smokers, Heavy Smokers, Total Oral Cancer, Low Stage Oral Cancer and High Stage Oral Cancer

sample t-test was performed to calculate significant differences in normal oral epithelium and oral cancer epithelium. All p values were obtained by two sided and values <0.05 were considered to be statistically significant. A receiver-operating characteristic (ROC) curve was used to test the feasibility of the COBRA Alu method of analysis of methylation status could distinguish between normal oral mucosa and oral cancer.

Results

Alu methylation status comparing normal oral mucosa and oral cancer tissues

In this analysis, we observed the frequency of each Alu methylation pattern compare between normal oral mucosa and oral cancer FPPE tissue samples. The results as shown in Table 1 and Figure 2 (A-D), indicate that overall methylation levels (%C) in oral cancer, was lower than normal oral mucosa (p=0.0584). Moreover, when same comparison was done using %mCmC values, this difference was significantly lower in oral cancer tissue (p<0.0002). In contrast, the partial methylation levels and the %CmC values in oral cancer tissues was higher than normal oral mucosa with p value=0.0014 and 0.0584. Overall, the data suggest that Alu hypomethylation were found in oral cancer tissue especially when observed in %mCmC value.

Comparisons of Alu methylation status between PBMCs from normal and oral cancer patients

As our previous analysis indicated that there was a clear difference in the methylation levels between normal oral mucosa and oral cancer tissue, we questioned if we would find a similar trend in PBMCs isolated from independent groups of normal and oral cancer patients. As shown when performing this analysis, Alu methylation levels and pattern were found to be only different between the two groups of PBMCs (p=0.2094; Figure 3). However, the decrease of methylation level was not found in the comparison of %mCmC and %CmC+mCmC.

Comparison of Alu methylation status in oral rinse samples from normal, light smoker, heavy smoker and oral cancer patients

Since our analysis using PBMCs showed only a marginal difference between normal and oral cancer patients, we sought to address if DNA from oral rinse may hold value. Cellular material from oral rinse from normal, smokers (light and heavy) and oral cancer patients was used to extract DNA and perform methylation analysis. The overall methylation level and the p value decreased respectively, from normal oral epithelium, light smoker (p=0.2129), heavy smoker (p=0.0017) and oral cancer (p<0.0001). Moreover, the %C level decreased from low stage (stage I and II) to high stage (stage III and IV) oral cancer (p=0.0150 and p=0.0008), respectively (Table 2). Conversely, hypomethylation pattern is observed to be highly elevated in patients with oral cancer and those who exposed smoking related carcinogens than in normal oral epithelium. However, no significant difference in the analysis of partial methylation pattern was observed. (Figure 4).

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Table 2. Percentage of Alu Methylation Levels in All Sample Groups

Sample groups		Level (Average±SD)			
		ᵐC	ᵐCᵐC	ᵐCᵐC+ᵐCᵘC	ᵘCᵘC
Paraffin-embedded tissue	Normal	60.62±5.65	24.63±6.79	35.99±3.04	39.38±5.65
	Oral cancer	56.46±4.77	13.65±4.57	42.81±5.16	43.54±4.77
PBMCs	Normal	66.68±3.52	16.25±6.11	50.43±5.26	33.32±3.52
	Oral cancer	65.56±3.67	16.48±7.89	49.08±6.16	34.44±3.67
Oral riase	Normal (Non-smoker)	60.41±3.37	16.44±3.93	43.97±4.30	39.59±3.37
	Light smoker	59.48±3.39	15.26±5.40	44.22±3.73	40.52±3.39
	Heavy smoker	57.81±2.52	13.53±3.80	44.28±3.13	42.19±2.52
	Oral cancer	56.58±4.84	11.08±5.27	45.50±3.43	43.42±4.84
	Low stage (I+II)	57.42±3.69	11.56±5.63	45.86±3.59	42.58±3.69
	High stage (III+IV)	56.17±5.31	10.84±5.18	45.33±3.40	43.83±5.31

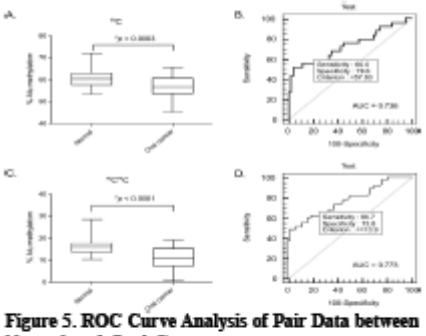

Figure 5. ROC Curve Analysis of Pair Data between Normal and Oral Cancer. Combined sensitivity (ᵐC or ᵐCᵐC) calculated by 100%-[false negative ᵐC×false negative ᵐCᵐC] and combined specificity (ᵐC or ᵐCᵐC) calculated by Specificity ᵐC×Specificity ᵐCᵐC. (AUC represent for Area under curve)

Receiver Operating Characteristic (ROC) curve analysis of ᵐC and ᵐCᵐC methylation pattern

Since our data suggested that ᵐC and ᵐCᵐC show the highest significant value, we chose to further analysis this sub-set. We selected methylation and hypermethylation results to perform ROC curve to assess if this was able to discriminate normal and oral cancer tissues. As shown in Figure 5, ᵐC pattern demonstrated a sensitivity and specificity of 60.0% and 78.6%, respectively. In the same way, 66.7% sensitivity and 73.8% specificity was observed for the ᵐCᵐC pattern. However, sensitivity and specificity determined individually for ᵐC and ᵐCᵐC are not suitable for use as tool for oral cancer detection. Fortunately, the combination of these two markers did improve the diagnostic power of the oral cancer detection (86.68% sensitivity and 56.68% specificity). With a high percentage of sensitivity, the methylation level of ᵐC or ᵐCᵐC in oral rinse sample has high potential for use as a screening tool for oral cancer detection from the oral rinse specimen.

Discussion

In this study, we have used COBRA Alu analysis, which is highly capable of detecting methylation levels of Alu elements at 2 CpG loci. This alone is the key advantage of this technique, providing information on multiple CpG loci rather than 1. For example, in the FFPE derived tissue, we not only found hypomethylation in both of the CpG loci (ᵐCᵐC), we were also able to examine the increase in ᵘCᵘC pattern which we determined to hold grater significance.

Since a previous study reported that Alu hypomethylation was correlated with aging when assessed in PBMCs (Jintaridth and Mutirangura, 2010), we chose to follow this approach for oral cancer, since it is not as invasive as tissue biopsy. Although our data did show that methylation was datable, the result of methylation patterns showed no significant changes. Nonetheless, decreasing trend in methylation level and increasing hypomethylation loci same as paraffined-embeded tissue were noted. The results are far from conclusive and this might have occurred by the possibility that the PBMCs could be with a high proportion of normal PBMCs and with very few circulating cancer cell DNA in the patient sample.

Finally, we observed notable differences in oral rinse samples which its self represents an excellent cost-effective and non-invasive technique for sample collection. We also investigated the component of potentially malignant condition arising from a smoking habit. Our results demonstrate a significant reduction in Alu methylation level in oral rinse samples from non-smoker, light smoker, heavy smoker and cancer patients (Figure 4). The level of Alu methylation is noted to be stepwise decrease concordant with the potentially malignant changes of the oral epithelium. Although the majority of the oral rinse contains cells from the normal oral epithelium, in smokers and cancer patients the oral rinse can likely include dysplastic/cancer squamous cells (Subbalekha et al., 2009; Wangsri et al., 2012). The DNA from dysplastic/cancer cells is capable to show reduced the Alu methylation level. This observation is confirmed by the Alu methylation level in high stage cancer is lower than low stage cancer, and collectively demonstrating the sensitivity of the COBRA Alu method of analysis.

The association between smoking status and Alu hypomethylation in tumors suggest that tobacco exposure may be causing genome-wide damage and contributing in epigenetic events including Alu methylation status. Smoking has been associated to promote methylation of several genes in different cancer, for example, SFRP in head and neck squamous cell carcinoma (HNSCC) (Marsit et al., 2006) and TSLC1/IGSF4 in non-small cell lung cancer (Kikuchi et al., 2006). Although smoking has not been previously shown directly to cause genome-

wide hypomethylation, there are reports suggesting that smoking can be associated with vitamin B12 reduction, which is required for the normal synthesis of S-adenyl-methionine (Gabriel et al., 2006), an important protein involved in methyl-transferase pathway. This may provide a clue in better understanding the association between Alu hypomethylation and smoking.

The result of this study clearly demonstrates that Alu methylation level and pattern in oral cancer was readily datable in oral rinse sample than in tissues or PBMCs. Here, we proposed to use the sample from oral rinse technique for developing a test for oral cancer detection. Supporting this is that when we performed ROC curve to evaluate the sensitivity and specificity for this test, we observe high sensitivity in combined ^{m}C or $^{m}C^{m}C$ methylation pattern, implying that, this technique may be suitable for oral cancer screening. However, some limitations of this study could be concerned. Firstly, in case of OSCC with ulcer, the results may disturbed by some blood cells contamination. Secondly, our experiment had limited sample size and unmatched age of the participants among normal, smoker and oral cancer patients. Therefore, further investigation should be age consideration and larger sample size evaluation. In conclusion, Alu methylation might be beneficial method for screening oral cancer in oral rinse sample.

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Alu Methylation in Serum of Nasopharyngeal Cancer Cases

RESEARCH ARTICLE

Alu Methylation in Serum from Patients with Nasopharyngeal Carcinoma

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Abstract

Background: Nasopharyngeal carcinoma (NPC) is a common cancer in Southern China and Southeast Asia. Alu elements are among the most prevalent repetitive sequences and constitute 11% of the human genome. Although Alu methylation has been evaluated in many types of cancer, few studies have examined the levels of this modification in serum from NPC patients. Objective: To compare the Alu methylation levels and patterns between serum from NPC patients and normal controls. Materials and Methods: Sera from 50 NPC patients and 140 controls were examined. Quantitative combined bisulfite restriction analysis-Alu (qCOBRA-Alu) was applied to measure Alu methylation levels and characterize Alu methylation patterns. Amplified products were classified into four patterns according to the methylation status of 2 CpG sites: hypermethylated (methylation at both loci), partially methylated (methylation of either of the two loci), and hypomethylated (unmethylated at both loci). Results: A comparison of normal control sera with NPC sera revealed that the latter presented a significantly lower methylation level (p=0.0002) and a significantly higher percentage of hypomethylated loci (p=0.0002). The sensitivity of the higher percentage of Alu hypomethyted loci for distinguishing NPC patients from normal controls was 96%. Conclusions: Alu elements in the circulating DNA of NPC patients are hypomethylated. Moreover, Alu hypomethylated loci may represent a potential biomarker for NPC screening.

Keywords: Alu element - methylation - nasopharyngeal cancer - serum marker

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Introduction

DNA methylation is an epigenetic modification that is essential for determining an individual's phenotype and regulating gene expression. DNA methylation involves the addition of a methyl (CH_3) group at the carbon-5 position of the cytosine ring and is frequently observed at CpG sites (Ramsahoye et al., 2000). In general, CpG sequences are distributed as interspersed repetitive sequences (IRS) and dispersed throughout the genome. DNA methylation participates in the regulation of many cellular processes, including embryonic development and differentiation. Furthermore, aberrant DNA methylation is implicated in several diseases (Robertson, 2005; Conerly and Grady, 2010). Changes in the DNA methylation status also contribute to a higher risk of carcinogenesis, including hypermethylation of tumor suppressor genes (Das and Singal, 2004) and genome-wide hypomethylation (Kitkumthorn and Mutirangura, 2011). Genome-wide hypomethylation can promote carcinogenesis, primarily by lowering the rate of replication-independent DNA double-strand breaks and consequently promoting genomic instability (Pornthanakasem et al., 2008; Kongruttanachok et al., 2010; Thongsroy et al., 2013).

Alu elements are members of the short-interspersed nuclear element (SINE) family. These elements are distributed throughout the genome, with over 1 million copies per genome, and they account for approximately 11% of the human genome. Therefore, a reduction of the Alu methylation level results in genome-wide hypomethylation. The methylation level of Alu elements is known to decrease during the aging process (Jintaridth et al., 2013) and has been observed in osteoporosis in post-menopausal women (Jintaridth et al., 2013). Alu hypomethylation has also been observed in cancers of the breast, colon, stomach, liver, lung, ovaries, urinary bladder, prostate gland and oral cavity (Choi et al., 2007; Rodriguez et al., 2008; Watts et al., 2008; Yoo et al., 2008; Bollati et al., 2009; Choi et al., 2009; Daskalos et al., 2009; Lee et al., 2009; Park et al., 2009; Cho et al., 2010; Xie et al., 2010; Hou et al., 2010; Kwon et al., 2010; Xiang et al., 2010; Yoshida et al., 2011; Puttipanyalears et al., 2013). Moreover, it has been suggested that the Alu methylation level is reduced, corresponding to the severity of oral cancer (Puttipanyalears et al., 2013), mucoepidermoid carcinoma of the salivary gland (Sirivanichsuntorn et al., 2013) and lymph node metastases of head and neck cancers (Kitkumthorn et al., 2012).

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Nasopharyngeal carcinoma (NPC) is a neoplasm that arises from the mucosal epithelium of the nasopharynx. In 2012, approximately 87,000 newly diagnosed cases were observed worldwide (GLOBOCAN, 2012). Southern China and Southeast Asia frequently report the highest incidence rate (Tiwawech et al., 2003). The 2-year survival rate is only 50% for patients with advanced NPC in stage III or IV. However, early detection of NPC is associated with a 2-year survival rate up to 90% or more (Sun et al., 2014). Currently, diagnosis is based on the patient's signs and symptoms and the radiographic and pathological features; however, most of these characteristics are only detected at a late stage (Raab-Traub et al., 1987). Consequently, searching for surrogate marker especially circulating serum DNA is considered for NPC screening.

In this study, we aimed to evaluate circulating tumor DNA by observing the Alu methylation levels in sera from NPC patients compared to normal controls.

Materials and Methods

Participants

The study protocol was reviewed and approved by the Ethics Committee of the National Cancer Institute, Bangkok, Thailand. Written informed consent was obtained from all participants. Fifty patients (39 males and 11 females with a mean age of 50 years, range 27-78 years) with histological confirmation of nasopharyngeal cancer between 2009 and 2011 were recruited for the study. One hundred and forty age-matched normal controls (80 males and 60 females with a mean age of 50 years, range 29-69 years) were enrolled in the study as controls. The health status of the controls was confirmed by direct interview and by reviewing the results of an annual medical check-up to ensure that there was no history of any malignancies.

Specimen collection and DNA extraction

Six milliliters of blood was collected from each participant. The blood samples were centrifuged at 1,600x g for 10 minutes to collect the serum. Then, the serum was transferred to new polypropylene tube and centrifuged at 14,000x g for 15 minutes. The supernatant was discarded, and the DNA in the remaining pellet was isolated using Tris/SDS and proteinase K and incubated at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation. The isolated genomic DNA was eluted and then treated with bisulfite. Bisulfite modification of the genomic DNA was performed using previously published methods (Chalitchagorn et al., 2004). Briefly, a total of 1μg of DNA from each sample was denatured in 0.22 M NaOH at 37°C for 10 min. After the addition of 10 mM hydroquinone (Sigma-Aldrich, Singapore) and 3 M sodium bisulfite (pH 5.0), the samples underwent an additional incubation at 50°C for 16-20 h. Then, the DNA was recovered using the Wizard DNA Clean-Up Kit (Promega, Madison, WI) following the manufacturer's protocol. The DNA samples were eluted from the columns with distilled water and precipitated with sodium acetate and 100% ethanol as described previously.

Quantitative combined bisulfite restriction analysis-Alu (qCOBRA-Alu)

The quantitative combined bisulfite restriction analysis-Alu (qCOBRA-Alu) technique is designed to detect 2 CpG loci among the thousands of Alu elements using one set of conserved primers. The qCOBRA-Alu, bisulfite-treated DNA was amplified using primers with the following sequences: Alu forward 5'- GGRGRGGTGGTTTARGTTTGTAA-3 and Alu reverse 5'- CTAACTTTTTATATTTTTAA TAAAAACRAAATTTCACCA-3'. The Alu sequence primers were based on the nucleotide sequences of the Alu Sx subfamily (Batzer MA, 1999). The PCR amplification of qCOBRA-Alu was performed according to the following cycling conditions: pre-denaturation at 95 °C for 15 min followed by 45 cycles of denaturation at 95 °C for 45 sec, annealing at 63°C for 45 sec, and extension at 72 °C for 45 sec, with a final extension at 72 °C for 7 min. After amplification, the Alu PCR products (133 bp in length) were digested with 2 U of the Tag1 restriction enzyme (Fermentas International Inc., Burlington, Canada). Each reaction was incubated overnight at 65 °C, and then, DNA fragments were separated on 8% polyacrylamide gels. Afterthat, the gel was stained using the SYBR green nucleic acid gel stain (Gelstar, Lonza, Rockland, ME, USA). The intensity of the DNA fragments was measured using a Phosphoimager with Image Quant software (Molecular Dynamics, GE Healthcare, Slough, UK). For normalization of the inter-assay variation between each experiment, we used DNA templates from the HeLa, Jurkat and Daudi cell lines as controls. The methylation levels of each cell line were standardized. Therefore, the results of all experiments were adjusted usingthe same control methylation levels.

Alu methylation analysis

An illustration of the qCOBRA-Alu technique and an example of gel electrophoresis are shown in Figure 1. The COBRA Alu loci were categorized into four groups based on the methylation status of 2 CpG dinucleotides in the 5' and 3' regions of the Alu sequence. These four groups were defined as follows: two unmethylated CpGs ($^{5'}$C$^{3'}$C); two methylated CpGs ($^{5'}$C$^{3'}$C); 5'-methylated

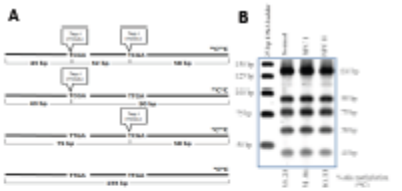

Figure 1. Determination of the Alu Methylation Pattern Using the COBRA-Alu Method. A) The detected Alu sequence contains two CpG dinucleotides. The amplified products were digested with Tag1 and presented 4 patterns: hypermethylation; $^{5'}$C$^{3'}$C (43, 32 and 58 base pair (bp) fragments), partial methylation; $^{5'}$C$^{3'}$C (43 and 90 bp fragments), partial methylation; $^{5'}$C$^{3'}$C (75 and 58 bp fragments) and hypomethylation; $^{5'}$C$^{3'}$C (133 bp fragment). B) Representative gel image from the COBRA-Alu assay. Lane 1: 25-bp markers. Lane 2: DNA from normal controls, Lanes 3-4: DNA from NPC patients

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and 3'-unmethylated CpGs (mCmC); and 5'-unmethylated and 3'-methylated CpGs (umCmC). We calculated the Alu methylation level and percentage of Alu loci in each group based on the intensity of the COBRA-digested Alu products. The DNA fragments derived from enzymatic digestion of the COBRA-Alu products were separated into 6 fragments of 133, 90, 75, 58, 43 and 32 bp, which represented different methylation states. The umCumC methylation state was represented by the 133-bp fragments. The mCmC methylation state was represented by the 90-bp fragment. The umCmC methylation state was represented by the 75-bp fragment. The mCmC and umCmC methylation states were represented by the 58-bp fragment. The mCmC and umCmC methylation states were represented by the 43-bp fragment. Finally, the mCmC methylation state was represented by the 32-bp fragment. The number of CpG dinucleotides was calculated by dividing the intensity of each band by the number of double-stranded bp of DNA sequence as follows: A=intensity of the 133-bp fragment divided by 133; B=intensity of the 58-bp fragment divided by 58; C=intensity of the 75-bp fragment divided by 75; D=intensity of the 90-bp fragment divided by 90; E=intensity of the 43-bp fragment divided by 43; and F=intensity of the 32-bp fragment divided by 32. After calculating the number of CpG dinucleotides and substituting this value in the formula, the Alu methylation levels were calculated as follows: Alu methylation level percentage (%mC)=100×(E+B)/(2A+E+B+C+D); percentage of mCmC loci (%mCmC) =100×F/(A+C+D+F); percentage of umCmC loci (%umCmC) =100×C/(A+C+D+F); percentage of mCmC loci (%mCmC) =100×D/(A+C+D+F); and percentage of umCmC loci (%umCmC) =100×A/(A+C+D+F).

Statistical analysis

The statistical analysis was performed using SPSS software for Windows version 17.0 (SPSS Inc., Chicago, IL). An independent sample t-test was performed to calculate significant differences between the normal controls and the NPC patients. A P value<0.05 was considered statistically significant. A receiver-operating characteristic (ROC) curve analysis was performed to test the ability of the qCOBRA-Alu method to distinguish between the sera of the normal controls and the NPC patients.

Results

Comparison of serum Alu methylation status

In this analysis, we compared the frequencies of each Alu methylation level and pattern between sera from normal controls and NPC patients. The results shown in Figure 2 indicate that the overall methylation levels (%mC) in the normal controls were higher than those in the NPC patients (p=0.0002). Moreover, when the same comparison was performed using the %umCmC values, this difference was significantly higher in the NPC patients (p=0.0002). In contrast, the other three methylation patterns were slightly less prevalent in the NPC patients, although the difference was not statistically significant (% mCmC: p=0.1189, %umCmC: p=0.0847 and %mCmC: p=0.1239).

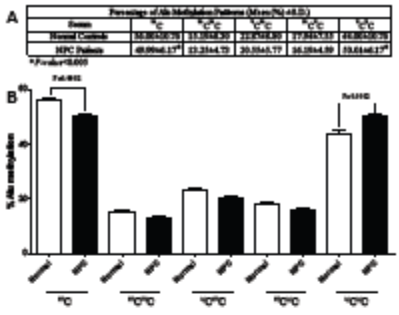

Figure 2. The Percentages of Each Alu Methylation Pattern in the Serum between Normal Controls and NPC Patients. A) Detailed description of the methylation level B) Histogram representation

Receiver Operating Characteristic (ROC) curve analysis of the mC and umCmC methylation patterns

A ROC analysis was performed to assess the ability of the Alu methylation pattern to distinguish between serum from normal controls and serum from NPC patients. Our data showed that mC and umCmC had the greatest diagnostic value, and therefore, these methylation patterns were chosen for this analysis. Both of these markers showed AUC values at 0.676. At a 58.58% cutoff, the sensitivity and specificity of mC were 94.00% and 42.90%, respectively. The cut-off value of umCmC was 40.21%, and the sensitivity and specificity values were 96.00% and 40.00%, respectively. These results imply that both of these Alu methylation patterns are potentially useful for screening purposes. Furthermore, when we combined these two markers (mC or umCmC), the specificity was not improved and there was a slight reduction in the sensitivity for NPC detection (90.00% sensitivity and 65.74% specificity).

Discussion

Alu elements are widely distributed throughout the genome, and previous studies have demonstrated Alu hypomethylation is correlated with aging and many types of cancer (Jintaridth and Mutirangura, 2010; Kitkumthorn and Mutirangura, 2011) To prevent interference related to the effect of aging, we therefore selected participants with age-matched controls. In this study, we used qCOBRA-Alu instead of the more commonly used pyrosequencing technique. Unlike pyrosequencing, qCOBRA-Alu can be used to measure Alu methylation levels and to classify Alu methylation patterns (Jintaridth and Mutirangura, 2010; Jintaridth et al., 2013; Puttipanyalears et al., 2013). Moreover, methylation patterns were shown to be more sensitive for detecting early carcinogenesis, for example, in mucoepidermoid carcinoma of salivary gland (Sirivanichsuntorn et al., 2013) Furthermore, for screening usage, we recently demonstrated that the number of Alu hypomethylated loci, particularly umCmC, could be useful for tumor DNA detection in an oral rinse and in

peripheral blood mononuclear cells from patients with oral cancer (Puttipanyalears et al., 2013). We then applied this approach in NPC, and the findings were consistent with the hypothesis that the Alu methylation level in NPC patients was significantly decreased compared with normal controls.

The results of this study also indicated the importance of Alu methylation patterns. The frequencies of the ^{m}C and $^{m}C^{m}C$ patterns were significantly different between the NPC patients and the normal controls. We performed an analysis of the ROC curve to evaluate the sensitivity and specificity of this test. Interestingly, a very high level of sensitivity was observed for the $^{m}C^{m}C$ level, followed by the ^{m}C level. Therefore, measurement of serum Alu methylation by qCOBRA-Alu is a potentially useful tool for NPC screening. In summary, we propose that $^{m}C^{m}C$ level may suggested to be a potential biomarker for screening and follow-up of NPC patients.

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20th January

Dear Dr. Kitkumthorn,

It is a pleasure to accept your manuscript entitled "Breast cancer up-regulating genes in stromal cells by LINE-1 hypermethylation and micrometastatic detection" in its current form for publication in Epigenomics. Thanks very much for the time spent revising the paper. Our production department will be in touch with the galley proofs some time in February or March - please do let me know if you are going to be away at any point and unable to check them.

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During the Ph. D. studying, he receive award in Good research award in Ph. D. level from parts of his thesis work. The award is received from Joint Conference in Medical Sciences 2015.

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