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Paysage épigénétique du cancer du sein

Aslihan Seda Dagdemir

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UNIVERSITÉ BLAISE PASCAL

UNIVERSITÉ D'Auvergne

2014

N° d'ordre

*ÉCOLE DOCTORALE
DES SCIENCES DE LA VIE ET DE LA SANTÉ*

N° d'ordre :

Thèse

Présentée à l'Université d'Auvergne
pour l'obtention du grade de DOCTEUR

Spécialité : Nutrition, Biologie Cellulaire et Moléculaire

soutenue le 29 Septembre 2014

DAGDEMIR Aslihan

THE EPIGENETIC LANDSCAPE OF BREAST CANCER

Président	:Pr. Yves-Jean Bignon	Université d'Auvergne, EA 4677, Centre Jean Perrin, Clermont Ferrand
Rapporteurs	:Dr. Pascale Rio	INSERM U 1069, Université François-Rabelais, Tours
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Département d'Oncogénétique du Centre Jean Perrin-CBRV, Ertica-EA 4677

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To Dr. Altan Yalciner...

Abbreviations

AdoHcy	: <i>S</i> -adenosylhomocysteine hydrolase inhibitor
AdoMet	: <i>S</i> -adenosyl-methionine
AF-1	: Activation Function 1
AI	: Aromatase Inhibitors
CBP	: CREB-Binding Protein
CH3	: Methyl
ChIP	: Chromatin Immunoprecipitation
CpG	: Cytosine Phosphate Guanine Islands
CREB	: cAMP Response Element-Binding Protein
DBDs	: DNA Binding Domains
DNA	: Deoxyribonucleic Acid
DNMT	: DNA Methyltransferase
DZNep	: 3-Deazaneplanocin A hydrochloride
E2	: Mammalian estrogen 17 β -estradiol
EGFR	: Epidermal Growth Factor Receptor
ER	: Estrogen Receptor
ErbBB	: Epidermal Growth Factor Receptor (EGFR/ERBB) Family
ERE	: Estrogen Response Element
EZH2	: Enhancer of Zeste Homolog 2
GFR	: Growth Factor Receptor
GPR30	: G Protein-coupled Receptor
H2A	: Histone 2A
H3	: Histone 3
H3K4ac	: Histone 3 Lysine 4 Acetylation
H3K9ac	: Histone 3 Lysine 9 Acetylation
H3K27me3	: Histone 3 Lysine 27 Methylation
HAT	: Histone Acetyltransferase
HDAC	: Histone Deacetylase
HDACi	: Histone Deacetylase Inhibitor
HER2	: Human Epidermal Growth Factor Receptor 2
HKMT	: Histone Lysine Methyltransferase
HMTi	: Histone Methylation Inhibitor

HRMT	: Histone Arginine Methyltransferase
miRNA	: MicroRNAs
IGFR	: Insulin-like Growth Factor Receptor
LBDs	: Ligand Binding Domains
NaBu	: Sodium Butyrate
NF- κ B	: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NR3C3	: Nuclear Receptor Subfamily 3, group C, member 3
NST	: No Special Type
QC	: Quality Control
Q-PCR	: Quantitative Polymerase Chain Reaction
O-DMA	: <i>O</i> -desmethylangolensin
PcG	: Polycomb Group Proteins
PR	: Progesterone Receptor
PRC2	: Polycomb Repressive Complex 2
R	: Arginine
RNA	: Ribonucleic Acid
SAHA	: Suberoyl Anilide Hydroxamic Acid
SBR	: Scarff-Bloom-Richardson
SERMs	: Selective ER Modulators
SIRT	: Sirtuin
TNBC	: Triple Negative Breast Cancer

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1. INTRODUCTION

Breast cancer remains the leading cause of cancer-related deaths in women. It is malignant cell growth in the breast. Breast cancer is noted for conflicting clinical behaviors and patient outcomes, despite common histopathological features at diagnosis. This can be explained by the high histological and molecular heterogeneity of the disease, making it hard to choose a therapy adapted uniquely to each patient.

In biology, epigenetics is study of heritable changes in gene expression or cellular phenotype caused by mechanisms, other than changes in the underlying DNA sequence. It refers to functionally relevant modifications to the genome that do not involve a change in the nucleotide sequence. Epigenetics refer to changes in phenotype and gene expression. Epigenetic modifications of the genome can be acquired *de novo* and are potentially inherited.

Epigenetic mechanisms work to change the accessibility of chromatin to transcriptional regulation locally and globally via modifications of the DNA and by modifications or rearrangements of nucleosomes. Epigenetics consist in several molecular mechanisms: histone modifications, small non-coding or antisense RNAs and DNA methylation; that are closely interconnected.

The incidence and mortality of breast cancer is high in the Western world as compared with countries in Asia. There are also differences in the regional cancer incidence rates in Western countries. Several studies involving immigrants to Western countries suggest that lifestyle and diet are two of the main causes of these differences. In Eastern countries, the incidence of breast cancer is approximately one-third that of Western countries, whilst their high dietary intake of phytoestrogens, mainly in the form of soy products, can produce circulating levels of phytoestrogens that are known experimentally to have estrogenic effects [1].

Phytoestrogens are plant-derived xenoestrogens functioning like the primary female sex hormone. They are not generated within the endocrine system, but consumed by eating phytoestrogenic plants. Also called “dietary estrogens”, they are a diverse group of naturally occurring nonsteroidal plant compounds that, because of their structural similarity to 17- β -estradiol, have the ability to cause estrogenic and antiestrogenic effects. An increasing number of epidemiological and experimental studies have suggested that the consumption of a

phytoestrogen-rich diet may have protective effects on estrogen-related conditions, such as breast cancer [2].

Based upon this information, we studied the effects of treatment phytoestrogens; genistein, daidzein and 17- β -estradiol on the post-translational modification of histones such as lysine methylation and acetylation of histones H3 and H4 in breast cancer cell lines.

Subsequently, we studied the effects of histone methylation inhibitor and histone deacetylase inhibitor on histone lysine trimethylation and acetylation in breast cancer cell lines. For this study, we used two breast cancer cell lines MCF-7 and MDA-MB-231. Each cell line was treated respectively with 3-Deazaneplanocin A hydrochloride (DZNep) [5 μ M] (HMTi), Sodium Butyrate (NaBu) [2 mM] (HDACi) and Suberoylanilide Hydroxamic acid (SAHA) [1 μ M] (HDACi) for 48 hours.

Finally, we completed studies in all cell lines with breast tumors to assess Chromatin ImmunoPrecipitation (ChIP) of selected histone modifications in cancer. The relative levels of three modified histones, including H3K27me3 (Histone 3 Lysine 27 Methylation), H3K9ac (Histone 3 Lysine 9 Acetylation), and H3K4ac (Histone 3 Lysine 4 Acetylation) will be determined in breast tumors compared to matched normal tissue according to the classification of Saint Gallen.

Today, ChIP has been coupled with promoter DNA microarrays to evaluate the mechanisms of human gene regulation on a genome-wide scale. ChIP-on-chip technology could be used to investigate the alterations of global gene expression in tumorigenesis. Here, we investigated differentially expressed genes associated with modified histones H3K27me3, H3K9ac and H3K4ac in breast tumors by Agilent SurePrint G3 400kX2 microarrays containing approximately 21,000 of human transcripts. We will scan the enriched regions at each gene promoter in thirty breast tumors compared with normal tissue samples. Breast tumor samples will be classified according to their clinical profiles, especially hormone receptor status.

2. BIBLIOGRAPHY

A. *Breast Cancer*

i. **General description**

Breast cancer is a malignant tumor that begins in the cells of the breast. A malignant tumor is a group of cancer cells that can grow into adjacent tissues or metastasize to distant areas of the body. The breast cancer occurs almost in women, but men can get it as well [3].

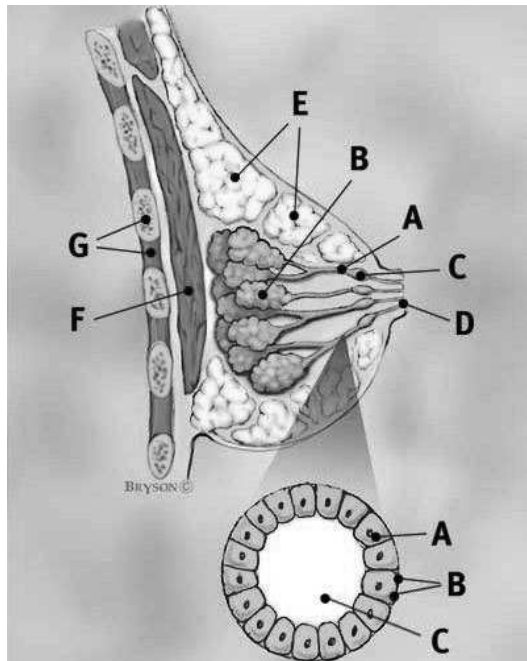
The female breasts are comprised mainly of milk-producing glands, ducts, and stroma (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels). Most breast cancers begin in the cells that line the ducts (ductal cancers). Some begin in the cells that line the lobules (lobular cancers), while a small number start in other tissues [4] (Figure 1).

The most common histologic type of breast cancer is ductal (70-80% of all breast cancer types) followed by the lobular type of cancer (5-10% of all types). Cancer can also be ductal-lobular, tubular, medullary and mucinous, and these class only a small minority of all breast cancers. Ductal cancer is easily detected by mammogram, but for the prognosis, the lobular type of breast cancer is better than ductal cancer.

It is also important to understand the lymph system because it is one way breast cancers can spread. This system has several parts. Lymph nodes are small, bean-shaped collections of immune system cells that are connected by lymphatic vessels. Lymphatic vessels are like small veins, except that they carry a clear fluid called lymph (instead of blood) away from the breast. Lymph contains tissue fluid and waste products, as well as immune system cells. Breast cancer cells can enter lymphatic vessels and begin to grow in lymph nodes [4].

Most lymphatic vessels in the breast connect to lymph nodes under the arm (axillary nodes). Some lymphatic vessels connect to lymph nodes inside the chest (internal mammary nodes) and those either above or below the collarbone (supraclavicular or infraclavicular nodes) (Figure 2).

If the cancer cells have spread to lymph nodes, there is a higher chance that the cells could have spread into the blood stream and spread (metastasized) to other sites in the body. Because of this, finding cancer in one or more lymph nodes often affects the treatment plan. Still, not all women with cancer cells in their lymph nodes develop metastases, and some women can have no cancer cells in their lymph nodes and later develop metastases.



Breast profile:

- A Ducts
- B Lobules
- C Dilated section of duct to hold milk
- D Nipple
- E Fat
- F Pectoralis major muscle
- G Chest wall/rib cage

Enlargement

- A Normal duct cells
- B Basement membrane
- C Lumen (center of duct)

Figure1. Section of Breast Imaging (a)

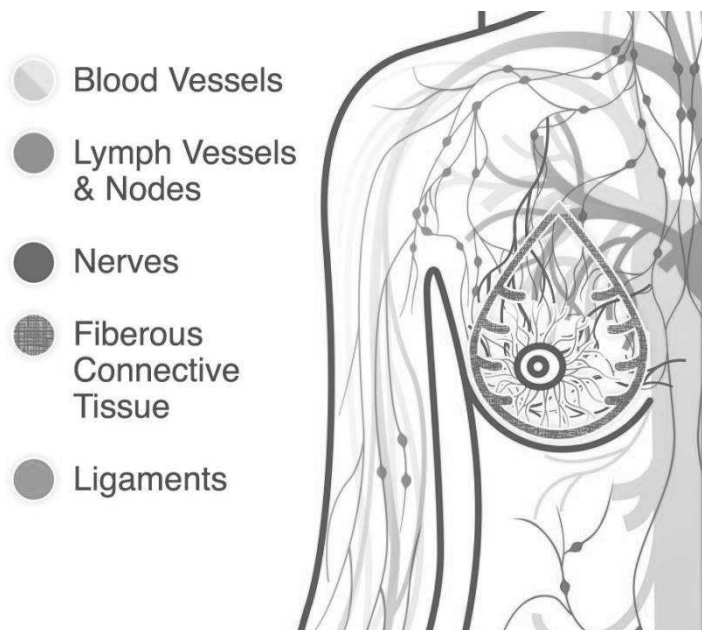


Figure 2. Section of Breast Imaging with Nodes and Vessels (b)

ii. Incidence and Mortality

Recent data show that breast cancer incidence and mortality in Europe are a key resource in both planning and assessing the impact of cancer control programs at the country and regional level. Europe carries a significant load of the global burden, with one quarter of the global burden of cancer observed in Europe in 2013 despite a total population that comprises one-ninth of the world's population. The International Agency for Research on Cancer (IARC), through its programs of collaboration with population-based cancer registries in Europe members of the European Network of Cancer Registries (ENCR, <http://www.enrcr.com.fr/>), has provided estimates of cancer burden at the European and European Union (EU) member state level over the last 25 years (Figure3-4) [1].

The breast cancer was the leading cancer site in women in all countries of Europe and also the leading cause of death of cancer in women in Europe. It is likely that the variation observed in breast cancer incidence across European countries maybe attributable to the variable extent and type of screening activities in operation, a differential in the prevalence and distribution of known risk factors for breast cancer. There is a 3- fold variation (49–148/100,000) with a clear geographical pattern. High incidence rates were estimated in Western European countries, notably in Belgium (147), France (137) and The Netherlands (131) and in Northern Europe, particularly in the United Kingdom (129) and in the Nordic countries, Denmark (143), Iceland (131) and Finland (121). In comparison, incidence rates in Eastern European countries such as the Ukraine (54) and Moldova (53) were much lower. The range of mortality rates varies two fold (15–36 per 100,000). Mortality rates were highest in the North (e.g. Belgium, 29 and Denmark, 28) and in the South (e.g. Serbia, 31 and Macedonia, 36). The high mortality rates in the northern countries reflect the high incidence, while in the south, there is a high mortality to incidence ratio, a proxy of low survival [5, 6].

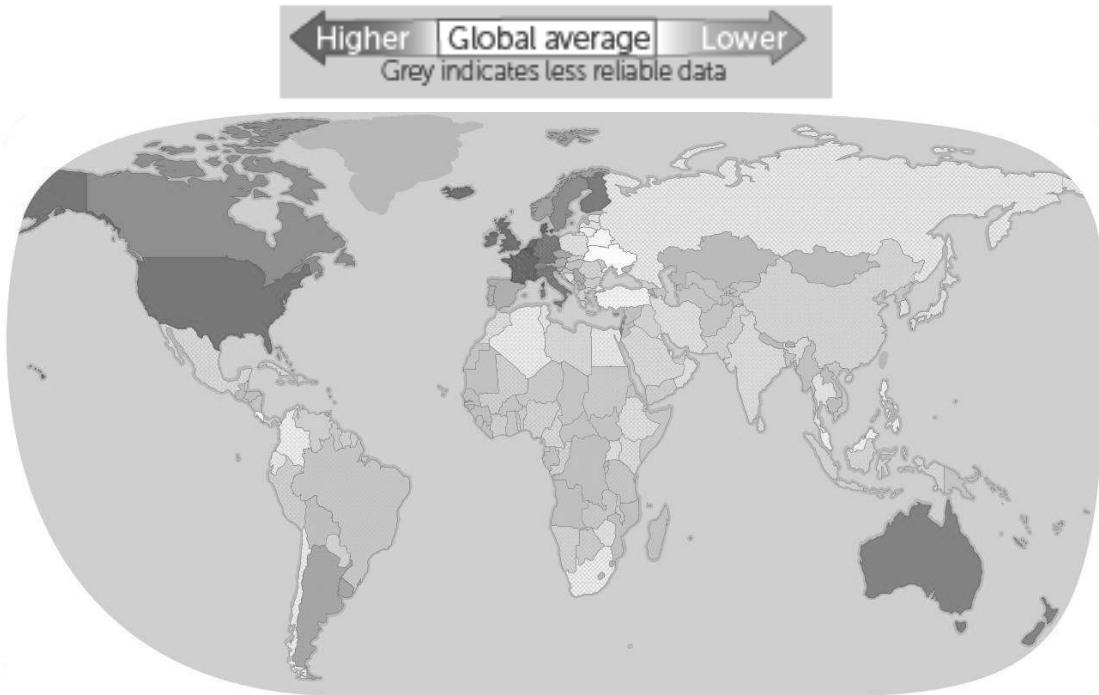


Figure 3. Incidence of breast cancer in the world (c)

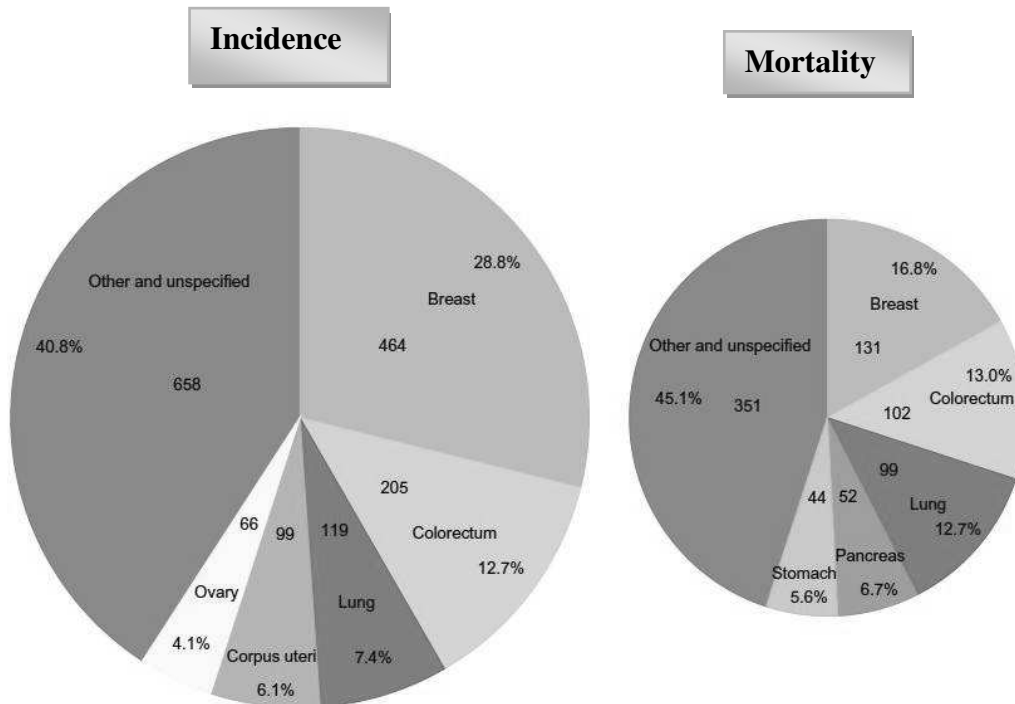


Figure 4. Incidence and mortality of cancer types (c)

iii. Breast cancer risk factors

The causes of breast cancer are not fully known and we know that breast cancer is multi-factorial disease. However, researchers have identified a number of factors that increase or decrease the chances of getting breast cancer. These are called risk factors. Breast cancer is complex and likely to be caused by a combination of factors.

Women have many different breast cancer risk factors and mechanisms over the course of their life. Some of these factors, like a family history cannot be changed, but many others are modifiable.

Well-established risk factors for breast cancer include reproductive factors such as; early age at menarche, late age at first birth, nulliparity, and late age at menopause; family history of breast cancer; alcohol intake; exposure to ionizing radiation; use of combined estrogen plus progestin postmenopausal hormone therapy; recent use of oral contraceptives; physical inactivity; and leanness in early life and obesity in later life.

Gender: Female gender is a strong risk factor for breast cancer. Women have a 150-fold higher breast cancer risk than men. This is evident due to female sex hormones. Conditions which lead to high estrogens levels in men are also associated with male breast cancer. This is probably because men have less of the female hormones, estrogen and progesterone, which can promote breast cancer cell growth [7].

Advanced age: Breast cancer incidence increases rapidly after age of 40, but after the age of 65 the incidence decreases. The risk of developing breast cancer increases with age. About 1 out of 8 invasive breast cancers are found in women younger than 40, while about 2 of 3 invasive breast cancers are found in women age 55 or older.

Age at menarche and menopause: Women experiencing menarche before 12 years have a 50% higher risk for breast compared to women who experience menarche at 14 years or older. Likewise, delayed menopause is associated with a risk elevation of 3% for each delayed year. Both early menarche and late menopause increase the length of lifetime exposure to endogenous female sex hormones which indicates the importance of these hormones in the development of breast cancer. The mechanisms underlying this relationship

are not well understood, but may involve higher levels of estrogen both earlier and later in life in girls with earlier menarche. Estrogen is thought to promote the growth of estrogen receptor-positive (ER+) breast cancer and may also have a role in the early development of ER+ and ER- breast cancers [8].

Many studies have found that age at menarche was associated with both hormone receptor-positive and hormone receptor-negative breast cancers, with one of the study reporting a stronger effect on hormone receptor-positive cancer [9]. In a pooled analysis of breast cancer patients from 34 studies, early age at menarche was less common among cases with progesterone receptor-negative (PR-) breast cancer than among cases with PR+ breast cancer. In the Multiethnic Cohort Study, age at menarche was associated with ER+/PR+ breast cancer, but not with ER-/PR- breast cancer [10].

Age at menarche is determined in part by hereditary factors, but body size, nutrition, and physical activity can also play a role.

Age at first birth and parity: Full-term pregnancy has a protective effect against breast cancer risk. During pregnancy, both estrogen and progesterone cause proliferation and differentiation of the ductal and lobular-alveolar epithelium, which ultimately reduces the risk for malignant transformation of the breast tissue. Human breast tissue also contains receptors for human chorionic gonadotropin and luteinizing hormones. Human chorionic gonadotropin and pregnancy may affect the expression of certain genes and growth factors which inhibit cell proliferation. Human chorionic gonadotropin may be the most important protective factor. The earlier the first full-term pregnancy occurs, the lower the risk. Women older than 30 at first delivery have a 2- 3.5 –fold higher risk for breast cancer, compared to women whose first delivery was before 21. The risk of breast cancer decreases by approximately 10% per birth. Even if the first birth is at age 30 or later, multi-party (5 deliveries) has a protective effect against breast cancer [7].

Benign breast disease: Heterogeneous groups of proliferative and non-proliferative breast lesions are defined as benign breast diseases. Non-proliferative lesions are not associated with breast cancer risk, but proliferative lesions, either with (3.5-5-fold) or without atypical (1.5-2-fold), are associated with an increased risk for breast cancer. Proliferative diseases account for 25-30% of all benign breast diseases, of which 5-10% show proliferative lesions with cellular atypia. Both benign and malignant breast disease can present similar symptoms with a palpable mass or an abnormal screening mammogram with no clinical findings [11].

Family history: Approximately 30% of all breast cancer patients have relatives with breast cancer. If a first-degree relative has breast cancer, the risk is elevated approximately 2-fold. The risk increases with the number of relatives affected and is greater for women with relatives affected at young age. The overall lifetime breast cancer risk for women without a family history of breast cancer is 7.8%. For those who have one first degree-relative affected, the risk is 13.3%, and for those having two, the risk is 21.1% [12].

Breast cancer genes: It has been determined that 5-10% of all breast cancers are caused by mutations in well-identified breast cancer susceptibility genes. The two most important mutations are the high-risk breast cancer genes *BRCA1* and *BRCA2*. However, these mutations only account for a part of the genetic susceptibility of breast cancer [13].

Alcohol use: Alcohol use is associated with an increased risk for breast cancer. The IARC classifies alcoholic beverages as carcinogenic to humans; alcohol causes cancers of the female breast, oral cavity, pharynx, larynx, oesophagus, liver, and colon and rectum. Relatively few studies have evaluated the impact of alcohol intake at young ages on risk of breast cancer. This elevation may be 9-11% with a daily consumption of one alcoholic drink (10 g/d), and the risk increase is linear up to 6 drinks. Further analysis of one of these studies, however, focused on alcohol intake during the interval between two important reproductive events: menarche and first full-term pregnancy. Among women with a longer interval between menarche and first pregnancy (10 years or longer), each 10 g/day increase in alcohol intake increased the risk of breast cancer by 21 %, independent of alcohol intake after first pregnancy. Among women with a shorter interval between menarche and first pregnancy, alcohol intake did not increase the risk of breast cancer. This suggests that a prolonged period of exposure at a stage when breast tissue is most vulnerable may increase the risk of breast cancer. The mechanism of alcohol-induced elevation in breast cancer risk is unknown, but increased levels of estrogen and androgen appear to be important. Alcohol may also enhance the susceptibility of mammary cells to carcinogenesis and increase the metastatic potential of breast cancer cells [14].

Race and ethnicity: Generally, white women are slightly more likely to develop breast cancer than are African- American women, but African-American women are more likely to die of this cancer. In women under 45 years of age, however, breast cancer is more common in African- American women. Asian, Hispanic, and Native American women have a lower risk of developing and dying from breast cancer [15].

Dense breast tissue: Women with dense breasts have a higher risk of breast cancer than women with less dense breasts. Unfortunately, dense breast tissue can also make

mammograms less accurate. However a number of factors can affect breast density, such as age, menopausal status, the use of drugs (such as menopausal hormone therapy), pregnancy, and genetics [16].

Size of a woman: Obesity is associated with a risk for breast cancer. However, obesity in childhood has not proven to have an effect on the risk of breast cancer later in life, but weight gain after the age of 18 or after menopause is associated with increased risk of breast cancer among postmenopausal women. On the contrary, a higher body mass index (BMI) at 18 years is associated with a lower risk of breast cancer in premenopausal life and, in some studies, in postmenopausal life as well. A high BMI (>31 vs. < 21) is also associated with a 46% lower risk for breast cancer in premenopause. One explanation for the increased risk for breast cancer after menopause in obese women is the high amount of endogenous estrogens produced in adipose tissue. Furthermore, obesity increases the circulating concentrations of insulin, which may be associated with the risk for breast cancer. Tall women appear to have a higher risk for breast cancer. Childhood energy intake, the cumulative exposure to growth hormone and insulin-like growth factor-I, or the number of ductal stem cells in the mammary gland have been proposed as potential biologic mechanisms associated with an increased breast cancer risk among tall women.

iv. Tumor markers

Breast cancer is a complicated, miscellaneous disease of presence that shows appreciable variation in morphological, clinical and molecular changes. Traditional classifications including histological assessment and clinical staging are used to guide patient management [17].

Historically, breast cancer classification systems have been based on histopathological assessment including histological type and grade.

At the present time, we use many markers for classification of breast cancer; such as, expression of estrogen receptor (ER), expression of progesterone receptor (PR) and over-expression and/or amplification of the human epidermal growth factor receptor 2 (HER2), tumor grade, lymph node metastasis and Ki-67. They have been included to set the classification for predicting prognosis as well as the potential response to endocrine treatment

and the humanized monoclonal antibody trastuzumab (Herceptin). ER and PR tests are usually done by immunohistochemistry whereas HER2/neu is accessed by FISH. This protein profiling of tumors helps to predict the eventual prognosis and can assist in the determination of the most appropriate treatment for the individual [18].

1. *Estrogen receptor (ER):*

The nuclear hormone family is activated by the hormone 17 β -estradiol, and ER is an intra-cellular receptor which is a member of this family. The main function of ER is as a DNA-binding transcription factor which regulates gene expression. There are two classes of estrogen receptor: ER, which is a member of the nuclear hormone family of intracellular receptors, and GPR30, which is a member of the rhodopsin-like family of G protein-coupled receptors. In addition, there are two different forms of ER; alpha (α) and beta (β), each encoded by a separate gene. Hormone-activated ERs form dimers. These two forms of ERs are co-expressed in various cell types including thyroid, bone, adrenals and female rat brain. This may lead to the formation of homodimer ER α ($\alpha\alpha$) or ER β ($\beta\beta$) or heterodimer ER $\alpha\beta$ ($\alpha\beta$). Estrogen receptors α and β show significant overall sequence homology, and both are composed of five domains. They share about 96% homology between their DNA binding domains (DBDs), but only 56% homology between their ligand binding domains (LBDs) and 28% homology between their amino-terminal activation functions 1 (AF-1s). ER α and ER β can homo- or hetero-dimerize, indicating that the two isoforms can act together or separately. The two ER isoforms share overlapping functions due, in part, to the significant homology of their DNA binding domains (Figure 5) [19].

The isoform α is encoded by the ESR1 and the β isoform is encoded by the ESR2 gene. The two ER isoforms are encoded from two separate genes in two different chromosomal locations. ESR1 is encoded on chromosome 6 (6q25.1) and ESR2 is encoded on chromosome 14 (14q). Both ERs are widely expressed in different tissue types, however, there are some differences in their expression patterns. ER α is expressed in endometrial, breast cancer cells, ovarian stroma cells and in the hypothalamus. ER β is expressed in kidney, brain, bone, heart, lungs, intestinal mucosa, prostate, and endothelial cells. The ER's helix 12 domain plays an important role in determining interactions with co-activators and co-repressors, thereby affecting the respective agonist or antagonist effect of the ligand. The ER α proteins are attributed to existence cytoplasmic receptors in their unliganded state, but

scanning research has shown that there are many ER α fractions in the nucleus of ER-negative breast cancer.

ERs are expressed in approximately 70% of breast cancer cases which are attended to as "ER-positive" tumors [20]. Binding of estrogen to ER stimulates proliferation of mammary cells, resulting in an increase in cell division and DNA replication and increases mutation rate. For these reasons, disruption of the cell cycle and apoptosis and DNA repair processes that eventually lead to tumor formation. Additionally, estrogen metabolism leads to the production of genotoxic by-products that could directly damage DNA, resulting in point mutations. ER α expression is associated with more differentiated tumors, while evidence that ER β is involved is controversial. However, recent research suggests that ER β is associated with a poor prognosis and proliferation. Different versions of the *ESR1* gene have been identified and are associated with different risks of developing breast cancer [21].

Patients with high levels of ER are treated with endocrine therapy. Endocrine therapy for breast cancer involves Selective ER Modulators (SERMS) which act as ER antagonists in breast tissue or as aromatase inhibitors. ER status is used to determine sensitivity of breast cancer lesions to tamoxifen and aromatase inhibitors. Raloxifene, which has anti-estrogenic behavior, has been used as a preventative chemotherapy for women determined to have a high risk of developing breast cancer [19].

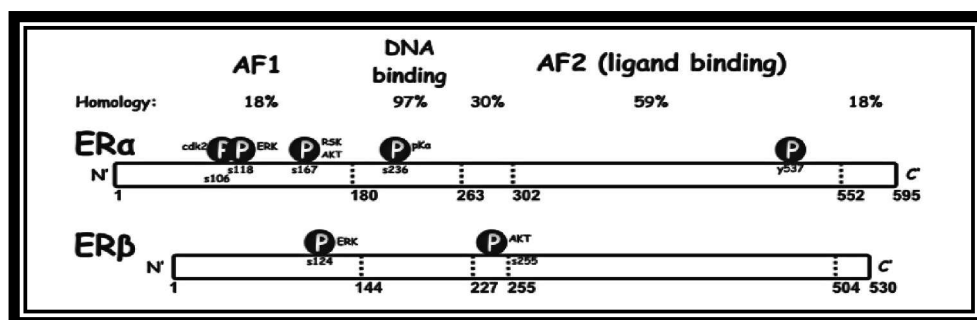


Figure 5: Domains of ER α and ER β (d)

2. Progesterone receptor (PR):

The human progesterone receptor (PR), also known as NR3C3 (nuclear receptor subfamily 3, group C, member 3), is an intracellular steroid receptor that binds progesterone. PR is encoded by the PGR gene which exists on chromosome 11 (11q22). PR is expressed as two isoforms, PR-A (94 kD) and PR-B (114 kD), which function as ligand-activated transcription factors. These two isoforms are transcribed from distinct ER-inducible promoters within a single copy PR gene [22].

The PRA form is a truncated version of the PRB form, lacking the first 164 N-terminal amino acids. In humans, PRA acts as a trans-dominant repressor of the transcriptional activity of PRB, glucocorticoid receptor, ER, androgen receptor and mineralocorticoid receptor. PRB functions mainly as a transcriptional activator. PRB is expressed strongly in endometrial glandular and stromal nuclei in the proliferative phase of the menstrual cycle and weakly during the secretory phase and early pregnancy (Figure 6) [23].

PR is expressed in reproductive tissue and has important roles in folliculogenesis, ovulation, implantation and pregnancy. Estrogen is necessary to induce the PRs activity. PRs become hyperphosphorylated upon binding of the steroid ligand. PR phosphorylation is complex, occurring in different cellular compartments and perhaps requiring multiple serine kinases. After progesterone binds to the receptor, restructuring with dimerization follows and the complex enters the nucleus and binds to DNA. There, transcription takes place, resulting in formation of messenger RNA that is translated by ribosomes to produce specific proteins [24].

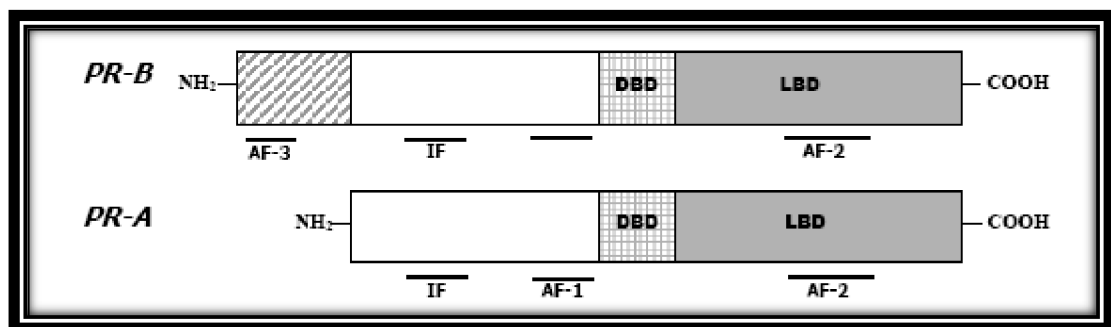


Figure 6: Domains of PRA and PRB (e)

3. *HER2/neu*:

Receptor tyrosine-protein kinase erbB-2, also known as CD340 (cluster of differentiation 340), proto-oncogene Neu, Erbb2 (rodent), or ERBB2 (human) is a protein that in humans is encoded by the *ERBB2* gene. The *HER2* gene is a proto-oncogene located at the long arm of chromosome 17 (17q11.2-q12). The *ERBB2* gene is also frequently called *HER2* (from human epidermal growth factor receptor 2) or *HER2/neu*.

HER2 is a member of the epidermal growth factor receptor (EGFR/ERBB) family. *HER2/neu* plays a significant part in the pathogenesis of breast cancer and as a target of treatment. It is a cell membrane surface-bound receptor tyrosine kinase and is normally involved in the signal transduction pathways leading to cell growth and differentiation. HER2 is thought to be an orphan receptor, with none of the EGF family of ligands able to activate it. However, ErbB receptors dimerise on ligand binding, and HER2 is the preferential dimerisation partner of other members of the ERBB family [25].

Today this protein has become an important biomarker and target of therapy for around 30% of breast cancer patients. Amplification or over-expression of this gene has been shown to play an important role in the development and progression of certain aggressive types of breast cancer and also associated with increased disease recurrence and worse prognosis. The poor prognosis may be due to global genomic instability as cells with high frequencies of chromosomal alterations have been associated with increased cellular proliferation and aggressive behaviour [25].

4. *Grade*:

Over the last years, histological grading has become extensively accepted as a powerful indicator of prognosis in breast cancer, especially of tumor grading systems currently missionary for breast cancer associated with nuclear grade, tubule formation and mitotic rate. On the whole, each element is given a score of 1 to 3; that is 1 being the best and 3 the worst, and the score of all three components are added together to derive the "grade". The lowest possible score (1+1+1=3) is given to well differentiated tumors that all form tubules and have a low mitotic rate. The highest possible score is 9 (3+3+3=9). The exact criteria for each component differ in each system and the systems are evolving as more

detailed data becomes available. Some studies even suggest that mitotic rate alone can be as predictive as the grading systems.

The United States uses the most common grading systems as described above; the original Scarff-Bloom-Richardson (SBR) system and the Black method, which accentuates nuclear grading and excludes consideration of tubules as criteria. In Europe, the Elston-Ellis modification of the SBR grading system is preferred and is becoming increasingly popular in the US. This modification provides somewhat more objective criteria for the three component elements of grading and specifically addresses mitosis counting in a more precision form. For example, hyperchromatic nuclei and apoptotic cells which are counted in the original SBR system are excluded in the Elston-Ellis modification and the area being assessed is specifically defined in square millimeters. These modifications have enhanced reproducibility of grading among pathologists and, to a considerable extent, have stimulated acceptance of grading by clinicians [26].

Criteria for grading is an active area of investigation, particularly in defining more objective criteria for assessing nuclear grade and we should expect image analysis to greatly contribute to this area in the future.

5. Lymph node metastasis:

Lymph node metastasis is considered an important prognostic parameter for use in determining treatment for breast cancer patients. The sentinel node is the first lymph node reached by metastasizing cells from a primary tumor. A sentinel node biopsy is a minimally invasive technique to identify lymph node metastases. Involvement of a lymph node in breast cancer significantly correlates with worse prognosis compared with no lymph node involvement. Such patients have a higher incidence of death due to disease and should therefore be treated more aggressively [27].

6. Ki67:

The choice of adjuvant systemic therapy is based on targeted therapy in line with the St. Gallen consensus meeting. In addition to the traditional parameters, the panel recommended the use of proliferation markers and multigene assays. The purpose of the present study was to evaluate the clinical significance of proliferative activity using the Ki-67

index as a prognostic marker and as a predictor of recurrence time in breast cancer patients. Ki-67 is present in all proliferating cells, and there is great interest in its role as a proliferation marker [28].

The Ki-67 antibody reacts with 395 kDa, which is a nuclear non-histone protein that is present in all active phases of the cell cycle, except the G₀ phase. Proliferation is a key feature of the progression of tumors and is now widely estimated by the immunohistochemical assessment of the nuclear antigen Ki-67. The expression of Ki-67 correlates with other markers of proliferation, including S-phase and bromodeoxyuridine uptake. High Ki-67 is a sign of poor prognosis associated with a good chance of clinical response to chemotherapy, but its independent significance is modest and does not rate measurements in most routine clinical scenarios. However, its application as a pharmacodynamic intermediate marker of the effectiveness of medical therapy holds great promise for rapid evaluation of new drugs [29].

v. Classification of breast cancer

Breast cancer is a complex disease with distinctive properties such as clinical, morphological and molecular. This heterogeneity cannot be explained just by clinical parameters such as tumor size, lymph node involvement, histological grade, age; or by biomarkers like ER, PR and HER2 routinely used in the diagnosis and treatment of patients [30].

There are more than 21 subtypes of invasive breast carcinoma defined in the fourth edition of the WHO (World Health Organisation) Classification of Tumours of the Breast. The most frequent is Invasive Carcinoma of No Special Type (NST), also known as invasive ductal carcinoma NST, and it comprises 40–75 % of cases. The remaining tumor types are morphologically distinct “special” types including invasive lobular, tubular, mucinous and metaplastic carcinoma and carcinoma with medullary, neuroendocrine or apocrine features.

The less common subtypes include mucinous, cribriform, micropapillary, papillary, tubular, medullary, metaplastic, and inflammatory carcinomas. These morphological subtypes of breast cancer can be further sub-divided into classifications based on their molecular signatures (*ie*, expression of protein biomarkers or gene expression profiles).

Routine histopathological subclassification of invasive ductal carcinomas is accomplished by immunostaining cancer tissues to detect expression of the estrogen receptor, the progesterone receptor and the human epidermal growth receptor 2, as well as HER1 and various cytokeratins (*eg*, CK5/6). The differential expression of these protein biomarkers provides a clinical classification for breast cancer [31].

1. *Luminal A:*

The luminal A breast cancer is the most common subtype, representing 50–60% of the total. It is characterized by the expression of genes activated by the ER transcription factors that are typically expressed in the luminal epithelium lining the mammary ducts. It also presents a low expression of genes related to cell proliferation. Based on their molecular profile, all cases of lobular carcinoma *in situ* are luminal A tumors, as are most of the infiltrating lobular carcinomas. The luminal A immunohistochemistry profile is characterized by the expression of ER, PGR and cytokeratin CK8/18, an absence of HER2 expression, a low rate of proliferation measured by Ki67 and a low histological grade [32].

Patients with this subtype of cancer have a good prognosis; the relapse rate is 27.8% being significantly lower than that for other sub-types. In addition, survival from the time of relapse is also longer. They have a distinct pattern of recurrence with a higher incidence of bone metastases and with respect to other localizations such as central nervous system, liver and lung which represent less than 10%. The treatment of this sub-group of breast cancer is mainly based on third-generation hormonal aromatase inhibitors (AI) in postmenopausal patients, SERMs like tamoxifen and pure selective regulators of ER like fulvestrant [33].

2. *Luminal B:*

Luminal B breast cancer occurs less frequently, approximately 10% and 20% of all breast cancers correlate with the luminal A. Luminal B breast cancers have a more aggressive phenotype, higher histological grade and proliferative index and worse prognosis. The pattern of regression also differs, and although the bone is still the most common site of recurrence (30%), this subtype has a higher recurrence rate in sites such as the liver (13.8%). Additionally, the survival from time of relapse is lower. Luminal A and B both express ER,

but since luminal B's prognosis is very different, a strong effort to find biomarkers that distinguish between these two subtypes has been made [30].

From the immunohistochemical point of view, the luminal B subtype has tumors with ER+/HER2- and high Ki67 or ER+/HER2+. It is worth noting that this definition does not include all luminal B subtype tumors (up to 6% of the luminal B tumors are clinically ER-/HER2-). Moreover, the technique used to determine Ki67 (cut-off point to distinguish luminal A and B set at 13.25%) has not been standardized adding a variability factor in the assessment of this marker. However, considering that this marker is the most widely used to measure cell proliferation; efforts are being made to reach a consensus on how to evaluate it. In fact, an international consortium has recently published a set of recommendations for Ki67 assessment in breast cancer [34].

Luminal B tumors have a worse prognosis than do luminal A tumors despite treatment with tamoxifen. However, they respond better to neoadjuvant chemotherapy achieving pathological complete response in 17% of the luminal B tumors (7% in luminalA). This is clearly lower than for the HER2+ and basal-like tumors with values of 36% and 43%, respectively. For these reasons, treatment of this subtype of breast cancer is currently challenging [35].

3. *HER2 Positive:*

Fifteen to twenty percent of all breast cancers correspond to this molecular subtype. They are characterized by a high expression of the HER2 gene and other genes associated with the HER2 pathway and/or HER2 amplicon located in the 17q12 chromosome. These cancers exhibit an over-expression of genes related to cellular proliferation. Although this sub-type does not express genes of the basal-like cluster, it may show a low expression of characteristic luminal genes. Morphologically, these tumors are highly proliferative, with 75% having a high histological grade and more than 40% have *p53* mutations. The immunohistochemical profile ER-/HER2+ does not correspond perfectly with the intrinsic subtype, since only 70% of HER2+ tumors by microarray have the protein over-expressed by immunohistochemical [36].

Conversely, not all tumors with HER2 amplification or over-expression are included in the cluster of HER2 in the analysis of microarrays. In addition, a significant number of

tumors clinically ER+/HER2+ are classified molecularly as luminal B. HER2 amplified tumors have been further sub-classified into three separate subtypes; one with a clearly worse prognosis (12% of 10 years survival compared to the 50–55% survival in the other two groups). In addition, it also had a strong prognostic value in tumors that over-expressed HER2 inside other subgroups of breast cancer [37].

From the clinical point of view, the HER2 subtype is characterized by a poor prognosis, although in the last decade, anti-HER2 treatment has substantially improved survival in, not only the metastatic diseases, but also in the initial stages. In neoadjuvant studies, this sub-type, as well as the basal-like subgroup, has a high chemosensitivity with higher response rates than that for luminal A and B tumors.

4. *Basal-like:*

The basal-like subtype represents 10–20% of all breast carcinomas. The term was coined because they express genes usually present in normal breast myoepithelial cells, including high molecular weight cytokeratins CK5 and CK17, P-cadherin, caveolin 1 and 2, nestin, CD44 and EGFR. They also express genes characteristic of luminal epithelium such as CK8/18 and Ki67, but at level significantly lower levels than those of luminal carcinomas. Clinically, they are characterized by their appearance at an early age, predominantly in women of African origin having a large tumor size at diagnosis, a high histological grade and a high frequency of lymph node affectation. One of the most relevant features of this type of tumor is the absence of expression of the three key receptors in breast cancer: ER, PGR and HER2. Therefore, in clinical practice, the terms basal-like and Triple Negative (TN) are often interchanged. They are not, however, equivalent terms since a discordance of up to 30% between the two groups has been described. Attempts to identify the basal-like group by an immunohistochemically profile have led to the selection of five markers (Basal Core Group): ER, PGR, HER2, EGFR and CK5/6. These markers classify this sub-type with a specificity of 100% and a sensitivity of 76%. Basal-like tumors have a worse prognosis than do luminal ones. Therefore, it is critical to identify new therapeutic targets and design appropriate treatment strategies [38].

5. *Claudin-low*:

After the initial molecular classification into sub-types of breast cancer, another intrinsic subtype was identified in 2007. It is characterized by a low expression of genes involved in tight junctions and intercellular adhesion; including claudin-3, -4, -7, cingulin, occludin, and E-cadherin hence the name claudin-low. This subtype is located in the hierarchical clustering near the basal-like tumors, suggesting that both subtypes share some characteristic gene expressions such as low expression of HER2 and luminal gene cluster. In contrast to the basal-like subtype, this new group over-expresses a set of 40 genes related to immune response indicating a high infiltration of tumor immune system cells [39].

Claudin-low tumors have a poor prognosis, albeit presenting a low expression of genes related to cell proliferation. Immunohistochemically, they are normally triple negative. Like basal-like tumors, the concordance triple negative/claudin-low is not 100%, and about 20% of claudin-low tumors are positive for hormone receptors. These tumors show poor long-term prognosis and an insufficient response to neoadjuvant chemotherapy with intermediate values between basal and luminal tumors [40].

The implications of the molecular classification in the therapeutic approach have been progressively accepted by some international panels. The St. Gallen International Expert Consensus for Early Breast Cancer 2013 recognized the usefulness of this classification in the therapeutic decision process. Note that the panel accepted that the different breast cancer subtypes can be defined, not only by genetic array testing, but by approximations to this classification using immunohistochemistry. This Expert Consensus established five clinicopathological definitions, luminal A, luminal B – HER2 negative, luminal B – HER2 positive, HER2 positive – non luminal and Triple Negative (ductal) (Table1).

CATEGORIES	CHARACTERISTICS
Luminal A	<ul style="list-style-type: none"> • ER and PgR positive • HER2 negative • Ki-67 low • Recurrence risk 'low' based on • Multi-gene-expression assay (if available)
Luminal B	<p>HER2 negative</p> <ul style="list-style-type: none"> • ER positive • HER2 negative <p>and at least one of:</p> <ul style="list-style-type: none"> • Ki-67 'high' • PgR 'negative or low' <p>HER2 positive</p> <ul style="list-style-type: none"> • ER positive • HER2 over-expressed or amplified • Any Ki-67 • Any PgR
HER2 Positive Non-Luminal	<ul style="list-style-type: none"> • HER2 over-expressed or amplified • ER and PgR absent
Basal like	<ul style="list-style-type: none"> • ER and PgR absent • HER2 negative

Table 1. Classification of Breast Cancer with Saint Gallen Criterias

B. Phytoestrogens

i. General description

When we investigated of the incidence and mortality of breast cancer, we saw that the breast cancer levels are high in the Western world compared with countries in Asia. There are also differences in the regional cancer-incidence rates in Western countries. Several studies involving immigrants suggest that lifestyle and diet are two of the main causes of these differences. This could be related to phytoestrogens. There are many hypotheses that phytochemicals in Asian diets, which are vegetarian or semi-vegetarian compared with western diets which are rich in animal proteins and fats, may affect cancer incidence by altering production, metabolism, and action of steroid hormones. We believed that the intestines and microflora had a central role in mediating the effects of diet on the disease pattern in western countries, whilst their high dietary intake of phytoestrogens, mainly in the form of soy products, can produce circulating levels of phytoestrogens that are known experimentally to have estrogenic effects [41].

Phytoestrogens are plant-derived formulates that structurally or functionally mimic mammalian estrogens, and are therefore under consideration to play an important role in the preclusion of cancers, heart disease, menopausal symptoms and osteoporosis. Phytoestrogens substitute a heterogeneous group of herbal substances, the structure of which is similar to that of 17- β -estradiol. They are called estrogen-like molecules or non-steroidal estrogens. On the other hand the structural similarity with estradiol; phytoestrogens are diphenolic yet non-steroidal compounds [41].

Currently the group of phytoestrogens include more than 100 molecules divided according to their chemical structure into;

- 1) Isoflavones (*genistein, daidzein, biochanin A, formonnetin*)
- 2) Lignans (*matairesinol, secoisolariciresinol-diglucoside*)
- 3) Coumestans (*coumestrol, 4-methoxycoumestrol*)
- 4) Stilbens (*resveratrol*)

ii. Phytoestrogen Signaling Pathways

Phytoestrogens are able to interact with enzymes and receptors, and also because of their stable structure and low molecular weight they can pass through cell membranes. These interactions allow them to bind to ERs, induce specific estrogen-responsive gene products, stimulate ER-positive breast cancer cell growth, interfere with steroid hormone metabolism or action and alter ER structure and affect transcription. Some genomic mechanisms of action include estrogenic and antiestrogenic effects on ERs, while other effects may not involve direct interaction with ERs. Nongenomic effects that do not involve ERs include: induction of cancer cell differentiation, inhibition of tyrosine kinase and DNA topoisomerase activities, suppression of angiogenesis and antioxidant effects of phytoestrogens. Other effects can take place at the cellular and molecular level and potentially influence the biosynthesis and metabolism of steroids and fatty acids, the serum steroid carrier proteins (sex steroid binding proteins and α -fetoprotein), and the intracellular and transmembrane transfer of hormones to a membrane and to nuclear receptors [42].

Phytoestrogens inhibit the enzymes needed for hormone conversions, which may reduce cancers by lowering the biological activity of sex hormones in target organs. As estrogen-like compounds, some phytoestrogens are able to induce estrus in mammals. The different activities and the bioavailability of phytoestrogens alter depending on such factors as the form of administration, dosage, individual metabolism and the ingestion of other pharmacological substances. Target tissue, concentration addition, number and type of ER, and the presence or absence of endogenous estrogens also influence the effect of phytoestrogen. Not only do phytoestrogens differ in their biological activity, but they also differ structurally because they come from diverse chemical classes, which may affect their influence on tissues and receptors. For these reasons the dissimilarity of chemicals that show estrogenic effects, it appears that estrogenic activity is often emphasized over chemical structure in defining phytoestrogens [43].

iii. Similarities and interaction between soy phytoestrogens and estradiol

There are lots of studies shown that phytoestrogens defined functionally are substances that promote estrogenic actions in mammals and structurally are similar to mammalian estrogen 17β -estradiol (E2) (Figure7) [44].

Other mammalian endogenous estrogens are estriol and estrone, which are weakly estrogenic compared with their mammalian counterpart, E2. The diverse biological activity of phytoestrogens is due in part to their ability to act estrogenically as estrogen agonists and antiestrogenically as antagonists. As estrogen agonists, phytoestrogens mimic endogenous estrogens and cause estrogenic effects. As estrogen antagonists, they may block or alter ER and prevent estrogenic activity, causing antiestrogenic effects.

As estrogen agonists and antagonists, phytoestrogens can also be classified as SERMs. SERMs are non-steroidal chemicals with a similar structure to E2 and an affinity toward estrogen receptors. They are unique in that they can function as agonists or antagonists depending on the tissue, ER and concentration of circulating endogenous estrogens. Tamoxifen and raloxifene are well-known SERMs. Tamoxifen has been used in clinical practice for breast cancer patients because it acts as an estrogen antagonist in breast tissue, slowing cancer cell proliferation and an estrogen agonist in bone tissue and in the cardiovascular system to prevent osteoporosis and heart disease. However, tamoxifen has shown estrogenic activity in the uterus and therefore may increase the risk of endometrial cancer.

Mechanistically phytoestrogens have been shown to bind to two types of estrogen receptors: as we talked about previously ER α and ER β , which was cloned in rats and in humans. The two receptors differ in their tissue distribution and affinity to ligands, yet there is some overlap. In rats, ER α and ER β both are clearly expressed in ovary and uterus tissue. ER β has been shown to have ligand specificity toward phytoestrogens and is distributed in humans in ovary, spleen, testis and thymus tissue and in rats in bladder, brain, lung, ovary, prostate, testis and uterus tissue. Phytoestrogens show a lower binding affinity than E2 and some show a higher binding affinity for ER β than for ER α , which may suggest different pathways for their actions and explain tissue specific variability of phytoestrogenic action.

The complexity of phytoestrogens and ERs appears to be further compounded because different transcriptional activities *in vitro* are activated depending on the ligands, as well as the environment of the promoter region of specific genes for translated ER α and ER β receptors. [45, 46].

iv. The Predominant Isoflavones: Genistein, Daidzein and Equol

Along with daidzein (4', 7dihydroxyisoflavone), genistein (4', 5, 7trihydroxyisoflavone) is the most widely studied isoflavone, universally found in soybeans. Genistein and daidzein are the main soybean phytoestrogens that have a specific chemical structure very close to the human estrogen and therefore to the estrogenic activity.

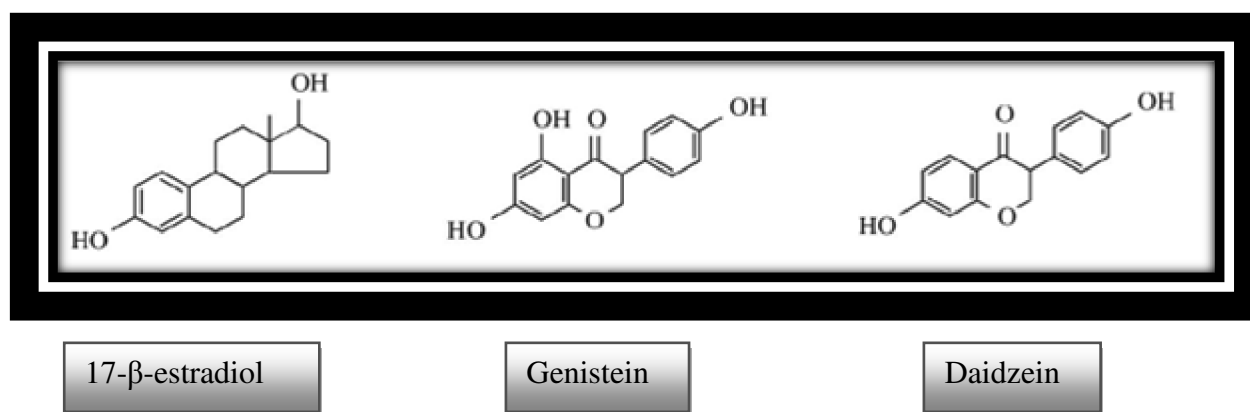


Figure 7. Molecular structure of 17-β-estradiol and isoflavones genistein and daidzine [47].

1. Genistein:

Genistein is one of the most active natural flavonoids and exercises various biological effects including chemoprevention, antioxidation, antiproliferation and anticancer. More than 30 clinical trials of genistein with various disease indications have been conducted to evaluate its clinical effectiveness. Based on many animals and human pharmacokinetic studies, it is well known that the most challenging issue for developing genistein as a chemoprevention agent is the low oral bioavailability, which may be the major reason for its ambiguous therapeutic effects and large inter individual variations in clinical trials [48].

In unfermented soy foods, genistein is the result of sugars forming β-glycosides that cannot be absorbed as such by human intestine. Following ingestion, β-glycoside of genistein is hydrolyzed by intestinal β-glucosidases to respective aglycone genistein, which is then

absorbed, probably by nonionic passive diffusion from the jejunum. Fermented soy products (miso, tempeh, natto) contain larger amounts of isoflavone aglycones due to bacterial hydrolysis, which may influence the bioavailability. Isoflavone aglycones are glucuronidated and, to a smaller extent, sulfated in the intestinal wall and liver.

A fraction of isoflavones undergo enterohepatic circulation in analogy to endogenous estrogens. In blood, the majority of isoflavones are in the form of glucuronide and sulfate conjugates, and a small amount of aglycones are present as both free as well as bound to plasma proteins. The main metabolites of genistein in humans are 7-OH-glucuronic acid and 4-OH-sulfate. These conjugates are eventually excreted in the urine in analogy of human estrogens [49].

Genistein has a diphenol structure that resembles stereo chemically human endogenous E2. The similar distance between the OH groups on the opposite sides of genistein and E2 molecules makes genistein capable of binding to ER subtypes α and β . Genistein, however, binds with higher affinity to ER β than ER α and also increases the binding of ER β to a genomic estrogen response element (ERE) more strongly than with ER α . Nevertheless, in human ER subtype specific reporter cells, genistein is a stronger agonist of ER α . Recently, some studies have shown genistein may promote carcinogenesis in mammary tissues. Therefore, it is crucial to understand the distribution of genistein in breast tissue in order to correlate exposure with its protective or adverse response to breast cancer [50]. Genistein showed relatively low concentrations in the breast tissue, as compared to plasma, after the intake of a high dosage of soy isoflavones in humans which indicates a weak estrogenic response on the breast. In addition to the differential tissue expression of the ER subtypes, ER β often has the opposite effect when compared to ER α . This may explain why the physiological net effect of genistein seems to be partly agonist, initiating estrogen-like actions, partly antagonist, inhibiting estrogen action; this is the case even though genistein has been classified to be a pure estrogen agonist in human cells.

Determining the actual biological net effect of phytoestrogens is complicated by different factors: the route of administration, bioavailability and metabolism, timing and level of exposure, endogenous estrogen state and the various non-hormonal effects. Moreover, genistein combined with DNA methylation inhibitors or other DNMTs can enhance the reactivation of genes silenced by methylation. As evidence of this, Li *et al.* found that

genistein inhibits DNMT1, 3a and 3b and inhibits the expression of hTERT. Genistein also increases acetylation by enhancing HAT activity [45].

2. Daidzein:

Daidzein belongs to the isoflavone family and is the most commonly ingested and most intensely studied type of phytoestrogen. It is often found in nuts, fruits, soybeans, and soy-based products. Previously, daidzein has garnered interest as a non-toxic compound capable of inducing tumor cell death in a variety of cancer types [51]. Also some studies have shown that daidzein causes cell cycle arrest at the G1 and G2/M phases in human breast cancer cells. They have also shown that, while caspase-9 activity was significantly increased by daidzein, cyclin D expression decreased [52, 53]. *In vivo*, 9, 10-dimethyl-1, 2-dibenzanthracene-induced mammary tumors in rats were notably inhibited by daidzein and tumor latency was significantly increased in mouse mammary tumor virus-neu mice. Moreover, one study has revealed that daidzein induced MCF-7 cell proliferation was blocked by treatment with antiestrogen antibody Faslodex (ICI 182780), demonstrating that daidzein-induced stimulatory effect was estrogen receptor (ER) mediated [54]. All of this data and the evidence from *in vitro* and *in vivo* studies indicate that the anti-cancer activity of daidzein in breast cancer is mediated through cell cycle arrest and apoptosis. However, the specific apoptosis mechanisms at work are not yet well understood. Because caspase-9 (an apoptosis biomarker) activity was significantly increased by daidzein, it prompted us to hypothesize that daidzein induces apoptosis through the intrinsic pathway, which regulates apoptotic cascades by signaling convergence in the mitochondrion. Therefore, the aim of the present study was to thoroughly authenticate the original research article of daidzein-induced apoptosis and explore the potential mechanisms at work.

Daidzein can cross the placenta and has been found in breast milk. It is unknown whether daidzein influences early onset of puberty in girls. *In vitro* and *in vivo* studies have found that daidzein stimulates the growth of estrogen-sensitive breast cancer cells. Epidemiologic studies have found conflicting evidence. Some studies have found an association between soy exposure and decreased breast cancer risk while others have found no association [55]. Some epidemiological evidence indicates that soy intake may be more protective when the exposure occurs prior to puberty. More research needs to be conducted on

the association between breast cancer risk and daidzein specifically before conclusions can be drawn. This fact sheet provides information about daidzein, one of three phytoestrogens being measured and examined by the Breast Cancer and the Environment Research Centers (BCERC) epidemiology studies, sources of exposures, effects on puberty, effects in the body, and research studies looking at daidzein as being associated with breast cancer risk [56].

Daidzein is an isoflavone aglycone and is produced in the body from plant isoflavones. Isoflavones are contained in soybean or soy foods in two chemical forms, i.e., aglycones (unconjugated form) and glucosides (bound to a sugar molecule). The main dietary source of daidzein is the biologically active glucoside daidzin. Fermentation or digestion of soybeans or soy products results in the release of the sugar molecule from the isoflavone glycoside, daidzin, leaving the isoflavone aglycone, daidzein [57]. Before daidzein can act it first needs to be released from daidzin. This normally happens in the stomach (acid hydrolysis) and intestine (action of bacterial enzymes). After daidzein is released from daidzin, it may be absorbed into the blood or it may be further metabolized by intestinal bacteria into the metabolites equol and O-desmethylangolensin (O-DMA) [56]. The extent of this metabolism appears to be highly variable among individuals and is influenced by the specific bacteria present in the intestine and other components of the diet. After consuming soy or daidzein, approximately 30%-50% of the population produces equol, and approximately 80%-90% produces O-DMA [56].

Daidzein is also an antioxidant. It is thought that daidzein is a less potent antioxidant than genistein; however, there are few studies comparing the antioxidant activity of the two isoflavones. Equol is a more potent antioxidant than daidzein.

3. *Equol:*

Equol is a metabolite of daidzein that has gained interest due to its possible effects on cancer risk. *In vitro* studies of equol found it to be more biologically active than daidzein, with a higher affinity for the estrogen receptor, and more potent antioxidant activity. This suggests that it may be advantageous to convert daidzein to equol to enhance its estrogenic potency. Equol is a chiral molecule and can exist as two isomers, R- and S-equol. S(-)-equol is the metabolite of daidzein by intestinal bacteria. Equol is expected to prevent hormone-dependent diseases, including breast cancer, due to its ability to bind both ER α and ER β . S(-)-equol is especially known to have a much stronger affinity for ER β compared to R-(+)-equol

[58], and moreover due to its superior anti-oxidative potential to all the isoflavones [59]. In addition, equol binds to sex hormone binding globulin and competitively inhibits estradiol and testosterone binding in a dose-dependent manner. Very little research focusing on equol specifically has been conducted, whereas mechanisms of soy isoflavone on breast cancer have been well done. *In vitro* studies have demonstrated that equol, both racemic and S-equol inhibited the growth of the breast cancer cell line MDA-MB-231 at higher concentrations ($\geq 10 \mu\text{M}$) but in contrast, equol at lower concentrations ($\leq 10 \mu\text{M}$) stimulated the proliferation of ER positive breast cancer cells. The compounds also showed effects in inhibiting the invasion of MDA-MB-231 cancer cells through matrigel [60]. Another study reported that (\pm)-equol had proliferative effects on MCF-7 cell growth *in vitro* within the concentration of plasma equol 2.10-3.21 μM .

v. Studies with Phytoestrogens on breast cancer

Considering the mechanism of action, the phytoestrogens on breast cancer may be mediated via many different mechanisms. Several isoforms of the estrogen receptor can be involved by mechanisms, such as ER β heterodimerisation with ER α , and a consequent reduction in estrogen effects. There are also many studies that have shown that genistein, soy, and rye bran can also cause apoptosis in cancer cells both *in vitro* and *in vivo*.

In addition, we now know that the chemical structure of phytoestrogen resembles that of E2 suggesting that ER-activated genomic and/or nongenomic signaling pathways and might mediate the principal function of phytoestrogen.

The signalling pathways play a role of the apoptosis, invasion, metastasis and proliferation of breast cancer cells. These pathways include nuclear ER (genomic ER)-initiated, membrane ER (non-genomic ER)-mediated, growth factor (GF)-transduction, G protein receptor (GPR)-directed and apoptotic signaling pathways.

To induce the modification of gene expression in breast cancer, nuclear ER α and/or ER β must be activated. Although encoded by unique genes, ER α and ER β have certain functional domains with a highly similar affinity to ligand- and DNA-binding sites. This suggests that these two receptors might play redundant roles. The mammary cell has a predominant distribution of ER α .

In the classic pathway to activate transcriptome, the activated ERs dimerizes and forms a complex for binding to a specific DNA sequence, the ERE.

In non-classic pathway, E2-ER complex interacts with several transcription factors such as Activator Protein 1 (AP1), Specificity Protein 1 (SP1) and NFkB to modulate gene expression without ERE. Another genomic pathway is through a ligand-independence, showing that Growth Factor (GF) activates in intracellular kinase pathway to phosphorylate ER at ERE-containing promoters [61]. Separate from nuclear genomic action, the membrane ER has been demonstrated as a G protein-coupled receptor. The activated membrane ER induces the dislocation of the G-protein α subunit to trigger the activation of Src kinases and PI3 kinase (PI3-K), respectively followed by downstream protein kinase C (PKC) and kinase cascade to extra-cellular regulated protein kinase (Erk), which are involved in proliferation and survival of breast cancer cells. The PI3-K activation also activates the recruitment of AKT, a serine-threonine kinase to control cell survival by activating downstream anti-apoptotic signals (e.g. Bcl-2) and transcription factors (e.g. NFkB and CREB) [62, 63]. In addition, growth factor receptors (GFR), including insulin-like growth factor receptor (IGFR) and epidermal growth factor receptor (EGFR), are the other E2-membrane signalling pathways within breast cancer cells. IGFR mediates the signal pathway through connecting ER α to plasma membrane, activating EGFR, and initiating PI3-K and Erk signalling [64].

In the ER-negative breast cancer cell, to induce the activation of downstream Src-coupled Erk and cAMP/PKA signaling networks via G $\beta\gamma$ subunit protein, E2 directly interacted with GPR30 [62]. The apoptotic signalling pathway is inactivated in most cancer cells, including a group of proteases such as caspase-8 and caspase-3 that cross-talk with EGF survival signalling network [63].

Finally, these mechanisms may occur as protection against cellular proliferation of breast cancers by intracellular mechanisms of phytoestrogen;

1. Binding to nuclear ER and inhibiting genomic ER-mediated gene expression
2. Interaction with membrane ER, blocking protein kinases and suppressing transcription factors
3. Inhibiting GFR activation and its downstream signalling networks
4. Activating caspases to initiate cellular apoptosis
5. Reducing the G-protein mediated signalling pathway in the ER-negative mammary cancer cell.

The nuclear ER interaction is the most completely studied mechanism of the effects of phytoestrogen. The phytoestrogen-rich pueraria mirifica showed a strong competitive binding ability to ER α and/or possibly synthesized suppressor of ER α in the therapy of rat mammary tumor [65]. Phytoestrogen has recently demonstrated to modulate ER α through the activation of ligand-independent pathway.

To summarize, breast cancer is a common malignancy with a high mortality rate in Western population. However, the people in the Asian societies have lower incidences of this cancer than the individuals from the Western societies. This speculation is supported by the finding that the higher the soy food consumption in adolescence, the lower is the risk of breast cancer. Phytoestrogens exhibit a wide array of pharmacologic properties, and recently, interest in the potential benefits of diets high in phytoestrogens has intensified, especially those related to chemoprevention. The link between phytoestrogens and breast cancer prevention has been the subject of numerous studies, and the epidemiology of breast cancer in relation to phytoestrogen consumption has recently been extensively reviewed. Generally, epidemiologic studies have been inconclusive, and the relationship between phytoestrogens and breast cancer prevention remains uncertain. Some studies have revealed the modest protective effects of phytoestrogens; others have detected no association between phytoestrogen intake and breast cancer risk; and a few have reported marked protective effects.

Nevertheless, some evidence suggests that soy intake must be high during certain windows of development, specifically pre-pubescence, in order to gain the protective effects of phytoestrogens. Despite intense investigation, it remains unclear whether phytoestrogens are actually chemo-protective agents or whether their presence is simply a biomarker indicative of a healthy diet.

Additionally, there are many studies about the phytoestrogens on breast cancer in our laboratory. Some of them, after genistein and daidzein 72h exposures, Satih *et al.* observed a restored expression of RAR α in MCF-7, but this was not the case in MDA-MB-231 cells. This observation may be due to the different ER status exhibited by these two cell lines. [66].

Several studies have shown that phytoestrogens may have an impact on the expression of BRCA2. To focus on these processes, they set up the BRCA2 specific

knockdown by RNA interference in two breast tumor cell lines (MCF-7 and MDA-MB-231) and also in a non-tumorigenic breast cell line (MCF-10a). After inhibition of *BRCA2* expression, cells were maintained in different conditions and treated with either daidzein or genistein or left untreated. Microarray analysis of mRNAs isolated from the *BRCA2* knocked down MCF-7, MDA-MB-231, and MCF-10a cell lines after being treated with phytoestrogens showed 35 differentially expressed genes between positive-ER β cells and negative-ER β cells. After genistein or daidzein treatments, *BRCA1* was found to be up-regulated when knocked down with *BRCA2*-siRNA MCF-7 and *BRCA2* was found to be up-regulated when knocked down with *BRCA2*-siRNA MDA-MB 231 cells. In MCF-10a, they observed a significant decrease in *BAX* and *BCL2* expressions with a greater effect of daidzein [44].

Also Bosviel *et al.* investigated the effects of equol on DNA methylation of *BRCA1* and *BRCA2* in breast cancer cell lines (MDA-MB-231 and MCF-7) and in a dystrophic breast cell line (MCF-10a). They demonstrated by quantitative analysis of methylated alleles that a significant decrease in the methylation of the cytosine phosphate guanine (CpG) islands in the promoters of *BRCA1* and *BRCA2* after the S-equol treatment in MCF-7 and MDA-MB-231 cells and a trend in MCF-10a cells. They also showed that S-equol increases *BRCA1* and *BRCA2* protein expression in the nuclei and the cytoplasm in MCF-7, MDA-MB-231 and MCF-10a cell lines by immunohistochemistry. They demonstrated the demethylating effect of S-equol on the CpG islands inside the promoters of *BRCA1* and *BRCA2* genes, resulting in an increase in the level of expressed oncosuppressors in breast cancer cell lines [13].

Additionally, there has been a second study by Bosviel *et al.* about the putative effects on DNA methylation by two naturally occurring isoflavones, genistein and daidzein, in a study of the *BRCA1* and *BRCA2* oncosuppressor genes in breast cancer cell lines (MCF-7, MDA-MB 231, and MCF10a) by methylated DNA immunoprecipitation coupled with PCR. Their results suggest that treatment with genistein or daidzein might reverse DNA hypermethylation and restore the expression of the oncosuppressor genes *BRCA1* and *BRCA2*. These observations, while requiring replication, provide new evidence on potential epigenetic mechanisms by which genistein and daidzein might contribute to regulation of the *BRCA1* and *BRCA2* [67].

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Before we began our first study, we considered the effects of phytoestrogen soybean on breast cancer. We present in this review a summary of the action of phytoestrogens in biological mechanisms and regulations of epigenetic breast cancer.

A high intake of dietary phytoestrogens has been suggested to account for the lower rates of climacteric complaints, cardiovascular diseases, breast and endometrial cancers, and osteoporosis-related fractures in Asian than in Western countries.

Phytoestrogens display an array of pharmacologic properties, and in recent investigation of their potential as anticancer agents has increased dramatically. In this article we review the published literature related to phytoestrogens and breast cancer as well as suggest the possible mechanisms that may underlie the relationship between phytoestrogens and breast cancer.

Review

Breast Cancer: Mechanisms Involved in Action of Phytoestrogens and Epigenetic Changes

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Abstract. *In this review, we consider phytoestrogens and different epigenetic modifications in breast cancer. Epigenetic phenomena are mediated by several molecular mechanisms comprising histone modifications, small non-coding or anti-sense RNA and DNA methylation. These different modifications are closely interrelated. Deregulation of gene expression is a hallmark of cancer. Although genetic lesions have been the focus of cancer research for many years, it has become increasingly recognized that aberrant epigenetic modifications also play major roles in breast carcinogenesis. The incidence and mortality of breast cancer are high in the Western world compared with countries in Asia. There are also differences in the breast cancer incidence rates in different Western countries. This could be related to phytoestrogens.*

Breast cancer remains the first cause of cancer-related mortality in women. This can be explained by the high histological and molecular heterogeneity of the disease, making it hard to choose a therapy adapted to each patient. Over this past year, several groups have evaluated the epigenetic component of breast cancer, as epigenetics appears to be important in carcinogenesis.

The progression through the multiple steps of breast cancer from epithelial hypertrophy to highly invasive breast carcinoma involves multiple coordinated changes in gene

expression programming. Such coordinated changes are bound to be controlled by global mechanisms of gene expression programming. The genome is programmed by the epigenome, which consists of the chromatin structure, a pattern of modification of DNA by DNA methylation, and a profile of expression of noncoding RNAs, such as microRNA. The revolution of epigenetics has revitalized cancer research, shifting focus away from somatic mutation towards a more global perspective involving the dynamic states of chromatin. Disruption of chromatin organization can directly and indirectly precipitate genomic instability and transformation. Epigenetic changes are reversible and may lead to loss or gain of biological functions. More importantly, many of the elucidated epigenetic changes are linked to the pathogenesis of human diseases, including cancer. Epigenetic aberrations arise early in carcinogenesis, before gene mutations in DNA supplies targets for early detection.

The incidence and mortality of breast cancer are high in the Western world compared with countries in Asia. There are also differences in the regional cancer incidence rates in Western countries. Several studies involving immigrants to Western countries suggest that lifestyle and diet are two of the main causes of these differences. In Eastern countries, the incidence of breast cancer is approximately one-third that of Western countries, whilst their high dietary intake of phytoestrogens, mainly in the form of soy products, can produce circulating levels of phytoestrogens that are known experimentally to have estrogenic effects.

Phytoestrogens

Phytoestrogens are plant-derived xenoestrogens functioning like the primary female sex hormone; however, not generated within the endocrine system but consumed by eating phytoestrogenic plants. Also called 'dietary estrogens', they

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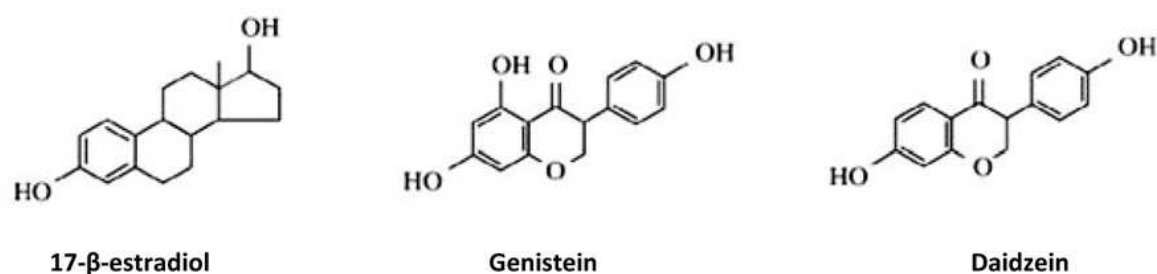


Figure 1. Molecular structure of 17-β-estradiol and isoflavones genistein and daidzein.

are a diverse group of naturally occurring nonsteroidal plant compounds that because of their structural similarity to estradiol (17-β-estradiol) have the ability to cause estrogenic and antiestrogenic effects. An increasing number of epidemiological and experimental studies have suggested that the consumption of a phytoestrogen-rich diet may have protective effects on estrogen-related conditions, such as prostate and breast cancer, osteoporosis and cardiovascular diseases (1). However, concerns have been raised about the potential dangers of consuming high levels of these compounds (2). Consequently, phytoestrogens are currently under active investigation for their role in human health. Phytoestrogen sources can be grouped into three: i: the naturally occurring of steroidal and non-steroidal estrogens; ii: human or animal steroidal estrogens (17-β-estradiol, estrogen sulfate); and iii: estrogenically active compounds arising from fungal attack (3).

Phytoestrogens are nonsteroidal compounds produced by many plants and contained in many natural dietary products, such as soybeans, wheat, barley, corn, alfalfa, and oats. Structurally, they are similar to endogenous estrogens and share a similar mechanism of action through their affinity for binding to estrogenic receptors. Although not steroids, phytoestrogens mimic or antagonize some of the actions of endogenous estrogens, but their potency is much lower than that of steroidal estrogens. Phytoestrogens have been ascribed certain putative health benefits against osteoporosis, heart disease, and some types of cancer (4).

Currently phytoestrogens include more than 100 molecules, divided according to their chemical structure into isoflavones (genistein, daidzein, biochanin A, formononetin) (Figure 1), lignans (matairesinol, secoisolariciresinol-diglucoside), and coumestans (coumestrol, 4-methoxycoumestrol), stilbens (resveratrol).

Some of these substances (*e.g.* resveratrol) act as natural antioxidants and findings concerning their effects in humans especially on the cardiovascular system, have been repeatedly reported in physiological research (4-6).

Mechanistically phytoestrogens have been shown to bind to two types of estrogen receptor: estrogen receptor α (ERα),

which was cloned in 1986, and estrogen receptor β (ERβ) cloned in rats and in humans. The two receptors differ in their tissue distribution and affinity for ligands, yet there is some overlap. In rats, ERα and ERβ both are clearly expressed in ovarian and uterine tissues. ERβ has been shown to have ligand specificity toward phytoestrogens and is distributed in humans in ovary, spleen, testis and thymus and in rats in bladder, brain, lung, ovary, prostate, testis and uterus. Phytoestrogens have a lower binding affinity than 17-β-estradiol and some exhibit a higher binding affinity for ERβ than for ERα, which may suggest different pathways for their actions and explain tissue-specific variability of phytoestrogenic action. The complexity of phytoestrogens and ERs appears to be further compounded because different transcriptional activities *in vitro* are activated depending on the ligands, as well as on the environment of the promoter region of specific genes for translated ERα and ERβ (7).

Isoflavones interact with sex steroids in multiple ways. Influence on the metabolism of sex hormones may be quite complex and may depend on several factors including species, sex, age, hormonal status, etc. Moreover, the dose and duration of isoflavone administration may not be linearly related to the treatment effect, which could add to the significant variability of research findings. Isoflavones were found to inhibit the activity of both 5α-reductase, which catalyzes the conversion of testosterone to 5α-dihydrotestosterone, and aromatase P450, which mediates the conversion of testosterone to 17-β-estradiol (6).

Isoflavones belong to the flavonoid group of compounds, the largest class of polyphenolic compounds. Isoflavones are found in a number of plants including soybeans, fava and beans. Several isoflavones have been investigated and indications are that they have antiangiogenic and anticancer properties. The three major isoflavones found in soybeans are genistin, daidzin, and glycitin. Their abundance in soy protein preparations varies widely and depends on the processing techniques used during production (8). Genistein, a phytoestrogen primarily found in soybeans, is perhaps the most studied of these bioactive compounds. This estrogen-like compounds acts as a chemopreventive agent in several

types of cancer (9). The affinity of genistein for ER β is about 20-30 times higher than that for ER α and is comparable to the affinity of 17- β -estradiol (10). The affinity of other isoflavones is approximately 100-500 times lower than that of 17- β -estradiol. Isoflavones act as agonists of ERs, but their activity is lower than that of 17- β -estradiol. At sufficiently high levels (over about 100 nmol/l for genistein), the effects of isoflavones may approach the effect of endogenous 17- β -estradiol at its physiological level.

Genistein has estrogenic properties in receptor-binding assays, cell culture and uterine weight assays. Genistein inhibits topoisomerase II, platelet-activating factor and epidermal growth factor-induced expression of c-Fos, diacylglycerol synthesis and tyrosine kinases. It also inhibits microsomal lipid peroxidation and angiogenesis. Most of these mechanistic data were derived from *in vitro* studies (11).

Not only do phytoestrogens differ in their biological activity, but they also differ structurally because they come from diverse chemical classes, which may affect their influence on tissues and receptors. Due to the diversity of chemicals that exhibit estrogenic effects, it appears that estrogenic activity is often emphasized over chemical structure in defining phytoestrogens (7).

Phytoestrogens and Breast Cancer

The weak estrogenic action of soy isoflavones and other phytoestrogens suggested the possibility that they could lessen the deleterious effects of more potent endogenous estrogens on breast and endometrial cancer. This hypothesis came from the low incidence of breast and endometrial cancer in Asian countries where soy products are prevalent in the diet and from certain animal models of breast and endometrial cancer showing the benefit of soy isoflavones (8).

In Western countries, breast cancer is the most common type of cancer affecting women. Historically, the risk of breast cancer was much higher in American women than in Asian women prior to the influence of the Western diet on Asian cultures (7). It is known that some types of tumors, such as breast, prostate and colon cancer, have a lower incidence in Asian countries compared to the population of Western countries (6).

At the beginning of the 1980s, it was suggested that lignans and isoflavonoids may prevent breast cancer. This idea led to numerous epidemiological, experimental, casecontrol, and prospective studies investigating the hypothesis (12). In epidemiological studies, associations varied between intake of soy foods and isoflavones and incidence of breast cancer. Much of the epidemiology of breast cancer can be explained by reproductive and hormonal factors; in relation to diet, the only factors definitely related to breast cancer risk are obesity in postmenopausal women and alcohol consumption. Several large prospective studies

have investigated whether high intakes of fruit and vegetables might be associated with a reduced risk of breast cancer, but overall the results are close to null. It seems unlikely that high intakes of fruit and vegetables in general have a significant protective effect, but it is still possible that specific vegetables rich in isoflavones, especially soya beans, might have a protective effect by reducing the estrogenic stimulation of breast cells (13).

Several studies have shown that in breast cancer cells treated with a low concentration of genistein, the promoter of the Glutathione S-transferase Pi 1 (*GSTP1*) gene was demethylated. Moreover, genistein combined with DNA methylation inhibitors or other DNA methyltransferases (DNMTs) can enhance the reactivation of genes silenced by methylation (14). As a result of research in that genistein inhibits DNMT1, 3a -3b and inhibits the expression of Telomerase reverse transcriptase (hTERT). Genistein also increases acetylation by enhancing histone acetyltransferase (HAT) activity. Furthermore, investigations have demonstrated that genistein-mediated hypomethylation and hyperacetylation reactivate the expression of tumor suppressor genes in prostate and breast cancer cells. Genistein and other isoflavones have also been found to regulate miRNA expression in several cancer cell lines (14, 15).

Animals fed with high doses of soybeans exhibited a lower incidence of breast and mammary gland cancer (16). In postmenopausal women, consumption of isoflavones was found to be associated with reduction of breast cancer incidence, mammary gland density and proliferative ability of mammary gland cells (17). These effects have been associated with the ability of isoflavones to increase the sex hormone-binding globulin (SHBG) concentration in serum, thereby reducing the bioavailability of sexual hormones in hormone-dependent tissues (17). Moreover, in peripheral tissues, isoflavones inhibit enzymes involved in the processes of cell proliferation (*e.g.* tyrosine kinase) and reduce estradiol availability through an inhibitory effect on aromatase P450 (6). On the other hand, it has been reported that high doses of genistein may activate cell proliferation in estrogen-dependent tumors (18).

However, despite the observation of inhibition of estradiol-forming enzymes *in vitro*, adding coumestrol or genistein to breast cancer cells in culture does not reduce or prevent estradiol formation (19). The conversion of estrogens to androgens in breast cells is thought to be important for the development of breast cancer. Genistein stimulates several antioxidative enzymes, such as catalase, superoxide dismutase, glutathione peroxidase and reductase, and it is also an inducer of tumor cell differentiation. Genistein down-regulates the epidermal growth factor receptor (EGFR) and (ERBB2/Neu) receptors in cancer cells, and may also inhibit tumor-cell invasion by inhibiting (MMP9) (92 kDa type IV collagenase) and up-regulating tissue inhibitor of

metalloproteinases (TIMP1), and various trypsin inhibitors. Daidzein may also enhance immune function (12).

At the present time, there is a growing number of studies showing that a high soy intake during childhood is associated with reduced breast cancer risk. But there is no convincing evidence to suggest that soy or isoflavone consumption in Western countries during adult life is protective against breast cancer (3, 8, 12). Soy consumption before puberty may have the same risk-lowering effects as an early pregnancy. It is suggested that phytoestrogens promote cell differentiation in the mammary gland, resulting in enhancement of mammary gland maturation.

Epigenetic Changes in Breast Cancer

The molecular mechanisms underlying the development and progression of breast cancer are far from understood. It is evident that the initiation of breast cancer, as well as its transition towards distinct breast cancer subtypes, is triggered by the accumulation of pathologically altered gene functions. As in other types of cancer, an increasing number of deregulated genes subsequently affect virtually all important cellular networks, such as cell cycle control, apoptosis, DNA repair, detoxification, inflammation, cell adhesion and migration. According to the somatic mutation theory, cancer has long been considered as a genetic disorder arising from the fatal acquisition of multiple mutations in key genes which coordinate these functional networks. Such mutations can result either in inactivation of tumor-suppressor genes [*e.g.* TP53, (*BRCA1*)] or activation of protooncogenes [*e.g.* (*MYC*)], both of which contribute to the malignant state of a transformed cell.

The somatic mutation theory of cancer has recently been revolutionized for it became evident that epigenetic misfunction also plays a role in cancer development. The concept of epigenetics describes mitotically stable states and changes of gene activity that do not involve alterations of the primary DNA sequence, thus providing a second layer of information over that of the pure genomic plan (20, 21).

Epigenetic alterations in transformed cells involve changes in DNA methylation including global hypomethylation and locus-specific hypermethylation, altered histone tail modification patterns and nucleosomal remodeling. DNA methylation is an enzyme-driven chemical change to the DNA sequence that most commonly occurs at CpG dinucleotides in mammals (22). DNA hypomethylation can be associated with gene reactivation and chromosomal instabilities and might lead to the up-regulation or overexpression of proto-oncogenes, increased recombination and mutation rates, skewed or loss of X-chromosomal inactivation and loss of imprinting (23). DNA hypermethylation is frequently associated with gene repression and genomic instability and can lead to the

suppression of tumor-suppressor genes and compaction of chromatin (20).

Associated with DNA methylation are post-translational histone tail modifications, another epigenetic mechanism that can modulate chromatin structure to regulate gene expression (24). In addition, it has been shown that some regulators that control nucleosomal remodeling are also involved in the regulation of DNA methylation and histone modification (25, 26). An understanding of all these epigenetic changes and their contributions to breast tumorigenesis is very important for further progress in the field of diagnosis, prognosis and therapy of breast cancer.

DNA Methylation in Breast Cancer

DNA methylation patterns highlight significant differences in breast cancer between tumor tissues and corresponding normal tissues (27). A common paradox observed in carcinomas is that despite regional hypermethylation of tumor-suppressor genes, the global 5-methylcytosine content is drastically-reduced in the bulk of the tumor genome (26). Less frequent than regional DNA hypermethylation, regional DNA hypomethylation can also occur in cancer, resulting in the activation of potential oncogenes (27, 28).

DNA hypermethylation. DNA hypermethylation indicates which genes are turned off in breast tumors and a unique pattern is observed in breast tumors. Interestingly, 40% loss of methylated cytosine is observed in breast tumors. Important genes in familial breast cancer are also epigenetically silenced. In sporadic tumors, *BRCA1* expression has been shown to be suppressed by a combination of gene deletion and epigenetic silencing *via* DNA hypermethylation (29).

Ectopic cytosine hypermethylation is generally associated with transcriptional repression and ultimately tumor formation. Collaboration between genetic and epigenetic breast cancer causes has been directly demonstrated by example in which one tumor-suppressor allele is inactivated by mutation and the other allele is transcriptionally silenced because of hypermethylation (30). In addition hypermethylation of tumor-suppressor genes may be an early event in cancer development (31, 32), suggesting that epigenetic and mutational cancer causes may collaborate from an early time point in disease progression (32). The list of tumor-suppressor genes found transcriptionally inactivated by hypermethylation in cancer is long and steadily growing (33), and includes genes that are part of every cancer-related pathway, including important genes such as (*CDKN2A*), (*pRB*), (*APC*), (*PTEN*), (*BRCA1*), (*VHL*) and (*CDHI*). By extension, epigenetic silencing may underlie genetic cancer causes. Epigenetic induction of a classical mutator phenotype *via* transcriptional inactivation of the DNA mismatch repair gene (*MLH1*) has been proposed to account for microsatellite instability in colorectal cancer and

silencing of the DNA repair gene coding for O-6-methylguanine-DNMT has been associated with specific mutations in (*K-RAS*) and *p53* (34, 35).

DNA Hypomethylation. Among solid tumor types, global DNA hypomethylation is most evident in breast cancer with up to 50% of cases showing reduced 5-methylcytosine content when compared with normal tissue counterparts (36). Hypomethylation in breast cancer mainly affects iterative DNA sequences and pericentromeric satellite DNA, which are normally heavily methylated in non-malignant cells (35).

Although a relatively rare event, DNA hypomethylation can also affect individual genes. In breast cancer, this is the case for the melanoma-associated cancer/testis antigens MAGE. The MAGE gene family encodes for (HLA)-restricted tumor-associated rejection antigens recognized by cytotoxic T-lymphocytes. Some of these target antigens may be potentially useful for cancer-specific immunotherapy. The expression of MAGE genes has been reported not only in melanoma but also in various other malignant tumors, such as hepatocellular carcinoma and germ cell tumors. Genes for MAGE antigens are methylated and silenced in adult tissues, but hypomethylated and expressed in several tumors and breast cancer cells (37). Other hypomethylated genes in breast tumors include the gene encoding the plasminogen activator uPA (*PLAU*), the breast cancer-specific protein 1/synuclein- γ gene (*SNCG*), and more recently reported, the multidrug-resistance 1 gene (*MDR1*) (38, 39).

Global DNA hypomethylation is a hallmark in human cancer, but its functional consequences are unclear (39). The mechanism of global hypomethylation is a long-standing question in cancer epigenetics (40).

Histone Modifications in Breast Cancer

A number of studies have investigated the use of histone modifications as biomarkers in tumors. High relative levels of global histone acetylation and methylation were associated with a favorable prognosis, and were detected almost exclusively in luminal-like breast tumors (93%). Clustering analysis identified three groups of histone status patterns which correlate with clinical outcome (41).

Histone acetylation. Histone acetylation is a dynamic process directed by HATs and histone deacetylases (HDACs). Normally, transcription factors recruit coactivators with HAT activity to regulatory DNA sites, whereas transcriptional repressors recruit co-repressors with HDAC activity.

Many HATs have also been shown to be involved in breast cancer. Among of them, p300/CREB-binding protein (CBP) and nuclear receptor coactivator family (NCOAs) are the most important and well-characterized HAT proteins associated with breast cancer (42).

p300 and its close homolog CBP are often referred to as a single entity. A role for p300 in tumor suppression has been proposed by the fact that disturbance of p300 function by viral oncoproteins is essential for the transformation of rodent primary cells and, consistent with this hypothesis, mutations of *p300* have been identified in certain types of human cancer, including breast carcinoma (43). Both the localization of p300 and the recruitment to aggresomes differ between breast cancer and normal mammary glands. The expression level of p300 in breast cancer epithelia is higher than that in normal mammary gland (44).

Cytoplasmic localization of p300 was also observed in tumor epithelia whereas nuclear localization was found in normal mammary glands in both animal models and in non-malignant adjacent areas of human breast cancer specimens. Proteasomal inhibition induced p300 redistribution to aggresomes in tumor but not in normal mammary gland-derived cells (45). The regulation of gene expression by nuclear receptors (NRs) controls the phenotypic properties and diverse biologies of target cells. In breast cancer cells, ER α is a master regulator of transcriptional stimulation and repression (46).

Upon 17- β -estradiol treatment, gene transcription is widely impacted, creating highly complex regulatory networks whose ultimate goal is the stimulation or suppression of specific biological processes. p300/CBP can function as a transcriptional cofactor of ERs and other nuclear hormone receptors (47).

The NCOA family, also named as p160 or steroid receptor coactivator, contains three homologous members: NCOA1 (SRC-1), NCOA2 (SRC-2, GRIP1 or TIF2) and NCOA3 (SRC-3, p/CIP, RAC3, ACTR, AIB1 or TRAM-1). These three members have an overall sequence similarity of 50-55% and sequence identity of 43-48%. As well as being NRs, NCOAs also serve as coactivators for many other transcription factors associated with breast cancer, such as (HIF1), (NF- κ B), (E2F1), p53, RB and (MRTFs) (48, 49). By regulating a broad range of gene expression controlled by NRs and non-NR transcription factors, NCOAs regulate diverse events in the development of breast cancer. NCOA1 is overexpressed in 19% to 29% of breast cancer cases and plays important roles in cell proliferation, lymph node metastasis, disease recurrence and poor disease-free survival (DFS) (50). Therefore, elevated expression of NCOA1 has been regarded as an independent predictor of breast cancer recurrence following therapy (51). Although the evidence is not conclusive, NCOA2 overexpression might also promote proliferation and invasion of breast cancer cells. In addition, NCOAs play important roles in the chemotherapy resistance of breast cancer. Increased expression levels of the ER-NCOA3 complex were found in tamoxifen-resistant cells, and such overexpression of NCOA3 could enhance the agonist activity of tamoxifen and, therefore, reduce its antitumor activity in patients with breast cancer (52).

The 18 HDACs identified so far can be categorized into four classes: class I (HDAC 1-3, HDAC 8), class II (HDAC 4-7, 9-10), class III (Sirtuin 1-7) and class IV (HDAC 11). Class I, II and IV HDACs share homology in both sequence and structure and all require a zinc ion for catalytic activity (53). HDACs remove the acetyl groups from histone lysine tails and are thought to facilitate transcriptional repression by reducing the level of histone acetylation. Like HATs, HDACs also have non-histone targets. Several HDACs have been found to be involved in breast cancer. In ER-positive MCF-7 breast cancer cells, expression of HDAC6 was increased after treatment by estradiol, and elevated expression of *HDAC6* increased deacetylation of alpha-tubulin and increased cell motility (54).

In vivo assays showed that patients with high levels of *HDAC6* mRNA tended to be more responsive to endocrine treatment than those with low levels, indicating that the level of HDAC6 expression might be used as both as a marker of endocrine responsiveness and also as a prognostic indicator in breast cancer (48, 55). However, HDAC1, Sirtuin3 (SIRT3) and SIRT7 are all overexpressed in breast cancer (56, 57). HDAC4 overexpression and mutations have also been found in breast cancer samples (57).

Histone methylation. Histones can be mono-, di-, or trimethylated at lysine or arginine residues by histone methyltransferases (HMTs). Many HMTs, including both lysine-specific HMTs (*e.g.* SMYD3) and arginine-specific HMTs (*e.g.* PRMT1 and CARM1), have been shown to act as ER coactivators and be involved in breast cancer.

Many histone lysine methyltransferase (HKMTs) have been isolated and characterized. Except for (Dot1), all HKMTs contains a conserved Su(var), enhancer of zeste, trithorax (SET) domain that is responsible for catalysis and binding of cofactor S-adenosyl methionine, and many have been shown to play roles in breast cancer (58).

Other methyltransferase mechanism attack of the breast cancer is arginine methyltransferase (HRMT). The protein arginine methyltransferase (PRMT) family is the major family of HRMTs to date. The PRMTs are classified into four groups depending on the type of methylarginine they generate: Type I (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8), type II (PRMT5, PRMT7 and PRMT9), type III (remained unclear) and type IV (only found in *Saccharomyces cerevisiae* as yet) (59).

Compared to HKMTs, the evidence for the involvement of HRMTs in human cancer is not forthcoming. However, underexpression of PRMT1 has been observed in breast cancer (60). PRMT4, also known as coactivator-associated arginine methyltransferase-1 (CARM1), is a coactivator for nuclear receptors and is overexpressed in prostate and breast cancer (61). PRMT4 plays an important role in estrogen-induced cell cycle progression in the MCF-7 breast cancer

cell line. Upon estrogen stimulation, the E2F1 promoter is subject to PRMT4-dependent dimethylation on H3R17, and this recruitment of PRMT4 by ER α is dependent on the presence of NCOA3 (62).

Histone phosphorylation. Phosphorylation of H3 on S10 and S28 is important not only during mitotic chromosome condensation but also in transcriptional activation of immediate early genes and growth factors stimulating the (RAS)/(MAPK) and increasing H3 pS10 at transcriptionally active loci may contribute to aberrant gene expression and breast cancer progression (63).

Other histone modifications in breast cancer. Besides acetylation, methylation and phosphorylation, there are some other modifications which occur of histone. These epigenetic changes include ubiquitination/sumoylation, ADP-ribosylation, deamination, and proline isomerization. Although the functions and mechanisms have not been demonstrated, some studies have shown that these modifications are also associated with breast cancer and other types of human cancer.

Recent studies revealed that E3 ubiquitin ligases play important roles in breast carcinogenesis. Ubiquitin-mediated protein degradation plays an important role in many cancer-related cellular processes. E3 ubiquitin ligases play critical roles because they control substrate specificity of histone. Accumulating evidence suggests that genetic and expression alteration of E3 ubiquitin ligases contributes to breast carcinogenesis (64).

(miRNAs) in Breast Cancer

miRNAs have been shown to play a critical role in the regulation of a wide range of biological and pathological processes. Recent large-scale profiling approaches have revealed that miRNAs are globally down-regulated in several cancer types, including breast cancer. Moreover, panels of miRNAs in breast carcinomas characteristic for the HER2/neu or ER status of the analyzed tumor have been detected (65). There is now increasing evidence that signatures of miRNA expression may not only be used in the future as tumor biomarkers for diagnosis and patient risk stratification, but since hypermethylation was identified as an important mechanism of miRNA silencing, deregulated miRNAs may also represent novel targets for an anticancer therapy. One study describing differential expression patterns of miRNAs in breast cancer also investigated their expression changes in relation to chromosomal localization. Interestingly, the authors found several miRNA candidates that reside in chromosomal regions which are either frequently deleted or amplified in breast cancer, *e.g.* down-regulation of miR-125b in the frequently deleted region 11q-23-24, or overexpression of miR-21 in 17q23, which is commonly amplified in breast cancer

(66). Another mechanism by which miRNA profiles may be altered in tumors lies within abnormalities in the miRNA-processing machinery. It was the same study that observed significant changes in expression of Dicer and (AGO1), both being involved in miRNA maturation processes (67). Decreased Dicer expression was recently observed in breast cancer, where loss of expression represented an independent prognostic factor in metastatic disease, and reduced expression of Dicer was associated with the highly aggressive mesenchymal phenotype (68). Whether genetic lesions such as (TARBP2) mutations in colorectal cancer also account for impaired miRNA processing in breast cancer remains to be determined in future studies. It is, however, conclusive that besides DNA hypermethylation of miRNA genes, structural genetic alterations also contribute to the observed dramatic changes of miRNA expression profiles in human cancer.

Conclusion

Phytoestrogens seem to protect against breast cancer if consumed throughout life, particularly before and during adolescence, and a low plasma enterolactone concentration is known to increase risk of breast cancer. Whether phytoestrogens are actually responsible for the protection is not known, it is more likely that the soybean products or grain-fiber complexes are protective in their entirety.

Research in phytoestrogens has increased dramatically in recent years as seen by the numerous publications. However, many questions remain. Research is still needed to evaluate the safety of phytoestrogens on human systems, beneficial and harmful doses, gender differences in response to phytoestrogens, differences in the chemical classes of phytoestrogens and the effects phytoestrogens may have with other drugs or dietary products. Due to the functional and structural differences of phytoestrogens, their biological activities are also highly variable and there may be other effects that have not yet been studied.

In summary, epigenetic modifications provide crucial regulatory functions in the process of gene transcription, and they play very important roles in the proliferation, metastasis, chemotherapy and other aspects of breast cancer, as well as in many other types of human cancer. An understanding of all these epigenetic changes and their contribution to breast cancer might allow great progress in the field of diagnosis, prognosis and therapy of breast cancer. We may thus hope that many of the open questions about the impact of phytoestrogen and epigenetics will be answered in the near future.

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Conclusion of Publication I:

In this review we reported which phytoestrogens were more important for breast cancer and what are the possible mechanisms of phytoestrogen on breast cancer. We reported that phytoestrogens soy, genistein and daidzein exert protective effects vis-a-vis breast cancer. However, some studies focus on the ambivalent role of phytoestrogens. It has indeed been shown that phytoestrogens can induce both proliferation and inhibition of the growth of breast tumor cells. Research is still needed to evaluate the safety of phytoestrogens on human systems, beneficial and harmful doses, gender differences in response to phytoestrogens, differences in the chemical classes of phytoestrogens and the effects phytoestrogens may have with other drugs or dietary products. Due to the functional and structural differences of phytoestrogens, their biological activities are also highly variable and there may be other effects that have not yet been studied.

After understanding the effects of phytoestrogens on breast cancer, we started to study epigenetics mechanisms and we also investigated the interaction between epigenetics and breast cancer. Different mechanisms are involved in the maintenance of epigenetic states. Studies discussed herein have shown that dietary factors are likely to contribute to epigenetic alterations and in some cases may be able to reverse abnormal epigenetic states. We saw that there are all three mechanisms are very important for breast cancers which are DNA methylation, histone modification and miRNAs. Nutriepigenomic studies focusing on personal responses to bioactive elements and personalized epigenetic diets consisting of bioactive dietary factors mentioned herein will be of particular interest in the future.

C. Epigenetic Changes

i. General description

The term epigenetics was first used by Conrad Hal Waddington as "the causal interactions between genes and their products, which bring the phenotype into being". This definition initially referred to understanding chromatin structure and its impact on gene function. The current definition of epigenetics is "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence". Epigenetic mechanisms that occur these expression patterns have been divided into three main categories: DNA methylation, covalent histone modifications and non-coding RNAs including microRNAs (miRNAs). Aberrant epigenetic alterations in the genome such as DNA methylation and chromatin remodeling play a significant role in breast cancer development [68].

DNA methylation is the most-studied epigenetic factor referring to the modification of cytosine residues by methylation at their carbon-5 position. In mammalian genomes, this modification primarily occurs on cytosine residues that are located 5' to guanine i.e. CpG dinucleotides. DNA methylation is catalyzed by DNMTs. In humans, at least, three DNMTs are involved in establishment and maintenance of DNA methylation patterns; *de novo* DNA methyltransferases DNMT3A and DNMT3B and maintenance DNA methyltransferase DNMT1 . A fourth enzyme previously known as DNMT 2 is not a DNA methyltransferase. CpG dinucleotides are not equally distributed across the human genome but are concentrated in distinct CpG-rich regions called "CpG islands" and in regions of large repetitive sequences [69]. DNA methylation also provides to other X-chromosome inactivation and genome stability [70]. DNA methylation patterns are reliably hereditary during both mitosis and meiosis. Also some studies have shown that while some CpG regions are stably methylated, a small number of dynamic methylated regions could play a major role in controlling the transcription network of cells [71]. Failure to maintain correct methylation patterns leads to aberrant DNA methylation, often observed in human diseases including neurodevelopmental defects, neurodegenerative, neurological and autoimmune diseases, and cancers.

Chromatin is a highly dynamic structure and must be kept in balance by being folded as much as needed and being accessible whenever necessary to cope with genome templated processes such as replication, transcription and DNA repair. The functional state of chromatin is partially regulated through posttranslational modifications of histones [72]. The amino-tails of histones in the nucleosomes can be modified in a number of modifications, for example; methylation, acetylation, phosphorylation, ubiquitination and sumoylation. Thereby these modifications are involved in regulating the gene expression.

The interactions between DNA methylation and histone modifications mediate gene silencing through recruitment of repressive complexes and methylated DNA binding proteins.

miRNAs are small non-coding RNAs of 20–22 nucleotides that inhibit gene expression at the posttranscriptional level. miRNAs are involved in the regulation of key biological processes, including development, differentiation, apoptosis and proliferation and play a critical role in the regulation of a wide range of biological and pathological processes. Each miRNA is expected to control several hundred genes. They have been implicated in cancer initiation and progression, and their expression is often down-regulated during carcinogenesis. Major mechanisms of miRNA deregulation include genetic and epigenetic alterations as well as defects in the miRNA processing machinery

Presentation of Publication 2:

Dagdemir A, Durif J, Ngollo M, Bignon Y-J, Bernard-Gallon D. Epigenetic Mechanisms that Operate in Mammals. Applied Cell Biology. 2013; 1(1):1-9.

Understanding the process of epigenetic reprogramming in development is important for studies of breast cancer. This landscape will summarize current knowledge on interactions between cancer cell metabolism and epigenetic modulation of gene regulation, and how both processes can be affected by dietary components. A greater understanding of these processes is also of clinical importance.

In this review, we will discuss the different layers of epigenetic regulation, including writer enzymes for DNA methylation, histone modifications, non-coding RNA, and chromatin conformation. We will highlight the combinatorial role of these structural and chemical modifications along with their partners in various cellular processes. The fate of any given cell is determined by epigenetic mechanisms. Each cell type in an organism has its own epigenetic signature that depicts genotype, developmental history and environmental influences and in the end leads to the phenotype of the organism. Various cell types, including neurons, muscle cell and lymphocytes, derive from a fertilized oocyte all carrying the same DNA sequence but are obviously distinct from each other. Once acquired a certain identity it is important for a cell to remember its status.

Although pluripotent stem cells or progenitor cells can be used in cell-replacement therapy to treat degenerative diseases, new drugs that target epigenetic regulators can be developed to treat developmental disorders or cancers that are caused by altered epigenetic states.

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Review

Epigenetic mechanisms that operate in mammals

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ABSTRACT

Epigenetic mechanisms act to change the accessibility of chromatin to transcriptional regulation locally and globally *via* modifications of the DNA and by modification or rearrangement of nucleosomes. Epigenetic changes can be defined as stable molecular alterations such as the gene expression they are heritable during cell divisions but do not involve changes in the DNA sequence. Epigenetics consist in several molecular mechanisms: histone modifications, small non-coding or antisense RNAs and DNA methylation; that are closely interconnected.

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KEYWORDS

Epigenetics;
DNA methylation;
Histone modification;
Non coding RNAs;
miRNA.

INTRODUCTION

Historically, the word “epigenetics” was used to describe events that could not be explained by genetic principles.

Conrad Waddington (1905–1975), who is given credit for coining the term, defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being”^[1].

Much of today epigenetic research is converging on the study of covalent and noncovalent modifications of DNA and histone proteins and the mechanisms by which such modifications influence overall chromatin structure. Chromatin, the complex of DNA and its intimately associated proteins, provides an attractive candidate for shaping the features of a cell epigenetic landscape.

Epigenetic phenomena have recently led researchers to conserved molecular mechanisms involving chromatin modification, a theme reinforced throughout this special issue. We favor the view that the macromolecular entities described below all significantly contribute to the physiologically relevant organization of most eukaryotic genomes. These entities, and possibly others yet unknown, should be considered collectively when exploring epigenetic mechanisms.

EPIGENETICS

In biology and specifically genetics, epigenetics is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms, other than changes in the underlying DNA sequence. It refers to functionally relevant modifications to the genome that

do not involve a change in the nucleotide sequence. Epigenetics refer to changes in phenotype and gene expression that occur without alterations in DNA sequence. Epigenetic modifications of the genome can be acquired de novo and are potentially heritable. These changes may remain through cell divisions for the remainder of the cell life and may also last for multiple generations. However, there is no change in the underlying DNA sequence of the organism, instead of non-genetic factors cause the organism genes to behave differently.

There are three major mechanisms of epigenetic regulation, including methylation of CpG islands, mediated by DNA methyltransferases, modification of histone proteins, and microRNAs. There are substantial interactions between these epigenetic mechanisms (Figure 1).

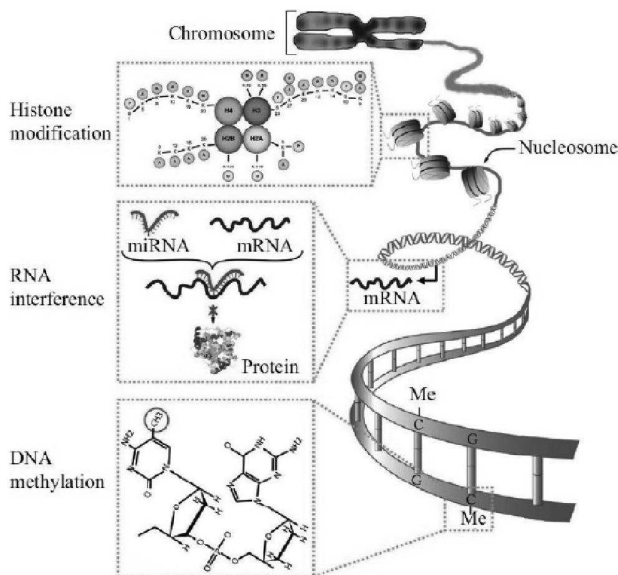


Figure 1 : Schematic of the mechanisms of epigenetic regulation. DNA methylation, histone modification and RNA-mediated gene silencing constitute three distinct mechanisms of epigenetic regulation. DNA methylation is a covalent modification of the cytosine (C) that is located 5' to a guanine (G) in a CpG dinucleotide. Histone (chromatin) modifications refer to covalent post-translational modifications of N-terminal tails of four core histones (H3, H4, H2A, and H2B). The most recent mechanism of epigenetic inheritance involves RNAs^[2].

DNA METHYLATION

DNA methylation is the most studied mechanism, provides suppression of gene expression, embryonic

development, transcription, chromatin structure, X-chromosome inactivation, genomic imprinting regulation and functional preservation of the stability of chromatin.

In mammals, methylated cytosine is predominantly observed in the context of CpG dinucleotides and is involved in a range of processes including embryogenesis, genomic imprinting and tumorigenesis.

DNA methylation is an essential component of the epigenetic machinery in regulating gene expression and interacting with nucleosomes that control DNA packaging, affecting entire domains of DNA^[2].

The only known epigenetic modification of DNA in mammals is methylation of cytosine at position C₅ in CpG dinucleotides^[3]. By contrast, the other main group of epigenetic modifications (the post-translational modification of histones) shows a high level of diversity and complexity^[4]. The mammalian DNA methylation machinery is composed of two components, the DNA methyltransferases (DNMTs), which establish and maintain DNA methylation patterns, and the methyl-CpG binding proteins (MBDs), which are involved in reading methylation marks^[5]. DNA methyltransferases (DNMTs) catalyze genome-wide DNA methylation and are associated with histone modifying enzymes (*e.g.* histone deacetylases (HDACs)), histone methyltransferases (SUV (39) H1/2 and EZH2), and ATP dependent chromatin remodeling enzymes^[6] (Figure 2). The methyl moiety of methyl cytosine resides in the major groove of the DNA helix, where many DNA-binding proteins make contact with DNA, and exerts its effect by attracting or repelling various DNA-binding proteins. A family of proteins that can bind to DNA containing methylated CpG dinucleotides, known as methyl-CpG-binding proteins, have been shown to recruit repressor complexes to methylated promoter regions and thereby contribute to transcriptional silencing. Certain transcription factors bind to CpG-containing DNA sequences only when they are unmethylated.

DNA methylation may affect the transcription of genes in two ways. First, the methylation of DNA itself may physically impede the binding of transcriptional proteins to the gene, and second, and more important, methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs). MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodeling proteins that can modify histones, thereby

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forming compact, inactive chromatin, termed heterochromatin^[7] (Figure 2).

Maintenance methylation activity is necessary to preserve DNA methylation after every cellular DNA replication cycle. Without the DNA methyltransferase (DNMT), the replication machinery itself would produce daughter strands that are unmethylated and, over time, would lead to passive demethylation.

DNMT1 is the proposed maintenance methyltransferase that is responsible for copying DNA methylation patterns to the daughter strands during DNA replication. Mouse models with both copies of DNMT1 deleted are embryonic lethal at approximately day 9, due to the requirement of DNMT1 activity for development in mammalian cells^[7].

It is thought that DNMT3a and DNMT3b are the *de novo* methyltransferases that set up DNA methylation patterns early in development. DNMT3L is a protein that is homologous to the other DNMT3s but has no catalytic activity. Instead, DNMT3L assists the *de novo* methyltransferases by increasing their ability to bind to DNA and stimulating their activity. Finally, DNMT2

(TRDMT1) has been identified as a DNA methyltransferase homolog, containing all 10 sequence motifs common to all DNA methyltransferases. However, DNMT2 (TRDMT1) does not methylate DNA but instead methylates cytosine-38 in the anticodon loop of aspartic acid transfer RNA^[7-9].

HISTONE MODIFICATION

Chromatin proteins serve as building blocks to package eukaryotic DNA into higher order chromatin fibers. Each nucleosome encompasses approximately 146 bp of DNA wrapped around an octamer of histone proteins. These octamers consist of double subunits of H2A, H2B, H3, and H4 core histone proteins (Figure 1). The histone proteins coordinate the changes between tightly packed DNA (heterochromatin) that is inaccessible to transcription and exposed DNA (euchromatin) that is available for binding and regulation of transcription factors^[10,11] (Figure 3). These changes occur due to structural characteristics of the nucleosome that are known as “histone tails,” which extend from the core octamer. These tails consist of N-termini of the histone proteins and are the major sites for post-translational modifications.

Chromatin is a highly dynamic structure and must keep the balance between being folded as much as needed and being accessible whenever necessary to cope with genome templated processes such as replication, transcription and DNA repair. The functional state of chromatin is partially regulated through post-translational modifications (PTMs) of histones. Thereby these modifications are involved in regulating the gene expression. Numerous types of histone modifications exist and they are divided into two groups. First group

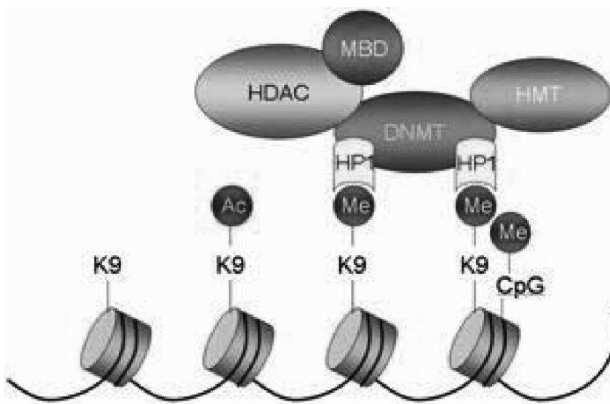


Figure 2 : Schema of the DNA methylation components^[35]

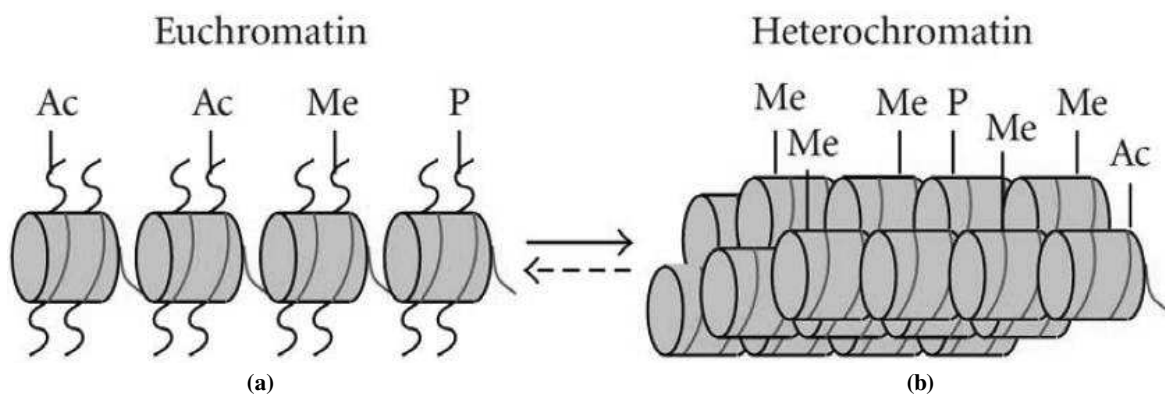


Figure 3 : Euchromatin and Heterochromatin. Histone tails have three types of modification including acetylation (Ac), phosphorylation (p), and methylation (Me)^[14].

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belong acetylation of lysines, phosphorylation of serines and threonines and methylation of arginines and lysines as they convey small chemical groups. Second, there are larger peptides such as ubiquitination and sumoylation of lysines and ADP-ribosylation of glutamic acid (TABLE 1). There are several mechanisms how histone posttranslational modifications can influence chromatin. First, histones and their modifications can alter the chromatin structure and thus regulate DNA accessibility. The other one is PTMs on histones facilitates the binding of a protein to chromatin by creating a specific binding site^[12,13].

Acetylation of histone proteins correlates with transcriptional activation and a dynamic equilibrium of histone acetylation is governed by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation status results from an intricate cross-talk between HATs and HDACs^[2]. HDACs are a class of enzymes catalyzing the opposite action to HATs. They influence a myriad of cell processes including signal transduction, apoptosis, cell cycle regulation, and cell growth^[15]. HDACs catalyze deacetylation of both histone and non-histone proteins and, similar to HATs, can be either nuclear or cytoplasmic. Aside from histones, many transcriptional regulators, chromatin modifiers, and intracellular signal transducers are posttranslational modified by acetylation. Importantly, HDACs are associated with a number of other epigenetic repression mechanisms, including histone methylation^[16], PcG-mediated repression^[17], and DNA methylation. Importantly, HDAC activity is often crucial to prepare the histone template for methyltransferases by removing acetyl groups obstructing methylation^[11].

Histone methylation also plays a major role in gene expression regulation^[14]. Histone methylation is associated with transcriptional repression or activation depending on the affected specific amino acid. For example, methylation of histone H3 lysines 4 and 36 is associated with active gene expression. However methylation of histone H3 lysines 9 and 27 is associated with gene silencing. Histone methylation is catalyzed by a large number of enzymes. Similar to acetylation/deacetylation, histone methylation is reversible and catalyzed by 2 families of histone demethylases (HDMTs), namely the lysine-specific demethylase 1 (LSD1) and the Jumonji domain-containing enzymes^[18,19]. Histone

methylases and HDMTs are usually part of large protein complexes that regulate gene transcription^[2,3,11].

Phosphorylation is in addition to acetylation and methylation of histone. Important progress has also been made in the studies of other types of covalent modifications including phosphorylation of histone H3 at Ser10 (H3-S10)^[20]. This has important implications regarding signal transduction. It suggests that the mainly cytoplasmic protein phosphorylation cascades that have dominated signal transduction processes for many years may have a more direct effect on gene expression through the phosphorylation of chromatin^[10]. Also phosphorylation in chromatin generates a barrier for the repair of DNA damage. Two phosphorylation sites on this histone have a role in doublestrand break repair via nonhomologous end joining: H2AS129 mediated by Mec1 and H4S1 mediated by Caesin kinase II^[21].

Ubiquitylation very large modification has been found on H2A (K119) and H2B (K20 in human and K123 in yeast)^[22]. A role for this modification has been demonstrated in transcriptional elongation by the histone chaperone fact^[23]. How ubiquitylation functions is unclear. It is likely to recruit additional factors to chromatin but may also function to physically keep chromatin open by a “wedging” process, given its large size^[10,24].

Sumoylation is like ubiquitylation. Sumoylation is a very large modification and shows some low similarity to ubiquitylation. This modification has been shown to take place on all four core histones, and specific sites have been identified on H4, H2A, and H2B^[25]. Sumoylation antagonizes both acetylation and ubiquitylation, which occur on the same lysine residue, and consequently this modification is a repressive for transcription in yeast.

ADP Ribosylation this histone modification is badly defined with respect to function. ADP ribosylation can be mono- or poly-, and the enzymes that mediate it are MARTs (Mono-ADP-ribosyltransferases) or PARPs (poly-ADP-ribose polymerases), respectively^[26]. In addition the Sir families of NAD-dependent histone deacetylases have been shown to have low levels of this activity, so they may represent another class of this family. There are many reports of ADP ribosylation of histones, but only one site, H2BE2ar1, has been definitively mapped. Although the function of the enzymes has often been linked to transcription, evidence that the catalytic activity is involved has been lacking.

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Recently, a role for PARP-1 activity in transcription has been demonstrated but only under conditions where DNA repair is induced. Double-strand breaks mediated by Topoisomerase II b activate the PARP-1 enzyme, which then directs chromatin changes to the estrogen-regulated *PS2* gene^[27].

TABLE 1 : Different classes of modifications identified on histones^[10,13,28]

Chromatin Modifications	Modified Residues	Regulated Functions
Acetylation	K-ac	Transcription Repair, Replication, Condensation
Methylation (lysines)	K-me1 K-me2 K-me3	Transcription Repair
Methylation (arginines)	R-me1 R-me2a R-me2s	Transcription
Phosphorylation	S-ph T-ph	Transcription Repair Condensation
Ubiquitylation	K-ub	Transcription Repair
Sumoylation	K-su	Transcription
ADP ribosylation	E-ar	Transcription
Deimination	R > Cit	Transcription
Proline Isomerization	P-cis > P-trans	Transcription

NONCODING RNAs

Non-coding RNA (ncRNA) molecules are those RNAs that do not encode proteins, but instead serve some other functions in the cell. Small noncoding RNAs refer to a family of RNAs that, by complementarity to the 3' untranslated region of messenger RNAs, lead to their degradation and subsequent inhibition of gene expression^[29]. Part of this family of noncoding RNAs are 20- to 22- nucleotide microRNAs (miRNAs), resulting from the sequential splicing of primary then pre-RNAs. miRNAs are involved in post-transcriptional control of gene expression. miRNAs transcripts are generated either by RNA polymerase II or III as long primary transcripts (pri- miRNAs) carrying the mature miRNA sequence in a stem loop structure^[30].

In the nucleus, cleavage of the pri-RNA stem loop by the RNase III endonuclease DROSHA releases a 60-70 nucleotide long precursor RNA, called pre-

RNA, which is subsequently transported into the cytoplasm and is further processed by Argonaute2 and DICER^[31]. One strand of the DICER cleavage product carrying the mature miRNA sequence is further incorporated into the RNA-induced silencing complex (RISC)^[32]. Due to the complementarities of miRNAs to the 3' UTR of mRNAs, the active RISC recognizes target transcripts and promotes translation inhibition or mRNA destabilization, both resulting in the reduction of target protein level^[32,33]. miRNAs are then incorporated in the RNA-induced silencing complex and transported back in the nucleus, where they exert their biological effect. Through Watson-Crick base pairing, miRNAs bind to complementary sequences of mRNAs and induce either degradation or translational silencing of the target mRNAs^[2]. Small RNA pathways are often entangled. Despite our growing understanding of the mechanism and function of small RNAs, their evolutionary origins remain obscure. It is interesting to note that miRNAs are also themselves epigenetically regulated at their promoter level, and target many genes that play important roles in such processes as cell cycle progression, apoptosis, and differentiation. A single miRNA can have hundreds of target mRNAs, highlighting the implication of this gene regulation system in cellular functions^[32]. The study of miRNAs has become the subject of intense interest, especially after the discovery of the fundamental role of these small, noncoding RNAs in a countless of cellular and biological processes ranging from development to disease states^[29,32,33].

ncRNAs appear to comprise a hidden layer of internal signals that control various levels of gene expression associated with physiological and developmental processes. ncRNAs, especially small ncRNAs, play a significant role in cellular physiology, specifically, epigenetic regulation of gene expression. Epigenetic regulation is a heritable change in gene expression that cannot be associated with genetic variation.

Approximately 1,000 miRNA genes have been computationally predicted in the human genome, with each miRNA targeting multiple protein coding transcripts. Although miRNA are vital to normal cell physiology their misexpression has been linked to carcinogenesis, and miRNA profiles are now being used to classify human cancers^[34]. A list of some of the miRNAs whose expression is altered during carcinogenesis is presented in TABLE 2. The influence of miRNA on the

epigenetic machinery and the reciprocal epigenetic regulation of miRNA expression suggest that its deregulation during carcinogenesis has important implications for global regulation of epigenetics and cancer.

TABLE 2 : MicroRNA alterations in various human cancers^[4,31,33]

MicroRNAs	Target Gene(s)	Cancer Type
miR-125	AKT, ERBB2-4, FGF, FGFR, IGF, MAPKs, MMP11, SP1, TNF, VEGF	breast
miR-205	VEGF-A, ErbB3	breast
miR-10b	Rho C	breast
miR-335	SOX4, TNC	breast
miR-29a	TTP	breast
miR-9	CDH1	breast cancer
miR-520	CD44	breast cancer
miR-146	NF-kB	breast, pancreas and prostate cancers
miR-10b	HOXD10	metastatic breast cancer
miR-372,miR-373	RAS, p53, CD44	testicular germ cell tumor and breast cancer
miR-342	ER, PR and HER2	breast and colon cancer
miR-145	ER	colon and breast cancer
miR-126	CRK1,PIK3R2,SPRED1, VCAM1	breast and lung cancer
miR-200 family	ZEB1, ZEB2	NCI-60 cell lines; breast, ovary
miR-218, miR-145	PXN	breast, lung and prostate cancer
miR-155	RHOA	Burkitt's lymphoma, breast, colon, and lung cancers
miR-21	PDCD4,PTEN,TPM1,RECK, TIMP3,BCL2	glioblastoma, breast, lung, prostate, colon and cervical cancer
miR-15a, miR-16-1	CCND1, Wnt3A	prostate
miR-101	Fos, EZH2	HCC, prostate
miR-127	Bcl-6	bladder cancer
miR-124	CDK6	colon cancer
miR-223	NFI-A, MEF2C	acute myeloid leukemia
miR-34b/34c	p53 network, CDK6, E2F3	colon cancer
miR-17, miR-92	c-MYC	lung cancer
miR-92b	PRMT5	brain primary tumors
miR-29c	ECM proteins	NPC
miR-127, miR-199a	BCL6, E2F1	cervical cancer
miR-421	CBX7, RBMXL1	gastric cancer
miR-32, miR345, miR-1228,miR-195, miR30b	CDKN2A,NF2,andJUN	Malignant mesothelioma (MM)
miR-190, miR-196	HGF	pancreatic cancer
miR-34a	c-Met	HCC
miR-146a, miR-146b	ROCK1, IRAK1,TRAF6	prostate cancer and papillary thyroid carcinomas
miR-340, miR-421, miR-658	MYC, RB, PTEN	lymph node metastasis and gastric cancer
let-7a-3	RAS, IGF-II	lung and ovarian cancer
miR-9	NF-_B	ovarian and lung cancer

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MicroRNAs	Target Gene(s)	Cancer Type
miR-221, miR-222	CDKN1C/P57 and CDKN1B/P27	hepatocellular carcinoma
miR-25, miR-32, miR-142	ITGA_1	lung cancer and solid tumor
miR- 124, miR-183	ITGB_1	lung cancer
miR-143	ERK5	cervical cancer
miR-372, miR-373	LATS2	testicular germ cell cancer
miR-370	MAP3K8 MzChA-1, KMCH-1,	cholangiocarcinoma downregulation
miR- 124, miR-183	ITGB1 _	lung cancer

Abbreviations: CDK6, cyclin D kinase 6; MEF2C, myocyte enhancer factor 2C; NFIA, Nuclear factor 1 A-type; p53, tumor protein 53; RAS, Rat Sarcoma; CD44, cluster differentiation 44; PDCD4, programmed cell death 4; TPM1, tropomyosin 1; PTEN, phosphatase and tensin homolog ; BCL2, B-cell lymphoma 2 protein; RECK, reversion Inducing cysteine rich protein kazal motif; ROHA, ras homolog gene family member A; NF- κ B, nuclear factor- κ B; PRNT5, protein arginine N-methyltransferase 5; HOXD10, homeobox D10; CDH1, Cadherin-1; CBX7, chromobox 7; RBMX L1, RNA binding motif protein X-linked; CDNK2A, cyclin-dependent kinase inhibitor 2A; NF2, neurofibromatosis, type 2; HGF, hepatocyte growth factor; ERBB2-4= or (HER4), human epidermal growth factor Receptor 4; JUN, janus N-terminal kinases; FGFR, fibroblast growth factor receptor; MAPKs, mitogen-activated protein kinase; MMP11, matrix metalloproteinase11; VEGF, vascular endothelial growth factor; TNF α , tumor necrosis factor-alpha; CRK1, Cdc2-related kinase1; PIK3R2, phosphatidylinositol 3-kinase regulatory subunit beta; SPRED1, sprouty-related, EVH1 domain containing 1; VCAM, vascular cell adhesion molecule; ROCK1, rho-associated, coiled-coil containing protein kinase 1; IRAK1, interleukin-1 receptor associated kinase-1; TRAF6, TNF receptor associated factor 6; Rb, retinoblastoma; IGF-II, insulin-like growth factor 2; PAX, paxillin; ITG β 1, integrin beta-1; ERK5, extracellular signal-regulated kinase 5; LATS2, large tumor suppressor homolog 2; ER, estrogen receptor; PR, progesterone receptor, TNC, tenascin C; HCC, hepatocellular carcinoma; BT-IC, breast tumor initiating cells; NPC, nasopharyngeal carcinoma.

CONCLUSION

In summary, recent studies revealed that deregulation of a number of epigenetic mechanisms may favour appearance of genetic alterations. Understanding the complexity of the epigenome and the actors involved in modulating its interactions within genomic sequences, it will open new horizons in our search to know all the mechanisms governing cellular fate.

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Conclusion of Publication2:

Latest approaches in epigenetic research have helped us with a better understanding of the dynamic of DNA methylation, histone modification and non-coding RNA mediated gene expression and mammalian cell development.

This review focused on DNA methylation, histone modification and non-coding RNA because these epigenetic events are widely implicated in cancer development and progression. In this review, we want to emphasize of epigenetic mechanisms to help for breast cancer therapy.

ii. Epigenetic modifications in breast cancer and epigenetic therapy

Epigenetic changes are critical for development and progression of cancers, including breast cancer. Significant progress has been made in the basic understanding of how various epigenetic changes such as DNA methylation, histone modification, miRNA expression, and higher order chromatin structure affect gene expression. Subsequently, it became clear that epigenetic modifications play important roles in diseases, including breast cancer. There is thus a pressing need to understand the functional genome; that is, the changes defined by regulatory mechanisms overlaying the genetic structure [73], [74].

It has been increasingly recognized over the past several years that CpG islands of a large number of genes, which are mostly unmethylated in normal tissue, are methylated to varying degrees in human cancers, thus representing tumour-specific alterations. In cancer cells, there is a deregulation of DNA methylation patterns that lead to genome-wide hypomethylation and hypermethylation of CpG islands associated with cancer-related genes. DNA methylation plays an essential role in development, chromosomal stability, and for maintaining gene expression states. DNA methylation occurs when methyl groups are added to cytosines in CpG dinucleotides, leading to a closed chromatin conformation and gene silencing. CpGs are often found at increased frequencies in promoter regions, forming CpG islands. Hypermethylation of CpG islands affects genes involved in cell cycle control, DNA repair, cell adhesion, signal transduction, apoptosis and cell differentiation. In tumour cells, local promoter hypermethylation is often accompanied by global hypomethylation. This results in more global patterns of methylation as compared with mutation spectra, which differ greatly in extent and patterns between tumours [75].

In addition, a large group of cancers are also associated with aberrant Histone deacetylases (HDAC) expression. Gene silencing and maintenance of cellular identity can also be mediated by histone modifications carried out by polycomb group (PcG) proteins. Enhancer of zeste homolog 2 (EZH2) is a core member of the polycomb repressive complex 2 (PRC2) that catalyses the histone mark characteristic for PcG-mediated silencing: trimethylation of lysine 27 on histone H3 (H3K27me3), which leads to the blocking of transcriptional activation factors and thereby gene silencing independent of promoter methylation. In cancer cells, the presence of PRC2 can lead to recruitment of DNMTs

resulting in *de novo* DNA methylation and more permanent repression of PRC2 target genes [76].

Moreover, many of the genes that undergo promoter methylation in breast cancer are already expressed at low levels in corresponding normal cells, suggesting that a large fraction of *de novo* methylation events in breast cancer cells are not subject to growth selection but instead reflect an instructive mechanism inherent of the normal cell from which the tumour originated.

Improving knowledge about epigenetic mechanisms is important for understanding prognosis and predicting of breast cancer and also promising for epigenetic therapy research. When we investigated therapeutic agents, we found that HDAC inhibitors (HDACi) have potential as anticancer therapeutics. HDACi are a new group of anti-tumor agents as a future function in the treatment of breast cancer. Currently, seven groups of HDACis have been reported; short-chain fatty acids, hydroxamic acids, benzamides, cyclic tetrapeptides containing a 2-amino-8-oxo-9, 10-epoxy-decanoyl (AOE) moiety, cyclic peptides without the AOE moiety, epoxides and psammoplins. Until now, two HDACi vorinostat (suberoylanilide hydroxamic acid-SAHA, Zolinza) and depsipeptide (romidepsin, Istodax) have been approved by the US Food and Drug Administration (FDA) for treatment of cutaneous T-cell lymphoma [77],[78].

Presentation of Publication 3:

Karsli-Ceppioglu S, Dagdemir A, Judes G, Ngollo M, Penault-Llorca F, Pajon A, Bignon Y-J, Bernard-Gallon D. Epigenetic Mechanisms of Breast Cancer: Update of Current Knowledge. Epigenomics (December 2014, in press).

In this review, we defined some epigenetic mechanisms and their effects of correlation with breast cancer.

Estrogen receptors are over-expressed in around 70% of breast cancer cases, referred to as "ER-positive", and can be demonstrated in such tissues using immunohistochemistry. Two hypotheses have been proposed to explain why this causes tumorigenesis, and the available evidence suggests that both mechanisms contribute. First, binding of estrogen to the ER stimulates proliferation of mammary cells, with the resulting increase in cell division and DNA replication, leading to mutations. Second, estrogen metabolism produces genotoxic waste. The result of both processes is disruption of cell cycle, apoptosis and DNA repair, and, therefore, tumour formation.

The PcG proteins are in charge of for maintaining the inactive expression of many genes. In the last days, three different polycomb repressive complexes have been identified: polycomb repressive complex 1 (PRC1), polycomb repressive complex 2 (PRC2), and pleiohomeotic-repressive complex (PhoRC). Enhancer of zeste homolog 2 (EZH2), is the most important subunit of the PRC2. Recent studies have shown that overexpression of *EZH2* is associated with breast cancer. *EZH2* over expression may promote cancer due to an increase in histone methylation which silences the expression of tumor suppressor genes. An *EZH2*-targeting drug inhibits the progression of breast cancer.

BRCA1 is human gene that produces tumor suppressor proteins. These proteins help repair damaged DNA and, therefore, play a role in ensuring the stability of the genetic material of cell. When either of these genes is mutated, or altered, such that its protein product is not made or does not function correctly, DNA damage may not be repaired properly.

As a result, cells are more likely to develop additional genetic alterations that can lead to cancer. Specific inherited mutations in *BRCA1* increase the risk of female breast and ovarian cancers, and they have been associated with increased risks of several additional types of cancer.

They are important key factors for cancer development and prognosis. Investigations related with aberrant epigenetic regulations in breast cancer focus on the initiating molecular mechanisms in cancer development, identification of new biomarkers to predict breast cancer aggressiveness and potential epigenetic therapy.

Epigenetic Mechanisms of Breast Cancer: Update of current knowledge.

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ABSTRACT

Epigenetic alterations are heritable changes in gene expression that occur without changes in DNA sequence. They are important key factors for cancer development and prognosis. Breast cancer is induced by the accumulation of altered gene regulation. Beside of genetic mutations, epigenetics mechanisms have an important role in breast cancer tumorigenesis. Investigations related with aberrant epigenetic regulations in breast cancer focus on the initiating molecular mechanisms in cancer development, identification of new biomarkers to predict breast cancer aggressiveness and potential epigenetic therapy. In this review, we will summarize the recent knowledge about the role of epigenetic alterations related with DNA methylation and histone modification in breast cancer. In addition, altered regulation of breast cancer specific genes and potential epigenetic therapy will be discussed according to epigenetic mechanisms.

Key Words: epigenetics; breast cancer; DNA methylation; histone modification; estrogen; *EZH2*; *BRCA1*; epigenetic therapy

INTRODUCTION

Cancer is a heterogeneous disease which is caused by multi-factorial processes. A vast majority of investigations are studying sequence alterations in the genome; however, thanks to the increased knowledge about gene expression mechanisms at last decades, it is found that epigenetic alterations are important key factors for cancer development and prognosis [1]. The term epigenetics describes the heritable changes in gene expression that occur without changes in DNA sequence and provides information about the effect of chromatin organization on transcriptional regulation.

Nucleosome is the structural subunit of chromosomes. It consists of 147 base pairs of DNA wrapped around a histone core octamer and comprised of two copies of H2A, H2B, H3 and H4. Nucleosome is composed of two parts; a highly structured part called the core domain, and the unstructured N- and C-terminal parts that stretch from the nucleosome core. The histone amino terminal tails are subject to many post-translational modifications. There are three main epigenetic modifications: DNA methylation, histone modifications and RNA-mediated gene silencing (FIGURE 1). DNA methylation is a covalent modification that takes place at the 5' position of cytosine rings in the CpG dinucleotide. Histone modifications are covalent post-translational modifications of N-terminal tails of four core histones. Small-non-coding RNA molecules can alter gene expression states by targeting mRNAs for degradation or by preventing their translation [2, 3]. Epigenetic alterations due to DNA methylation and histone modifications have gained particular importance for cancer prognosis and therapy.

Breast cancer is a prominent cause of cancer-related deaths in women. Deaths caused by female breast cancer comprise 6.1% of total deaths in women. According to The American Cancer Society's predictions of breast cancer for 2013; about 232,340 new cases of invasive breast cancer will be diagnosed in women and 39,620 women will die from breast cancer in United States [4]. Breast cancer is induced by the accumulation of altered gene regulation and function which cause abnormal cell growth and expansion. In addition to genetic mutations, it has become obvious that epigenetics play an important role in breast cancer tumorigenesis based upon increasing amount of

research. These studies focus on initiating molecular mechanisms in cancer development; identification of new biomarkers to predict breast cancer aggressiveness and potential epigenetic therapy [5-7]. In this review, we aim to discuss the recent knowledge about the role of epigenetic alterations related with DNA methylation and histone modification in breast cancer.

DNA Methylation

DNA methylation is a covalent chemical modification, occurring with the addition of a methyl (CH₃) group from S-adenosylmethionine (SAM) at cytosine residues of the DNA template [1]. DNA methyltransferases (DNMT) are the main activators of DNA methylation. They either catalyze the *de novo* methylation or establish methylation in hemimethylated DNA. The discovered DNMT protein family in mammals are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, DNMT3B with its isoforms, and DNMT3L, however just three of them have catalytic methyltransferase activity (DNMT1, DNMT3A, and DNMT3B) [8, 9]. The mammalian DNMTs comprise of two parts: the catalytic C-terminal domain which is responsible for cofactor binding and catalysis; and the regulatory N-terminal part which is important for differentiation between hemimethylated and unmethylated DNA [3]. DNMT1 is the most abundant DNMT in mammalian cells that mainly catalyzes methylation for hemimethylated CpG sites during DNA replication. The DNMT2 shows tRNA methyltransferase activity and furthermore, it detects DNA damage, DNA recombination, and mutation repair [10, 11]. *De novo* methyltransferases DNMT3A and DNMT3B are mainly expressed during embryo development [12]. DNMT3A can catalyze methylation of hemimethylated and unmethylated CpGs at the same rate; its DNA methyl-transferase activity is lower than DNMT1, but greater than DNMT3B. DNMT3A and DNMT3B are mainly considered as *de novo* methyltransferases, due to knock-out of these genes in mice they die early in development or shortly after birth [10, 13]. DNA methylation takes place at mechanisms associated with genomic integrity and gene expression. It was often assumed as an epigenetic alteration responsible for gene repression, which results in transcriptional silencing. However, increasing research in this field has clarified that DNA methylation plays an important role in transcriptional activation as well [10].

Regions of high CpG content, called CpG islands, are located in the promoters of 60% of protein-coding genes in the human genome. Methylation of CpG islands was the first evidence for aberrant epigenetic change in cancers. Whereas most CpG islands are unmethylated in healthy cells; CpG islands become hypermethylated in cancer cells which repressed the transcription of tumor suppressor genes. On the other hand, CpG sites may become hypomethylated, leading to transcriptional activation of normally repressed genes like oncogenes and retrotransposons [3].

The family of methyl-CpG-binding proteins (MBDs) mediates silencing of tumor suppressor genes besides DNMTs. These proteins recognize and bind specifically to methylate DNA containing regions of CpG islands with their methyl-CpG-binding domain. MBDs establish a connection between DNA methylation and histone modification enzymes associated with transcriptional repression. The family of MBD proteins consists of five members: MeCP2, MBD1, MBD2, MBD3 and MBD4. These proteins are related with hypermethylation of tumor-suppressor gene promoters at CpG islands and their silencing [3, [14].

DNA Methylation and Breast Cancer

Aberrant methylation plays an important role in breast cancer development. DNA hypomethylation has been generally demonstrated on a genome-wide scale in cancer researches while hypermethylated DNA regions of certain genes, especially normally unmethylated CpG islands, were frequently reported. A growing number of studies have indicated hypermethylated genes in breast cancer which have crucial roles in cell-cycle regulation, apoptosis, tissue invasion and metastasis, angiogenesis and hormone signaling [6, 15-17]. Aberrant methylation profiles of these genes are associated with breast cancer stage and prognosis; therefore, they have been proposed to be a diagnostic marker. In a study, Esteller et al. investigated the hypermethylation profiles of 12 tumor suppressor genes which are undergoing epigenetic inactivation in human tumors [6]. According to their results, tumor suppressor genes *BRCA1* and *p16*, DNA repair genes *GSTP1* and *CHD1*, which all are linked with metastasis and invasion, were found hypermethylated in breast tumor samples. In addition, the methylation status of *ADAM23* gene, which is a member of surface

molecules and involved in the cell adhesion process, was analyzed. It was shown that, the degree of *ADAM23* promoter hypermethylation gets higher along with elevated tumor grade [15]. Oshiro et al. evaluated the epigenetic silencing of *DSC3* expression in 32 breast tumor samples [16]. Desmocollins belong to cadherin superfamily and *DSC3* is mainly expressed in the basal layers. They demonstrated that *DSC3* expression was downregulated in tumor samples and *DSC3* silencing was related with cytosine methylation in 41% of the tumor samples. Wnt signaling plays an essential role in cell differentiation and proliferation; however, it is strongly associated with tumorigenesis. The SFRP family consists of five glycoproteins which are putative inhibitors of Wnt signaling. Several studies emphasized aberrant methylation of Wnt antagonist genes in breast cancer. Their results indicated that frequent promoter methylation of SFRP family genes was the main factor for silencing in breast cancer. Moreover, *SFRP1*, *SFRP2* and *SFRP5* were methylated in both cell lines and breast tumor samples at a quite higher frequency. They asserted that majority of breast tumors represent aberrant methylation of at least one Wnt antagonist gene and silencing of these genes would be the main factor of Wnt signaling in breast cancer [18-20]. Holm et al. determined the methylation status of 807 selected cancer-related genes according to molecular subtypes of 189 breast tumors [17]. They found that basal-like, luminal A and luminal B tumors have different methylation profiles, however, tumors of normal-like and HER2-enriched subtype represent similar methylation profiles. The methylation frequency of genes, especially *RASS1* and *GSTP1*, was significantly higher in luminal B tumors, but low methylation frequency was seen in basal-like tumors. Moreover, among *BRCA1* and *BRCA2* mutation carriers, tumors of *BRCA2* mutation carriers were significantly more methylated than *BRCA1* mutation carriers.

Epigenetic silencing of *RAS-association domain family 1 (RASS1F1)* gene by promoter hypermethylation is observed in a considerable number of cancers and it is widely investigated in breast cancer. In recent studies, high frequency of *RASS1F1* promoter methylation was found in breast cancer tumors [21-24]. Moreover, downregulated expression of *RASS1F1* gene was reported concordant with its methylation status by Alvarez et al [21]. Significant association between

hypermethylated *RASSF1A* and hormone receptor status was demonstrated. Especially, ER positive tumors were found to be more frequently methylated than ER negative tumors [21, 22, 24].

Genome-wide analyses of aberrant methylation in breast cancer take care of clarifying the entire genomic distribution and the underlying molecular mechanisms. These studies also give the opportunity to understand the methylation profiles of breast cancer subtypes. Li et al. investigated the DNA methylation status of 12 pairs of ER+/PR+ and ER-/PR- breast cancer samples by genome-wide profiling and different methylation profiles of *FAM124B*, *ST6GALNAC1*, *NAVI* and *PER1* was represented according with receptor status [25]. In another study, extensive hypermethylation was determined in ER positive tumors in comparison with ER negative tumors [26]. In addition, 40 CpG loci were found to be associated with ER-subtype. Accordingly Fang et al. observed that CpG island methylation was more prevalent in ER+/PR+ tumors and those tumors demonstrated better prognosis than ER+/PR+ tumors which did not possess CpG island methylator phenotype [27]. Hill et al. showed the impact of methylation profile on hormone receptor status in various breast cell lines [28]. They pronounced that, hypermethylation of *RECK*, *SFRP2*, *ITR*, *UGT3A1*, *ACADL* and *UAP1L1* was significantly related to the worst survival and 18 genes/loci was correlated with ER+/PR+ status.

Global DNA hypomethylation is prevalent in breast tumors and up to 50% of the cases represent reduced 5-methylcytosine content when compared with matched-control normal tissues [29, 30]. Whole genome distribution of aberrant DNA methylation in eight breast cancer cell lines and normal human mammary epithelial cells were analyzed by Ruike et al [31]. It was shown that hypomethylation was distributed throughout the entire genome and simultaneous hypermethylation occur at CpG-rich regions. In addition, hypomethylation was three to five times more frequent than hypermethylation. Genome-wide DNA-methylation was investigated in familial breast cancer cases to demonstrate methylation profiles of the different mutation groups (*BRCA1*, *BRCA2* and non-*BRCA1/2*) [30]. In this study, genome-wide correlation was not observed between gene expression and methylation, however, expression of ~800 genes were inversely correlated with DNA

methylation. In addition to these studies, which demonstrated the genome-wide hypomethylation status of breast cancer, there are several investigations that reported hypomethylated genes. Paredes et al. found that methylation of *P-cadherin* gene exists in all normal breast epithelial cells while unmethylation was demonstrated in 42% of invasive carcinomas [32]. Furthermore, promoter hypomethylation of *FEN1* gene, which is associated with DNA replication, repair and apoptosis, was reported by Singh et al. [33] and hypomethylation of *N-acetyltransferase NAT1* promoter was found at significantly higher levels in primary breast tumors by Kim et al. [34].

Histone Modification

Histone modifications are another main epigenetic mechanism, which are responsible for post-translational modifications at N-terminal tails of histones in order to regulate chromatin structure and gene expression. These modifications are carried out by acetylation, methylation, phosphorylation, sumoylation, poly (ADP)-ribosylation and ubiquitination. Each histone modification constitutes a particular mark that affects the chromatin structure [2]. Chromatin can be found in two states: heterochromatin or closed, and euchromatin, also called open or noncondensed. Genes in open chromatin are transcriptionally active; genes in heterochromatin are transcriptionally silenced (FIGURE 2).

Histone acetylation, the most studied histone modification, catalyzes the transfer of acetyl group from acetyl-CoA to the Σ amino group of lysine residues. This modification alters the electrostatic charge by neutralizing positive charges of histones, which induces open chromatin structure. Acetylation of histones catalyzed by enzymatic actions of histone acetyl transferases (HATs) and acetyl groups are removed by histone deacetylases (HDACs), which leads to gene inactivation and silencing. HATs can be categorized in five families: the GNAT family; the MYST family; the p300/CBP family; the SRC family and the TAFII250 family. In addition to histone proteins, HATs catalyze the acetylation of a number of nonhistone proteins such as GATA1, E2F1, PRB or TP53 [3]. Eighteen HDACs have been identified in human and classified into four groups according to their homology with yeast HDACs and enzymatic activities. Class I HDACs (HDACs 1, 2, 3 and 8)

are nuclear proteins and associated with various transcriptional repressors and cofactors. Class II HDACs are divided into two sub-classes based on their protein structure; Class IIa with one catalytic domain (HDACs 4, 5, 7 and 9) and Class IIb with two catalytic domains (HDACs 6 and 10). Class III HDACs contain seven HDACs (SIRT1 to SIRT7) and they require co-factor nicotinamide adenine dinucleotide (NAD⁺) for catalytic activity. Recently identified HDAC11 is the only member of Class IV HDAC group [35].

Histone methylation induces the methylation of arginine and lysine residues and does not change histone's charge. Catalytic activity of histone methylation conducts with histone methyltransferases (HMTs). The HMTs enzyme family has a conserved catalytic domain called as SET (Suppressor of variegation, Enhancer of Zeste, Trithorax). Histone lysine methylation exists within the tails of histone H3 and H4 either in a mono-, di- or trimethylation state [36]. According to their effects on gene regulation, H3K4me_{2/3} and H3K79me₃ are generally associated with transcriptional activity, while methylation of H3K9me_{2/3}, H3K27me_{2/3} and H4K20me₃ are associated with repression [37, 38].

Histone Modifications in Breast Cancer

In breast cancer, investigations carried out on histone modifications are relatively newer than DNA methylation studies. Post-translational histone modifications have a critical role in breast tumorigenesis and aggressiveness of prognosis. In addition, they are potential therapeutic targets. Since different breast cancer subtypes represent distinct gene expression profiles, it is important to clarify the effect of histone marks on gene expression levels at breast tumors. However, there are limited studies about histone modification patterns in breast cancer tumors, though the numbers of investigations are increasing in this field.

Elsheikh et al. investigated the frequency of global histone modifications in breast cancer tumors by using microarray technology and immunohistochemistry [39]. They evaluated the relative levels of histone lysine acetylation (H3K9ac, H3K18ac, H4K12ac and H4K16ac), lysine methylation (H3K4me₂ and H4K20me₃) and arginine methylation (H4R3me₂) in 880 primary operable

invasive breast carcinoma cases. According to their results, levels of all seven histone marks were low compared to the high tumor grade. Particularly, levels of H4R3me2, H3K9ac, and H4K16ac were detected at significantly low levels relevant to large tumor size. Also, levels of H4R3me2 and H3K9ac were detected at high levels when the lymph node stage was low. Histone modification levels were significantly higher at steroid receptor (estrogen receptor, progesterone receptor, and androgen receptor)-positive tumors. They have concluded that reduced detection of these histone marks would be correlated with poor prognostic characteristics.

Suzuki et al. evaluated the expression levels of acetylated histone H4 (ac-H4), ac-H4K12, ac-tubulin, HDAC1, HDAC2 and HDAC6 in 58 breast surgical samples of normal mammary epithelium, ductal carcinoma *in situ* and invasive ductal carcinoma by immunohistochemistry [7]. They have showed that acetylation of ac-H4, ac-H4K12, ac-tubulin, HDAC1, HDAC2, and HDAC6 were decreased in ductal carcinoma *in situ*, and invasive ductal carcinoma in comparison with normal breast epithelium. The levels of ac-H4 and ac-H4K12 were decreased extremely at ductal carcinoma *in situ* compared to normal epithelium.

Guendel et al. investigated the arginine and lysine methylation of *BRCA 1* both in breast cancer cell lines and 4 breast tumor tissue samples [40]. Their results indicate that *BRCA 1* is methylated at arginine and lysine residues both in cell lines and tumor samples. However, lysine methylation was only detected in MDA-MB-231 cells, which are triple negative and possess a mutant p53, while arginine methylation was detected in MDA-MB-231 and MCF-7 cells. MCF-7 cells are estrogen, progesterone positive, HER2 negative and show wild-type 53. Further, *BRCA1* was methylated at both K and R residues in four breast tumor patient samples. *In vivo* BRCA1 binding to the *APEX*, *ARHG* and *GADD45G* promoters was increased along with methylation inhibition. According to these results, they proposed that methylation may affect the ability of BRCA1 binding to specific promoters or affects protein-protein interactions that alter the recruitment of BRCA1 to these promoters.

Choe et al. demonstrated the histone modification patterns of H3K4me1, H3K4me3 and H3K9/14ac in breast cancer cell line MCF-7 and normal mammary epithelial cell line, MCF-10A by ChIP-seq analysis [41]. No significant differences were seen in studied regions. This study showed that the H3K4me1 distribution, which covers the entire human genome, was 53% of the gene body, 42% of the intergenic region, and 5% of the promoter. The promoter regions covered 54% and 52% of total sequence reads in H3K4me3 and H3K9/14ac, respectively. As a result, H3K4me3 and H3K9/14ac are greatly co-localized in the same cells, but their correlation in different cells is moderate. In another study of the same group, they analyzed the histone modification profiles of histone H3K4me1, H3K4me3, H3K9/16ac, and H3K27me3 in MCF-7 cells in human chromosomes 8, 11 and 12 [42]. The results demonstrated that the promoters were the most abundant regulatory elements and H3K4me3 and H3K9/14ac were highly enriched within ± 1 kb from transcription start sites.

It is clear that modified histones gain importance as biomarkers of breast cancer prognosis. The investigations carried out on mechanisms of histone modifications are also promising for the development of efficient HDAC inhibitor therapies.

Estrogen and Epigenetic Mechanisms

Expression level of estrogen receptors (ERs) in tumors is a crucial factor for predicting the prognosis and the response to therapy in breast cancer patients. Two isoforms of ER, ER α and ER β , have been characterized. ER α is encoded by *ESR1* and ER β is encoded by *ESR2*. These isoforms show similar affinities for estradiol and have different roles in the regulation of gene expression. Estrogens normally regulate growth, reproduction and neural development. However, exposure to elevated levels of estrogen increases the breast cancer incidence and proliferation [43]. ER negative tumors establish more aggressive progression with higher histological grade and are associated with a higher recurrence rate and decreased overall survival. About 80% of breast cancers are ER α positive, however, a considerable part of them lose ER expression during tumor progression and become resistant to drug therapies [44]. ER complexes regulate gene transcription by the

recruitment of co-regulator and transcription factors like HDAC1 and DNMT1 [45]. Several studies focused on hypermethylation of *ESR1* promoter silencing, which is resulted in decreased expression of ER α protein. Ramos et al. investigated the methylation of *ESR1* and *CXCL12* genes in breast cancer cell lines and 69 breast tumor samples of Brazilian women. According to their results, *ESR1* promoter methylation was shown in samples with methylated *CXCL12* islands [46]. Wei et al. evaluated the promoter methylation patterns of ER α and the ER α expression levels of 113 familial breast cancer patients in Han Chinese Population [47]. ER α methylation was demonstrated in 41.6% samples of familial breast cancer patients. Moreover, ER α promoter methylation was significantly correlated with tumor size, progesterone expression, p53 nuclear accumulation, *BRCA1* and *BRCA2* statuses. Prabhu et al. demonstrated the hypermethylation of ER promoter in 98 breast cancer tumor specimens of Indian women [48]. They found that ER promoter was methylated in 27% of triple-negative tumors. On the other hand, less than 2% of ER-positive tumors were hypermethylated.

There are limited studies about epigenetic regulation of *ESR2*. In a recent study, Zhao et al. researched hypermethylation of ER β in 178 sporadic breast cancer and 14 benign breast hyperplasia cases of Chinese women [49]. They determined ER β promoter methylation at 44.9% of studied tumors, which was a higher frequency in comparison with methylation status of benign breast diseases.

Enhancer of Zeste Homolog 2 (EZH2) and Breast Cancer

Enhancer of zeste homolog 2 (EZH2) is a catalytic subunit of Polycomb Repressive Complex 2 (PRC2). Expression levels of *EZH2* are upregulated in variety of cancers including prostate, bladder, gastric, lung, hepatocellular and breast. As a histone methyltransferase, EZH2 catalyzes lysine 27 tri-methylation of histone H3 which results in a repressive histone mark [50].

Expression level of *EZH2* is associated with aggressiveness of breast cancer and overexpression affects the tumor invasion and cancer progression. Kleer et al. analyzed the expression levels of

EZH2 at 917 samples from 280 patients by tissue microarray and they reported that EZH2 levels were higher in patients with invasive carcinoma and breast cancer metastases in comparison with normal breast tissues [51]. At their follow-up study, Dig et al. assessed the EZH2 protein expression levels to detect precancerous state in 13 women carrying *BRCA 1* heterozygous mutation and 25 breast cancer patients with no personal or family history of breast cancer. According to their results, EZH2 levels were increased in *BRCA1* mutation carriers and they suggested that elevated EZH2 levels would be associated with higher risk for breast cancer.

EZH2 expression mostly increases in basal-like breast cancers, as well as in ER-negative breast cancer cell lines. Besides, EZH2 has a crucial role in the regulation of progesterone signaling through histone methylation. Doherty et al. demonstrated that after exposing endocrine-disrupting chemicals such as diethylstilbestrol and Bisphenol-A in mice utero, mammary tissue expression of EZH2 was increased [52]. Moreover, Pal et al. investigated the epigenetic mechanisms associated with *EZH2* in mammary physiology by determining genome-wide histone methylation profiles [53]. They have found that EZH2 regulates mammary progenitor activity and alveolar development, and coordinates in this way the progesterone signaling with global changes.

Holm et al. assessed the global levels of H3K27me3 and EZH2 protein expression in various molecular subtypes of breast tumors [54]. They have found that EZH2 levels were statistically high in tumors whose histological grade were 3, ER/PR negative, and with high S-phase fraction; while H3K27me3 levels were low in these tumor groups. Furthermore, small tumor size was correlated with high H3K27me3 levels. Protein levels of EZH2 were highest in triple negative tumors and basal-like tumors, while expression was lowest in ER+/HER2-/Ki67 low and luminal A and normal-like tumors. The trimethylation of H3K27 was low at triple negative and HER2+ tumors; also it was low at triple negative and ER+/HER2-/Ki67 high tumors. In conclusion, they indicated that high levels of EZH2 and low levels of H3K27me3 were associated with poor survival.

EZH2 affects the invasion and progression of breast cancer by regulating a group of genes which are linked with cell proliferation and invasion. These genes; *RAD51*, *RUNX3* and *CKD1C* ($p57^{KIP2}$)

are associated with cell proliferation and *FOX*, *CDH1* (E-cadherin) are related with metastasis. *RUNX3* was reported as tumor suppressor gene in gastric cancer [55]. It was also shown that its expression is inactivated by DNA methylation in various cancers, such as prostate, lung and pancreas. H3K27 trimethylation through EZH2 plays an important role in downregulation of *RUNX3*. *RUNX3* expression was recovered along with the reduced expression level of EZH2 in MCF-7 cells, [56]. Moreover, a decline in the *RUNX3* concentration reduces *CKD1C* (*p57^{KIP2}*) expression levels [57]. Over-expression of EZH2 tends to transcriptional repression of *FOX C1*, which is a member of the Forkhead box transcription factor family. Increasing levels of *FOX C1* expression lead to reduced cell migration and invasion in breast cancer [58]. In addition, it is reported that elevated levels of EZH2 reduce E-cadherin expression levels, which is related with the invasiveness and malignant progression of epithelial tumors [59].

Breast Cancer 1 (BRCA1) and Breast Cancer

Inherited germline mutations in the *BRCA1* tumor suppressor gene lead to development of aggressive breast tumors at an early age with high histological grade, expression of proliferation markers and hormone receptor negativity. In addition, promoter methylation of the *BRCA1* gene is reported in 10-30% of sporadic early-onset breast cancer, which is associated with *BRCA1* gene silencing and aggressive pathological features of breast tumors [47, 60, 61, 62].

Esteller et al. investigated the methylation patterns of *BRCA1* promoter in series of breast cancer cell lines and 215 breast and ovarian tumor samples [63]. Promoter hypermethylation of the *BRCA1* gene was detected in 13% of 84 primary breast carcinomas; on the other hand, abnormal methylation was not determined in breast cancer cells. Turner et al. analyzed promoter methylation of *BRCA1* to investigate *BRCA1* silencing and downregulation in basal-like cancers [64]. They have demonstrated that methylation-induced *BRCA1* silencing was frequent in metaplastic tumors. Also, *BRCA1* expression levels were significantly lower in basal-like cancers. Stefansson et al. investigated the relationship between subtype-specific markers and promoter methylation of *BRCA1* in sporadic breast cancer by tissue microarrays [65]. Their results revealed that CpG island

hypermethylation plays a critical role at the development of basal/triple-negative breast cancers in sporadic cases. Moreover, they showed that there is a correlation between the expression levels of BRCA1, pRb, p16, PTEN and p53. These markers determine a subgroup of triple-negative breast cancers related with defects in *BRCA1* gene. Wong et al. assessed the BRCA1 promoter methylation in peripheral blood and tumor samples obtained from 255 women to predict early-onset breast cancers [66]. They demonstrated that approximately 4% of subjects have detectable *BRCA1* promoter methylation and their tumor samples were highly methylated at the *BRCA1* promoter, so they have approximately 3.5-fold increased risk of breast cancer before 40 years-old. Jung et al. examined the promoter methylation of 24 tumor suppressor genes in tumor and normal tissue samples of 60 Korean patients with primary breast cancer [67]. They found that hypermethylation of *BRCA1* was linked with negative estrogen and progesterone receptor expression. Iwamoto et al. investigated the relationship between *BRCA1* promoter methylation and breast cancer pathogenesis [68]. For this reason, they analyzed promoter methylation of *BRCA1* in peripheral blood samples of 200 cases, 200 controls and 162 tumor tissues. They determined *BRCA1* promoter methylation in peripheral blood samples of 21.5% of breast cancer patients and 13.5% of controls ultimately; and significantly higher risk of breast cancer was shown in women with *BRCA1* promoter methylation. In addition, *BRCA1* promoter methylation was found in 31 of 162 breast tumors. Al-Moghrabi et al. analyzed the promoter methylation of *BRCA1* in 47 breast tumor samples from Arab female patients and 73 peripheral blood samples of healthy controls by methylation-specific PCR [69]. They demonstrated that frequency of hypermethylated *BRCA1* promoter was high in primary breast carcinomas of study population. Besides, *BRCA1* promoter methylation was strongly associated with the early-onset of cancer (≤ 40 years) and high-grade tumors, but no correlation was shown with estrogen status. Sturgenon et al. evaluated the expression levels of 12 tumor suppressor genes, which were frequently methylated in breast cancers, in serum of 325 breast cancer cases and 249 benign breast disease controls [70]. Their results showed that the median methylation level of *BRCA1*, *CCND2*, *CDH1*, *ESR1*, *HIN1*, *P16* and *TWIST* were higher in breast cancer cases with

negative lymph nodes than benign breast disease subjects. Bosviel et al. investigated the clinical and prognostic role of *BRCA1* gene in sporadic breast cancer patients [71]. They analyzed the site-specific DNA methylation of *BRCA1* in peripheral blood DNA of patients and demonstrated increasing trend toward *BRCA1* promoter hypermethylation in breast cancer patients compared with controls, however the differences were not statistically significant. In addition, they have assessed the methylation of the CpG islands present at the *BRCA 2* promoter region in the same breast cancer and control population [72]. They did not find a statistically significant difference between methylation statuses of study populations. All these results support the aspect of *BRCA 1* promoter methylation being an important risk factor for breast cancer and thus, its affiliation with hormone expression.

Relationship between EZH2 and BRCA1

As mentioned above, linked with somatic mutations or promoter methylation, *BRCA 1* gene silencing cause basal-like breast carcinomas. In addition, elevated EZH2 concentrations are associated with the aggressive basal-like tumors. Gonzalez et al. demonstrated that BRCA1 is regulated by EZH2 expression in benign breast cells and ER negative breast cancer cells [73]. They also observed that EZH2 over-expression in MCF10A cells resulted in aberrant mitoses and genomic instability. Wang et al. recently reported that BRCA1 directly binds to EZH2 in both human breast cancer and mouse embryonic stem cells [74]. They found that genetic deletion of one allele of *BRCA1* or transient knockdown of *BRCA1* in mouse embryonic stem cells increases the EZH2 activity and H3K27me3 levels at PRC2 target loci concordantly, which causes the inhibition of embryonic stem cell differentiation and result in aggressive breast cancer phenotype. As a result, decreased expression level of BRCA1 leads to an enhancement in EZH2 activity which induces aggressive progression of breast carcinomas.

Developments of Epigenetic Therapy in Breast Cancer

Improving knowledge about epigenetic mechanisms is important for prognosis and predicting the course of breast cancer. Moreover, it is also promising for epigenetic therapy research. Since

modifications at HDAC activity have been reported in various tumor types, HDAC inhibitors (HDACi) have potential as anticancer therapeutics. HDACi cause transcriptional upregulation of relevant genes by interfering with the catalytic domain of HDACs to block substrate recognition of these enzymes. HDACi inhibit proliferation of breast cancer cells by activating genes that produce cell-cycle arrest and inducing the apoptosis. HDACi can be classified into four groups: hydroxamic acids, cyclic peptides, short-chain fatty acids and benzamids [75]. Until now, two HDACi vorinostat (suberoylanilide hydroxamic acid-SAHA, Zolinza) and depsipeptide (romidepsin, Istodax) have been approved by the US Food and Drug Administration (FDA) for treatment of cutaneous T-cell lymphoma. In addition, depsipeptide has obtained FDA approval for treatment of peripheral T-cell lymphoma in 2011 [76, 77].

Various studies have showed that, vorinostat, inhibitor of HDAC I and II, suppress the growth of both ER α positive and negative breast cancer cell lines by stimulating G1, G2/M cell cycle arrest and apoptosis. HDACis are more effective on transformed cells in comparison with normal cells and the expression levels of more than 10% of genes in transformed cells are altered with vorinostat treatment [78]. The evidence from clinical trials demonstrated that vorinostat has limited therapeutic activity with single treatment. However, it has the potential to modulate ER expression and provides novel opportunity to reverse the resistance to hormone therapy in breast cancer. In breast cancer therapy, the mechanisms of vorinostat action are investigated with various studies. For instance, Zhou et al. demonstrated that ER expression was reactivated with vorinostat treatment and EGR expression was inhibited due to impairment of its mRNA stability in ER-negative human breast cancer cells [79]. In another study it was reported that vorinostat stimulates apoptosis by elevating expression of genes related with p53 signaling in MCF7 cells. On the other, hand its treatment in MDA-MB-231 cell line induces genes which take part in cell death pathways such as TNFSF10 (TRAIL) caspase. Lauricella et al. found that vorinostat treatment sensitized both MCF7 and MDA-MB-231 cells to TRAIL-induced apoptosis and decreased the level of both EGFR and its phosphorylated form [80]. ER-negative breast cells are resistant to apoptosis and after treatment

with vorinostat/TRAIL combination, MDA-MB-231 cell lines became sensitive to apoptosis associated with EGFR downregulation. Vorinostat treatment caused cytotoxicity in tamoxifen-resistant human breast cancer cells and induced G2/M phase arrest. Moreover, the acetylated histone H3 and H4 levels were increased and expression of HDAC 1, 2, 4 and 7 were reduced with the treatment.

Clinical trials that were held on breast cancer epigenetic therapy have focused on combination of vorinostat and hormone therapy [81]. As a result of this phase II trial, tumor regression or prolonged disease stabilization was observed in 40% of the patients with hormone receptor-positive breast cancer. In another phase I–II clinical trial, combination therapy of vorinostat with paclitaxel and bevacizumab was evaluated in metastatic breast cancer patients [82]. In addition, effects of vorinostat on acetylated histones and non-histone proteins were detected. It was reported that combination of vorinostat with paclitaxel and bevacizumab did not increase the toxicity of paclitaxel-bevacizumab. Moreover, vorinostat was effective inducing hyperacetylation of histone and non-histone proteins. According the early results of clinical trials, it could be concluded that vorinostat has potential to recover hormone therapy resistance in breast cancer. The hyperacetylation effects of HDACis were observed in breast cancer patients and evaluation of vorinostat-induced acetylation on histone and non-histone proteins would be predictors for biological activity and clinical benefit.

Various HDACis are being evaluated for their therapeutic potential. For instance, panobinostat (LBH489) was reported to activate hyperacetylation of histones H3 and H4, decrease proliferation and induce apoptosis and G2/M cell cycle arrest in triple negative cell lines with minimal toxicity [83]. HDACi sodium butyrate has crucial functions on inhibition of cell growth and apoptosis. It was shown that sodium butyrate induced the activity of caspase-3,-8 and -10 in a time- and dose-dependent manner in human breast cancer cell line MRK-nu-1. Moreover, sodium butyrate treatment caused DNA fragmentation in a dose-dependent manner [84].

With the increasing interest among epigenetic therapy, various research projects are reviewing the combination treatment of HDACis to sensitize breast cancer cell lines to radiotherapy, hormone therapy or chemotherapy. Cho et al. investigated the combined effects of sodium butyrate and demethylation agent, 5-Aza-2'deoxyctidine (5-aza-dC) on radiosensitivity in MCF-7 breast cell lines [85]. They reported that sodium butyrate was more effective for enhancement of radiosensitivity in MCF-7 cell lines. Moreover, combination therapy with sodium butyrate and 5-aza-DC increased the effect. Several studies were focused on combination treatment of trichostatin A/5-aza-DC in triple negative cell lines that could activate the expression of silenced genes in cancer [86-88]. They also demonstrated that ER-negative cells became sensitive to tamoxifen therapy after combine treatment with trichostatin A/5-aza-dC [86, 87]. In another study, entinostat showed stimulation of aromatase expression by upregulating ER α expression in ER α -negative cell lines [89].

Epigenetic drugs, which are effective on HAT mechanism, were also tested for their therapeutic potential. 3-Deazaneplanocin A (DZNep) is an inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase and inhibits histone methylation by downregulation of PRC2 complex [90, 91]. Tan et al. demonstrated that DZNep treatment regulates expression of genes which are transcriptionally repressive in breast cancer, by inversion of PRC2 and histone methylation-mediated gene silencing. They clarified that DZNep induces apoptosis of PRC2 target genes [92].

Consequently, it is clear that evolving investigations about epigenetic therapy will represent further opportunity to treat particularly basal-like tumors by repairing ER α expression. HDACis have critical ability to restore the resistance to hormonal therapy. Further investigations will elucidate underlying mechanisms of those synergistic interactions.

Conclusion & future perspective

Breast cancer is a significantly heterogeneous disease in histology, genetics and prognosis. It is clear that epigenetic alterations have crucial role in breast cancer tumorigenesis. In the last two decades, with increased knowledge about epigenetic mechanisms, we could provide insight into

molecular mechanisms of breast cancer. According to the investigations carried out in epigenetic field, novel approaches are propounded about new biomarkers to predict breast cancer aggressiveness and potential epigenetic therapies. In addition to the genome-wide analyses that identify epigenetic regulation of multiple genes; detection of specific genes such as *EZH2*, *BRCA1* and *RASSF1A*, whose expression levels are altered epigenetically, will be beneficial for determining prognosis and treatment in breast cancer patients. Technological advancements in epigenetic researches, which clarify the role of additional mechanisms and complex epigenetic regulations, lead to a better understanding of underlying mechanisms of breast tumorigenesis and the approaches to the prevention and treatment of breast cancer. In the near future, finding correlations between the early epigenetic changes and clinical profiles could provide a better clinic outcome. Difficulties in the availability of suitable tissue material limit the research; however, investigating the aberrant epigenetic patterns in primary tumors is essential to demonstrate epigenetic profiles of breast cancer and to correlate them with clinical status. In conclusion, further investigations could improve our aspects in epigenetic mechanisms of breast cancer tumorigenesis and its clinical outcome.

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Executive summary

Breast Cancer

- Breast cancer is a significantly heterogeneous disease in histology, genetics, and prognosis. Epigenetic alterations gained importance at reflecting the molecular mechanisms of tumorigenesis.

DNA methylation and breast cancer

- DNA methylation is one of the most common molecular alterations in cancer, refers to the covalent addition of a methyl (CH₃) group from S-adenosylmethionine (SAM) onto cytosine residues of the DNA template.
- Genome-wide DNA methylation analysis demonstrates that global DNA hypomethylation is highly frequent in breast cancer. In addition, hypermethylation of breast cancer specific genes such as *BRCA 1*, *RASSF1A* and *Cadherin superfamily* genes are also reported very often.

Histone Modifications in Breast Cancer

- Histone modifications are post-translational modifications at N-terminal tails of histones in order to regulate chromatin structure and gene expression, which are carried out by acetylation, methylation, phosphorylation, sumoylation, poly (ADP)-ribosylation and ubiquitination.
- Post-translational histone modifications are associated with breast tumorigenesis, aggressiveness of prognosis, and they are potential therapeutic targets.

Estrogen, EZH2 and BRCA1

- Hypermethylation of estrogen receptor genes *ESR1* and *ESR2* promoters results in with gene silencing, which is associated with decreased expression levels of ER α and ER β proteins in cancer cell lines. However, there are limited number of studies that demonstrated *ER α* and *ER β* gene silencing in breast tumors.

- Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase and its overexpression is associated with the tumor invasion and cancer progression.
- In addition to inherited germline mutations, promoter methylation of *BRCA 1* tumor suppressor gene is also associated with developing aggressive breast tumors.

Developments of Epigenetic Therapy in Breast Cancer

- Epigenetic therapy will represent further opportunity to treat breast cancer tumors by reversing the resistance to hormonal therapy.

Figures and Legends

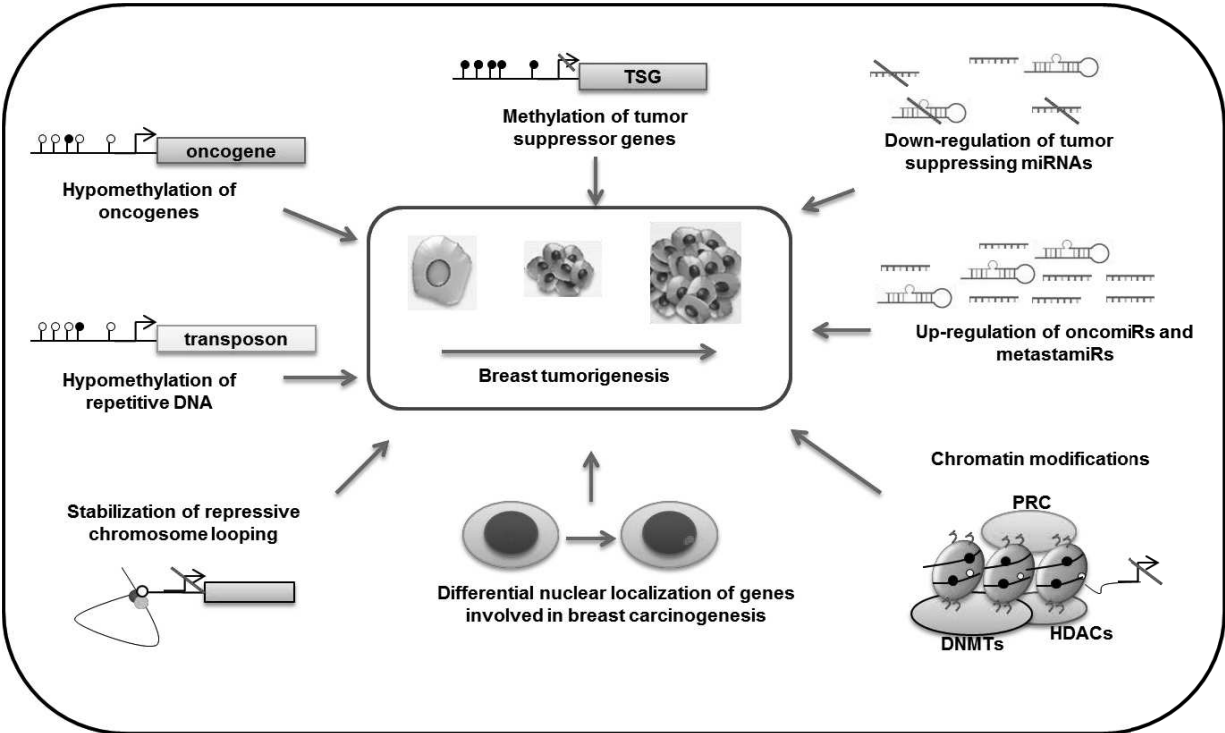


Figure 1. Epigenetic alterations in breast cancer [93] (*Reproduced by permission of the publisher*).

TSG: tumor suppressor gene; miRNAs: microRNAs; DNMTs: DNA methyltransferases; HDACs: histone deacetylases; PRC: polycomb repressor complexes.

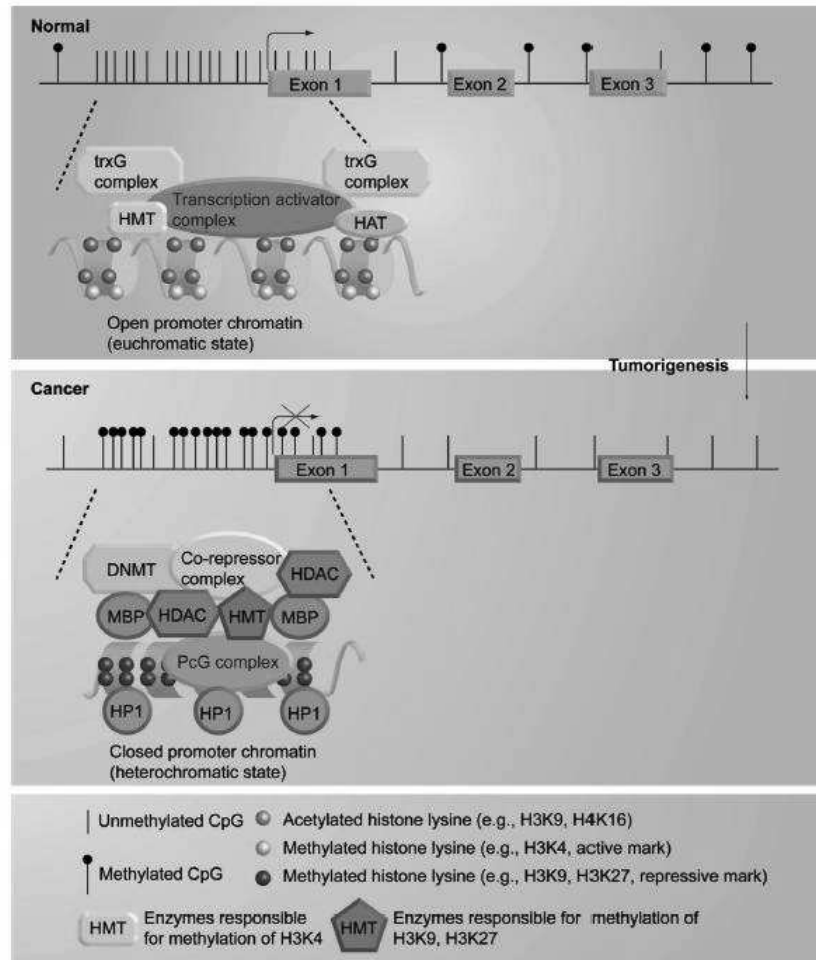


Figure 2. DNA methylation, histone modifications and chromatin remodeling are occurring in tumor suppressor genes during tumorigenesis. a. Most CpG islands of tumor suppressor genes are unmethylated in healthy cells, and are transcriptionally active associated with the active histone 3 lysine 4 (H3K4) methylation and histone 3 lysine 9 (H3K9) acetylation chromatin marks. b. The family of methyl-CpG-binding proteins (MBDs) recognize and bind specifically to methylated DNA containing regions of CpG islands with their methyl-CpG-binding domain. Repressive proteins, such as histone deacetylases (HDAC) and histone methyltransferases (HMT) which deacetylate H3K9, methylate H3K9 and histone H3 lysine 27 (H3K27) residues, respectively, are recruited by MBDs and lead to gene inactivation and silencing [8] (*Reprinted by permission of the publisher*).

DNMT: DNA methyltransferases; HAT: Histone acetyltransferases; HDAC: Histone deacetylases; HMT: histone methyltransferases; HP1: Heterochromatin protein 1; MBP: Methyl-CpG binding proteins; PcG: Polycomb group; trxG: Trithorax group.

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Conclusion of Publication3:

After the review we understood that *ER*, *EZH2* and *BRCA1* expressions are very important development of breast cancer. They are very consequential and they have got truly critical effect themselves and also its very significant correlation among each other.

Previous studies have shown that mutation in *BRCA1* causes of increase *EZH2* concentration particularly in the basal like breast carcinomas. Generally *EZH2* is overexpressed in ER-negative breast cancer and inhibits BRCA1 phosphorylation. For this reason transmission from G2 to M is accelerated in cell cycle and increased cell proliferation.

Also, it has been shown that before *BRCA1* inhibits ligand-induced ER α signaling and blocks subsequent ER α transcriptional activation. In many breast cancers, estrogen-mediated signaling has a very important function of adjusting to evolution and endurance signaling pathways and can directly drive tumorigenesis by leading the expression of genes. Some authors have reported the ER β is down-regulated in breast tumorigenesis and another recent study has shown the regulation of ER β expression by promoter methylation. *In vitro* studies point out ER β plays a role in the modulator of proliferation and invasion of breast cancer cells, so the loss of ER β expression could be one of the reasons to breast cancer development.

Two enzymes are associated with in histone deacetylation; histone acetyltransferase (HAT) and histone deacetylase (HDAC). These enzymes catalyse the transfer of an acethyl group from acethyl-CoA molecules to the lysine-amino groups on the N-terminal tails of histones. HDACi induce G1-phase cell cycle arrest with downregulation of cyclin D1 and upregulation of *p21* in breast cancer cells. When HDAC activity is inhibited, expressions of only 2% of the mammalian genes are affected.

3. OBJECTIVES OF THE STUDY

The first goal of the project was to understand the mechanisms by which phytoestrogens act on chromatin in breast cancer cell lines. For this study, we used chromatin immunoprecipitation (ChIP) coupled with QPCR to follow soy phytoestrogen effects on specific histone marks with selected genes. For the second project, we investigated the effects of histone methylation inhibitors (HMTi) and histone deacetylase inhibitors (HDACi) on histone lysine trimethylation and acetylation in breast cancer cell lines.

Subsequently, further investigations in epigenetics were performed with breast tumor and matched normal tissues. The aim was to find some epigenetic differences between breast tumors classified according to Saint Gallen and normal tissue breast. For that analysis, ChIP-QPCR technique also was used to follow the histone modification patterns associated with breast cancer.

Finally, histone modifications patterns in breast tumor and normal samples were analyzed using promoter microarrays. After validation in independent sets of samples, the identified methylation and acetylation histone signature would be helpful for the diagnosis of breast cancer and/or for the assessment of risk to develop breast cancer.

4. RESULTS

A. Effects of Phytoestrogens and Estrogen on Breast Cancer Cell Lines

Presentation of Publication 4

Dagdemiir A, Durif J, Ngollo M, Bignon Y-J, Bernard-Gallon D. Histone Lysine Trimethylation or Acetylation Can be Modulated by Phytoestrogen, Estrogen or Anti-HDAC in Breast Cancer Cell Lines. Epigenomics. 2013 Feb;5(1):51-63. doi: 10.2217/epi.12.74.

In this study, we studied phytoestrogens because they have been investigated as natural alternatives to hormone replacement therapy and their potential as chemopreventive agents. The goal of the project was to understand the mechanisms by which phytoestrogens which act on chromatin in breast cancer cell lines. We suggest that phytoestrogens possess anti-estrogenic properties may be also responsible for their chemopreventive effects in epigenetics.



Histone lysine trimethylation or acetylation can be modulated by phytoestrogen, estrogen or anti-HDAC in breast cancer cell lines

Aim: The isoflavones genistein, daidzein and equol (daidzein metabolite) have been reported to interact with epigenetic modifications, specifically hypermethylation of tumor suppressor genes. The objective of this study was to analyze and understand the mechanisms by which phytoestrogens act on chromatin in breast cancer cell lines. **Materials & methods:** Two breast cancer cell lines, MCF-7 and MDA-MB-231, were treated with genistein (18.5 μM), daidzein (78.5 μM), equol (12.8 μM), 17β -estradiol (10 nM) and suberoylanilide hydroxamic acid [1 μM] for 48 h. A control with untreated cells was performed. 17β -estradiol and an anti-HDAC were used to compare their actions with phytoestrogens. The chromatin immunoprecipitation coupled with qPCR was used to follow soy phytoestrogen effects on H3 and H4 histones on H3K27me3, H3K9me3, H3K4me3, H4K8ac and H3K4ac marks, and we selected six genes (*EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3* and *P300*) for analysis. **Results:** Soy phytoestrogens induced a decrease of trimethylated marks and an increase of acetylating marks studied at six selected genes. **Conclusion:** We demonstrated that soy phytoestrogens tend to modify transcription through the demethylation and acetylation of histones in breast cancer cell lines.

KEYWORDS: acetylation ■ breast cancer ■ histone ■ methylation ■ soy phytoestrogens

Breast cancer is the most common cancer for women in developed countries and urban zones from developing countries [1]. Breast cancer incidence is lower in Asian women in comparison with Westerners. This difference had been attributed to the fact that the traditional Asian diet contains important soy intakes compared with the western diet. Phytoestrogen chemicals found in soy share similarities with the natural human estrogen, 17β -estradiol, and are able to tighten the estrogen receptors [2–4] with a higher affinity for *ER β* [3–5].

Breast cancer progression is not well understood, however, it is likely due to a great amount of genetic mutations leading to widespread changes in gene-expression profiles [6,7], especially the expression of tumor suppressors and oncogenes [8]. Besides the genetic mutations, we need to further investigate epigenetic modifications in breast cancer, such as DNA methylation [9,10] and histone marks [11,12]. Many post-translational modifications of histones, lysine acetylation and methylation, and arginine methylation seem to be implicated in gene regulation [13]. A combinatorial histone code is able to recognize these and other modifications on chromatin regions and create transcription activation or repression of genes [14]. Although the epigenetic code is has not been completely elucidated, lysine acetylation (H3K4ac, H3K9ac and H4K8ac)

and trimethylation (H3K4me3) modifications are able to generate transcriptionally active gene promoters [15–17], whereas other marks, such as lysine methylation (H3K9me3 and H3K27me3), are associated with repressed chromatin [13,14]. Thus, there is evidence that epigenetic alterations in breast cancers can be followed using small-molecule epigenetic modulators. Therefore, we assessed the effects of soy phytoestrogens, 17β -estradiol or anti-HDAC (suberoylanilide hydroxamic acid [SAHA]) in breast tumor cell lines using chromatin immunoprecipitation (ChIP) of selected histone modifications. The relative levels of five modified histones, including H3K9me3, H3K27me3, H3K4me3, H4K8ac and H3K4ac, were determined in MCF7 and MDA-MB 231 breast cancer cell lines exhibiting different estrogen receptor statuses. In order to identify the presence of variations in global levels of histone marks after treatment, we proposed to follow ChIP with qPCR of six genes implicated in breast cancer and for which the decrease or increase in their expression can be the result of bulk histone modifications. We chose the *EZH2* and *P300* genes that code, respectively, for a HMT and a HAT, which are chromatin-modifying enzymes implicated in gene regulation by catalyzing reversible post-translational modifications of histones [18–20]. *SRC3*, which is coded by the *ncoa* gene, is a steroid receptor coactivator

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belonging to the p160 family (SRC1, SRC2 and SRC3), constituting a platform for other coactivators such as P300 [21], BRCA1, which is a transcription factor tumor suppressor gene [22], ER α , which is a nuclear receptor activated by 17 β -estradiol that stimulates proliferation, and ER β , which binds phytoestrogens and induces cellular proliferation and invasion [23].

Materials & methods

■ Cell lines

MCF7 and MDA-MB 231 breast tumor cell lines were collected from a pleural effusion of patients with invasive breast carcinoma [24,25]. Both human cell lines were provided by the American Type Culture Collection. MCF7 were cultured in RPMI-1640 medium supplemented with 20 μ g/ml of L-glutamine (Invitrogen), gentamycin (20 μ g/ml; Panpharma), 10% fetal bovine serum (Invitrogen) and insulin (1–4 mg/ml; Novo Nordisk) in a humidified atmosphere at 37°C containing 5% CO₂. This cell line had a positive ER status (ER α ⁺/ER β ⁺). MDA-MB-231 cells were grown in Leibovitz L-15 medium with 15% fetal bovine serum (Invitrogen), gentamycin (20 μ g/ml; Panpharma) and L-glutamine (20 μ g/ml) in a 37°C humidified atmosphere without CO₂. This cell line ER α ⁺/ER β ⁺ status. ER status of cell lines has been previously confirmed by immunohistochemistry [26].

■ Treatments

Cells (1 \times 10⁶ per T75 flask) were seeded in the medium and treated with 12.8 μ M S-equol synthesized by the ENITA Unité Micronutriments-Reproduction-Santé, genistein (18.5 μ M; Sigma-Aldrich), daidzein (78.5 μ M; Sigma-Aldrich), 17 β -estradiol (10 nM; Sigma-Aldrich) and suberoylanilide hydroxamic acid (SAHA; 1 μ M; Sigma-Aldrich) and dissolved in dimethyl sulfoxide and ethanol for 17 β -estradiol. For the controls, each cell line was conditioned in the medium without any treatment for 48 h. The doses of genistein and daidzein were previously determined as corresponding to the 50% inhibition of the proliferation (IC₅₀) [26]. SAHA [27], 17 β -estradiol [28] and S-equol concentrations were chosen according to studies by other authors and adapted to our studies [5,29–31].

■ Cell collection & DNA–protein cross-linking for the ChIP assay

Before 80% confluence was achieved, cells were trypsinised and counted by Millipore Scepter 2.0

Cell (Fisher Scientific). We used 1 \times 10⁶ cells for each treatment. Formaldehyde (36.5%; Sigma-Aldrich) was diluted to 1% in the culture media and cells were incubated at room temperature for 8 min for fixation. Cross-linking was finished at room temperature with 1.25 M glycine for 5 min. Hereafter, all chromatin preparations and ChIP reactions were handled at 4°C.

■ Cell lysis & chromatin shearing by sonication

The cross-linked cells were washed with PBS-Inhibitor solution (NaBu 20 mM) and cell membranes were lysed using HighCell ChIP Kit (Diagenode). The cross-linked cells were then ready for chromatin shearing. The chromatin was prepared in TPX tubes (Diagenode) with shearing buffer S1 and 1X protease inhibitor (Diagenode). The samples could be submitted to sonication using Bioruptor UCD-200 (Diagenode) for 5 runs of 10 cycles (30 s 'ON', 30 s 'OFF') for MCF7 and 4 runs of 10 cycles for MDA-MB 231 at 200 watt, at 4°C at all times during the sonication process. Between each run, samples were spun and vortexed. Fragments of sizes suitable for ChIP are of 100 at 500 bp. The sheared chromatin was frozen at -80°C for later use.

■ Chromatin immunoprecipitation

ChIP reactions were carried out on SX-8X IP-STAR compact automated system (Diagenode) and Diagenode Kits for all immunoprecipitation procedures. ChIP experiments were performed with 2 μ g of rabbit polyclonal antibodies (anti-H3K9me3 [pAb-056–050, Diagenode], -H3K27me3 [pAb-069–050, Diagenode], -H3K4me3 [pAb-003–050, Diagenode], -H4K8ac [pAb-103–050, Diagenode] or -H3K4ac [pAb-165–050, Diagenode]) and nonimmune rabbit IgG (Kch-504–250, Diagenode; e.g., negative control). ChIP reactions were brought up to 200 μ l. The IP DNA was purified with DNA Isolation Buffer according to the HighCell ChIP kit protocol. Each Auto-ChIP sample was performed using Auto Histone ChIP-seq kit reagents and contained 1 μ g of input chromatin. The reaction was incubated for 2 h for antibody coating with protein A-coated magnetic beads, then for 10 h at 4°C for IP reactions (for eight strips).

■ Quantitative real-time PCR

Real-time PCR was performed in triplicate using a 96-well optical tray with optical adhesive film, at a final reaction volume of 25 μ l containing

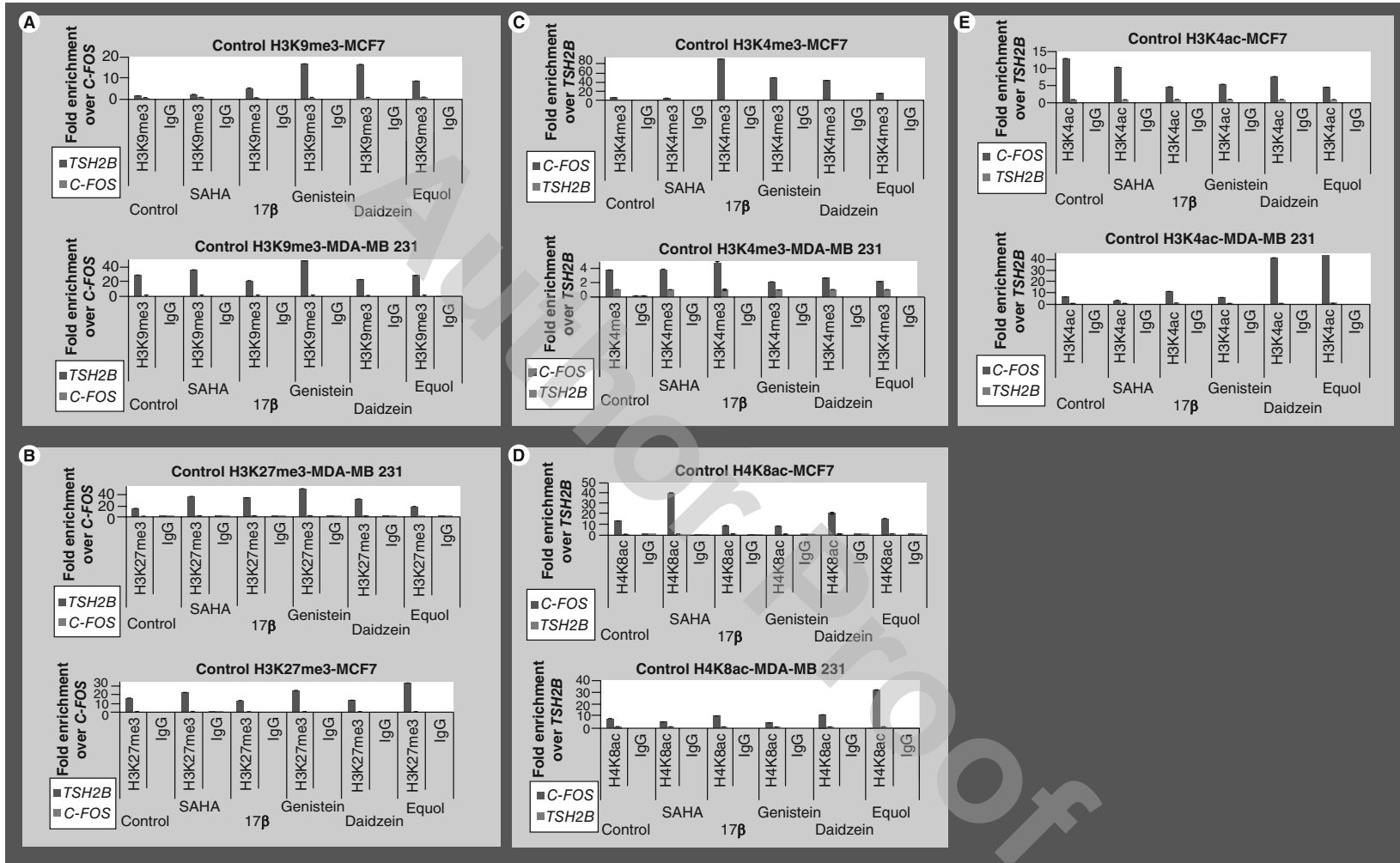


Figure 1. Control of repressive and activating epigenetic marks with *TSH2B* or *C-FOS* genes. MCF7 and MDA-MB 231 were treated by genistein (18.5 μM), daidzein (78.5 μM), 17β-estradiol (10 nM) or SAHA (1 μM) for 48 h and controls corresponded to untreated cells. Chromatin immunoprecipitation was controlled with rabbit IgG. Control of specific antibody (A) H3K9me3; (B) H3K27me3; (C) H3K4me3; (D) H4K8ac; (E) H3K4ac. The vertical axis represents the fold enrichment of H3K9 and H3K27 trimethylated on positive gene *TSH2B* over negative gene *C-FOS*. The y-axis represents the fold enrichment of H3K9me3, H3K27me3, H4K8ac, H3K4me3 and H3K4ac over *C-FOS* or *TSH2B* genes, respectively. SAHA: Suberoylanilide hydroxamic acid.

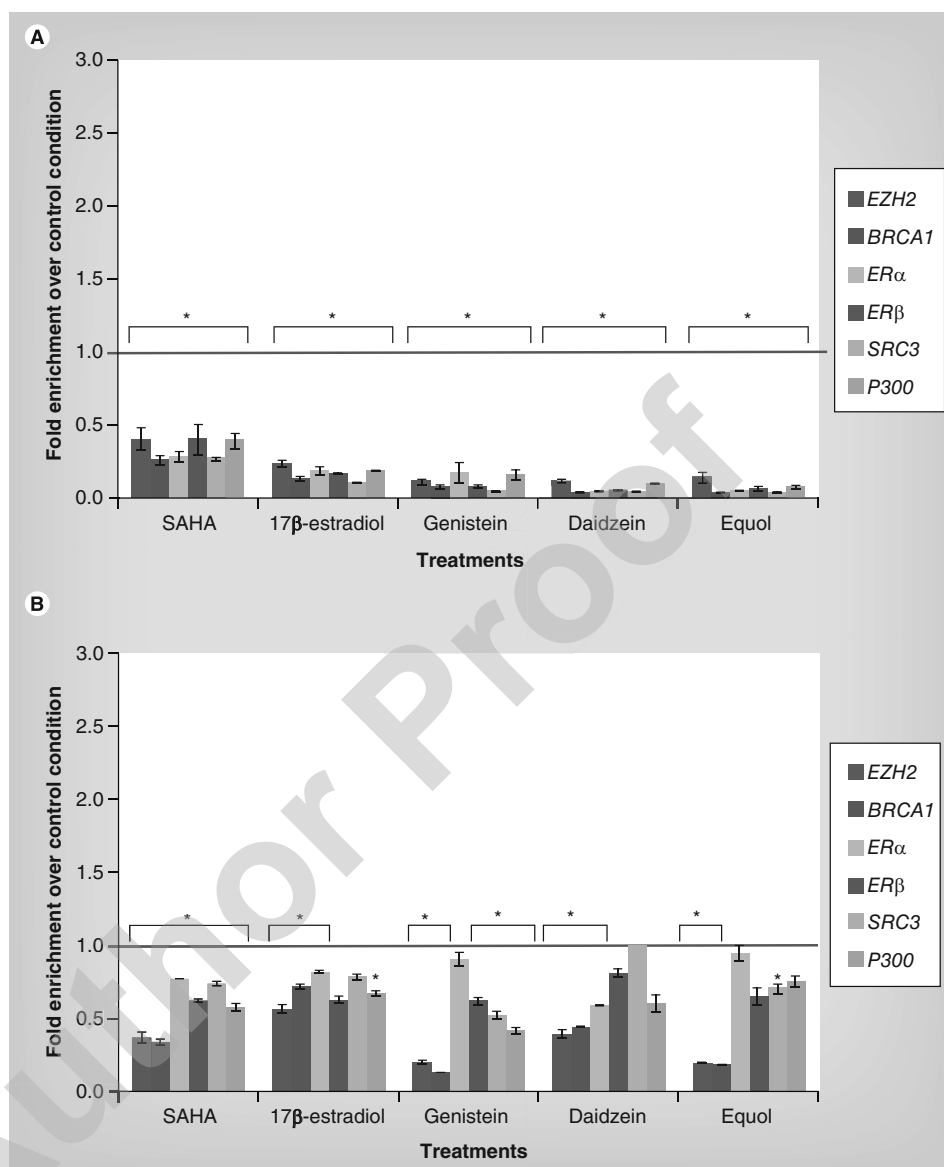


Figure 2. Decrease in H3K9me3 marks in breast cancer cell lines after different treatments.

Real-time PCR of *EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3* and *P300* after chromatin immunoprecipitation on repressive H3K9me3 marks. **(A)** MCF7 cell line after treatment with SAHA, 17 β -estradiol, genistein, daidzein, equol and controls without any treatment. **(B)** MDA-MB 231 cell line. The y-axis represented the fold enrichment of H3K9me3 marks over the control condition without any treatment, was normalized to 1 on the graph (red bar).

*p < 0.05.

SAHA: Suberoylanilide hydroxamic acid.

DNA IP (e.g., immunoprecipitated DNA) or DNA Input (e.g., total DNA; 5 μ l), 1X SYBR Green Supermix (Applied Biosystems) and 200 nM each of *C-FOS* (pp-1004–500, Diagenode; positive control for acetylation) or *TSH2B* (pp-1041–500, Diagenode; positive control for methylation) promoters. For other genes, Taqman Universal PCR Master Mix was used at 1X, 400 nM each of forward and

reverse primers and 250 nM of probe. Initial denaturation at 95 $^{\circ}$ C for 10 min to activate DNA polymerase was followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 15 s, and annealing and extension at 60 $^{\circ}$ C for 1 min (7900HT, Real-Time PCR System; Applied Biosystems). Primer and probe sequences were selected with the help of Primer Express software (ABI). Primer and probe sequences are:

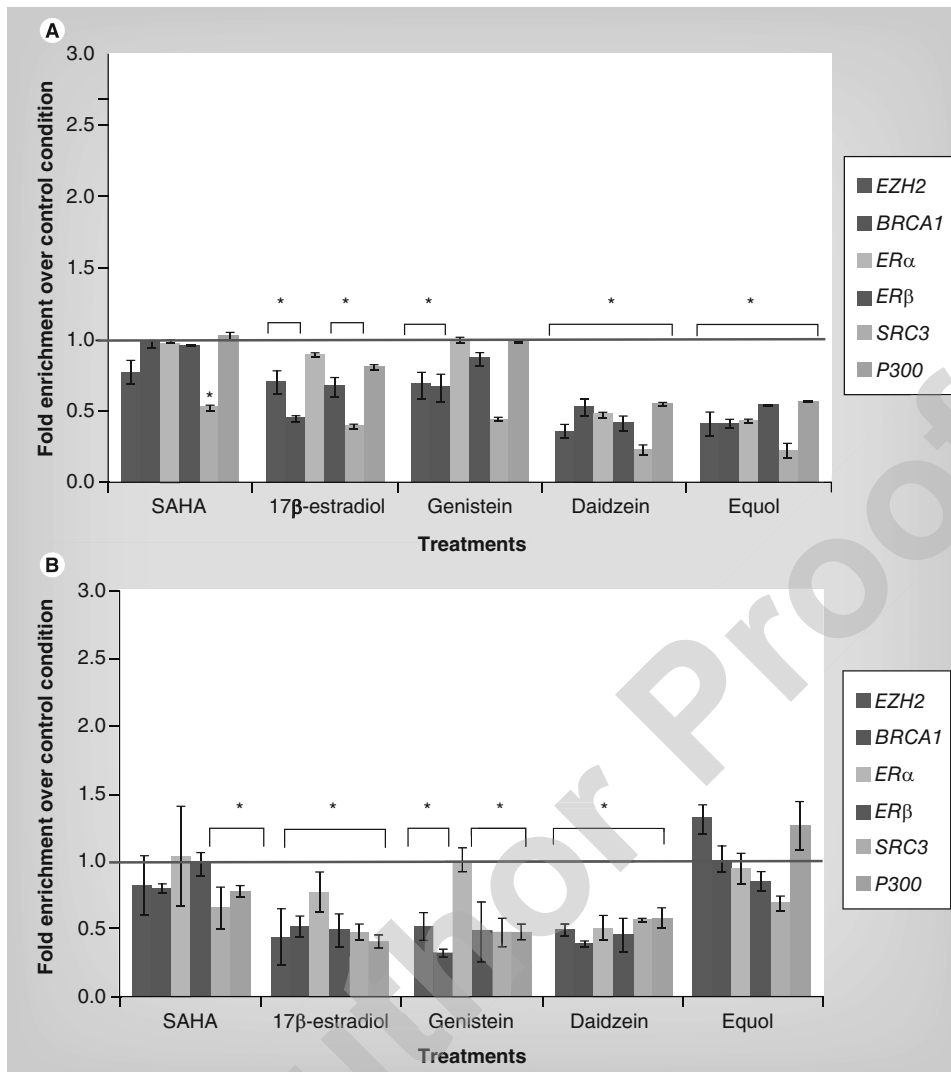


Figure 3. Decrease in H3K27me3 targets in breast cancer cell lines after treatments.

Chromatin immunoprecipitation with H3K27me3 targets analyzed by quantitative PCR on *EZH2*, *BRCA1*, *ER α* and *ER β* , *SRC3* and *P300* genes. (A) MCF7 cell line. (B) MDA-MB 231 cell line. The y-axis represented the fold enrichment of studied marks over control condition without any treatment, was normalized to 1 (red bar).

*p < 0.05.

SAHA: Suberoylanilide hydroxamic acid.

- for *ER α* , forward primer: 5'-CCCTACAT-TGGCTTAAACATCA-3'; reverse primer: 5'-TCTTTGGATCGCTCCAAAT-3'; probe: 5'-6FAM-TCCAGGCACAACCTC-MGBNFQ-3';
- for *ER β* , forward primer: 5'-GAGAGGCT-TTGGGTTTGTCAAAT-3'; reverse primer: 5'-CCTCTAGTCCACGGCTTTGC-3'; probe: 5'-6FAM-CAGCAAACGTAAC-CTCGGGCCCTG-TAMRA-3';
- for *P300*, forward primer: 5'-CGATGGCA-CAGGTAGTTTCG-3'; reverse primer: 5'-GCGCACCAGTAGAAAAGATTA-A-3'; probe: 5'-6FAM-CAGCCCCGGCC-TTCCACGTT-TAMRA-3';
- for *SRC3*, forward primer: 5'-AAAATTAA-GGGCAGGGCTAGGA-3'; reverse primer: 5'-GTGCGGCCGCTTTCG-3'; probe: 5'-6FAM-TCCGGATCCCGAGGGAGC-TCC-TAMRA-3';
- for *EZH2*, forward primer: 5'-CCCTCCAG-AAACACAATCAATAGA-3'; reverse primer: 5'-CCGCTGGTCTGGCTTTAT-3'; probe: 5'-6FAM-CAGAGCAGCTCGACTCTTCCCTCAAACCTT-TAMRA-3';

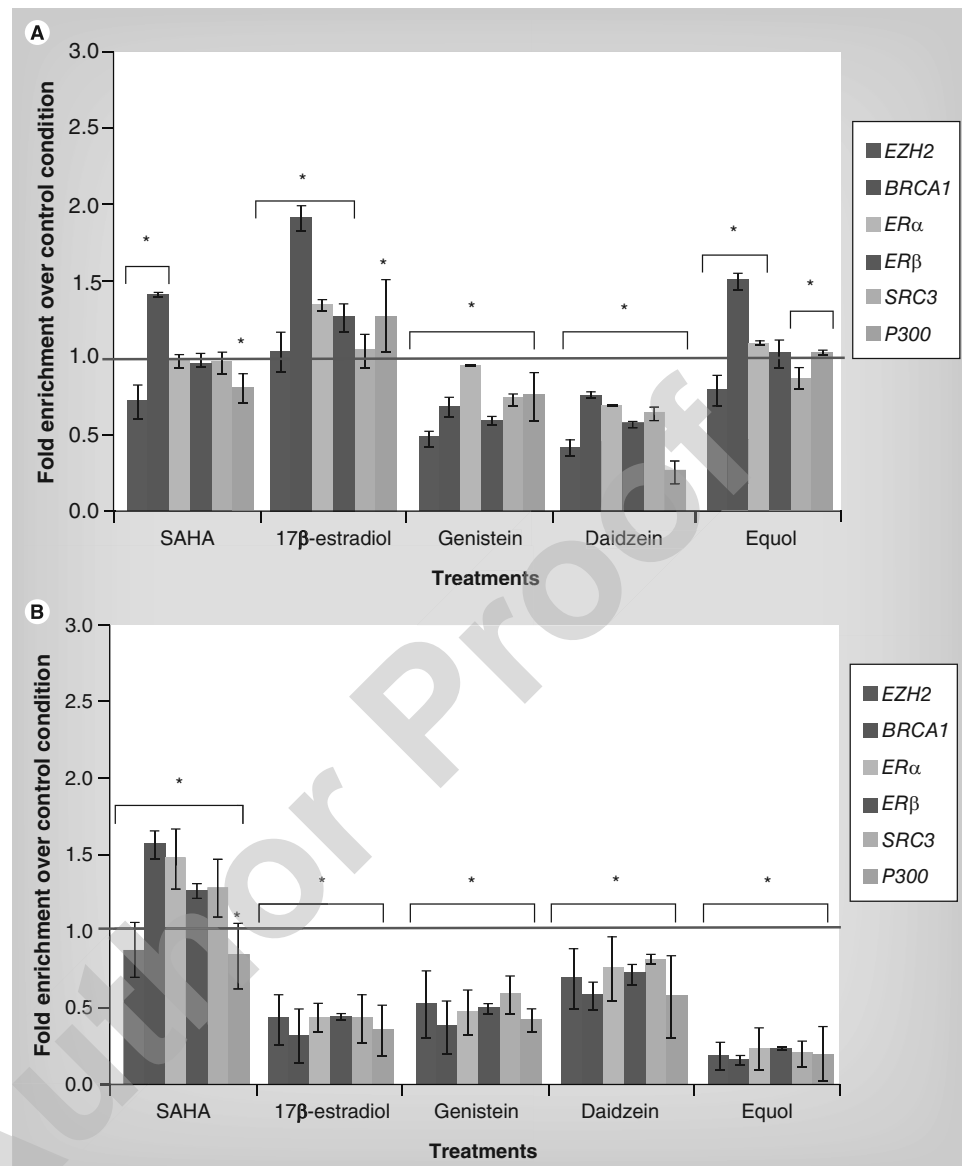


Figure 4. Global assessment of activation modification H3K4me3 in breast cancer cell lines after treatments. Data analyses after ChIP-qPCR with H3K4me3 activatrice marks. **(A)** MDA-MB 231 cell line. **(B)** MCF7 cell line. The y-axis represented the fold enrichment of the studied mark over controls without any treatment, were normalized to 1 (red bar).

*p < 0.05.

SAHA: Suberoylanilide hydroxamic acid.

- for *BRCA1*, forward primer: 5'-CCCCGTC-CAGGAAGTCTCA-3'; reverse primer: 5'-GCGCGGGAATTACAGATAAATT-3'; probe: 5'-6FAM-CGAGCTCACGCCGCGCAG-TAMRA-3'.

The amount of FAM fluorescence released during the PCR was measured by the real-time PCR system and is directly proportional to the amount of the PCR product generated. The cycle number at which the fluorescence signal crosses a detection threshold is referred to as Ct. The level

of methylation or acetylation was disclosed by the rate of IP relative to input. The efficiency of chromatin immunoprecipitation of a particular genomic locus can be calculated from qPCR data and reported as a percentage of starting material:

$$\%(\text{ChIP}/\text{total input}) = 2^{\left(\left[\text{Ct}(\text{x\% input}) - \frac{\log(\text{x\%})}{\log 2} \right] - \text{Ct}[\text{ChIP}] \right)} \times 100\% \quad (1)$$

All p-values were calculated using Student's test; p < 0.05 was considered to be statistically

significant.

■ Immunohistochemistry

A total of 4 μm alcohol–formalin–acetic acid-fixed and paraffin-embedded sections of MDA-MB 231 human breast cancer cell pellets were cut using a microtome, mounted on silanized glass slides (Starfrost) and dried overnight at 37°C. An automated Benchmark XT immunohistochemical instrument (Roche) was used to process the slides. Deparaffinization and rehydration of sections using EZ Prep (Roche), and a heat-induced antigen retrieval method was performed for 30 min with CC1 or CC2 Buffer (Roche). The slides were then incubated at 37°C for 40 min with anti-EZH2 (1:100 Polyclonal Rabbit; Diagenode) or anti-P300 (1:200 Polyclonal Rabbit; Santa-Cruz) primary antibodies. Subsequent incubations with a secondary antibody were carried out with the UltraView universal DAB detection kit (Roche) according to the manufacturer's protocol. The signal was amplified using the Roche amplification kit. Slides were then counterstained with hematoxylin, cleaned in distilled water, and coverslipped with an aqueous Faramount mounting media (DAKO). A negative control was done with replacement of the primary polyclonal antibody with PBS.

Results

■ Soy phytoestrogen decrease trimethylation at H3K9

First, we compared repressive histone modification of histone H3 with H3K9me3 marks in MCF7 and MDA-MB 231 cell lines without any treatment, versus cell lines treated by soy phytoestrogens. We performed positive control of activating marks of *C-FOS* and positive control of repressive marks, *TSH2B*, and negative control of ChIP reaction as rabbit IgG (FIGURE 1A). We carried out ChIP assays using an antibody against H3K9me3 analyzed by real-time PCR performed with *EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3* and *P300* primers. When MCF7 and MDA-MB 231 were treated by phytoestrogens the fold enrichment of H3K9me3 over control condition decreased significantly, such as 17 β -estradiol treatment whose action is the same as phytoestrogens (FIGURE 2A & 2B).

■ Loss of repressive trimethylation at H3K27

We performed the same control as previously and the fold enrichment over *C-FOS* increased with *TSH2B* (FIGURE 1B). ChIP on cells derived from

breast cancer without any treatment confirmed the previously described predominance of H3K27 alterations in cancer [32]. In MCF7 and MDA-MB 231 cells that were treated with soy phytoestrogens and the natural hormone, 17 β -estradiol, the H3K27me3 marks were found to decrease significantly over controls without any treatment on transcriptional genes (FIGURE 3A & 3B).

■ Global decrease of activating H3K4me3 marks in MCF7 & MDA-MB 231

Thus, we investigated the methylation of activating marks. We kept the controls with *C-FOS*, the *TSH2B* genes, and polyclonal rabbit IgG. The fold enrichment on *C-FOS* over *TSH2B* was found to be increased in all treatments in two cell lines (FIGURE 1C). Results of the treatment by the anti-HDAC inhibitor (SAHA) showed a significant increase on activating mark H3K4me3. Despite activating action on the studied mark [33], the treatment by Genistein and Daidzein leads to significant loss of methylation at K4 of H3 in MCF7 and MDA-MB 231 cell lines, and by 17 β -estradiol and S-equol in MCF7 cell lines (FIGURE 4A & 4B).

■ Soy phytoestrogens & SAHA increase acetylation at H4K8 & H3K4

We performed controls with *C-FOS* and *TSH2B* genes, and polyclonal nonimmune rabbit IgG. We examined the enrichment of acetylation modification on *C-FOS* (positive control) over *TSH2B* (negative control; FIGURE 1D & 1E). ChIP experiments revealed the acetylating effect of SAHA in MDA-MB 231 and MCF7. Phytoestrogens and 17 β -estradiol increased acetylation significantly at K8 and K4 of H4 and H3, respectively (FIGURE 5A, 5B, 6A & 6B).

■ Immunohistochemistry correlates with histone methylation & acetylation at selected silenced or enhanced genes

Activating marks were increased by treatment. Histone acetyltransferase P300 establishes the formation of the acetylated mark H4K8ac. An extensive increase in P300 staining was found by immunohistochemistry in the cytoplasm in MDA-MB 231 cell line after 18.5 μM Genistein exposure for 48 h compared with untreated cells (FIGURE 7A). By contrast, repressive marks were found to be decreased by treatment with 17 β -estradiol and SAHA. The H3K27me3 mark, the most studied in breast cancer, was

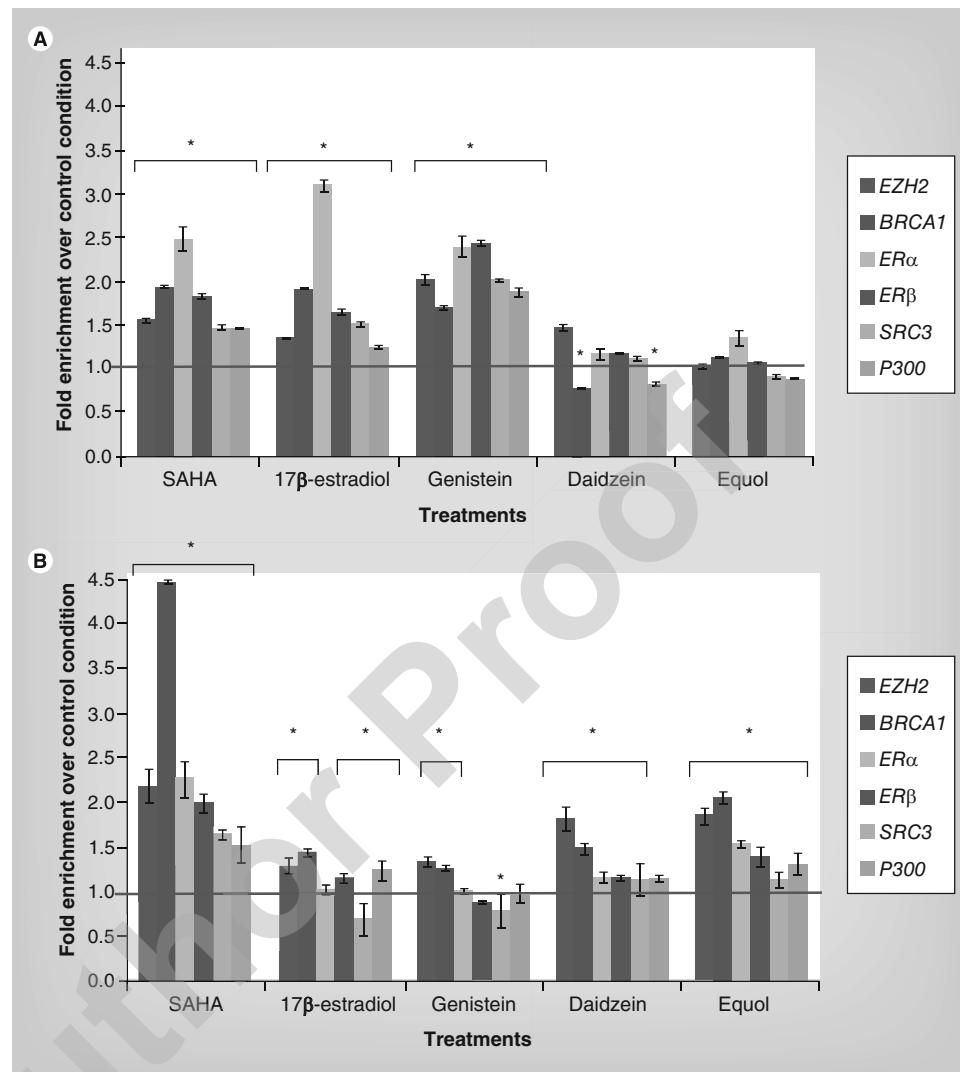


Figure 5. Histone modification enrichment with H4K8ac in breast cancer cell lines after treatments. Chromatin immunoprecipitation was performed with anti-H4K8ac. **(A)** MDA-MB 231 cell line. **(B)** MCF7 cell line. The vertical axis represented the fold enrichment of the studied mark over controls condition without any treatment, were normalized to 1 (red bar).

*p < 0.05.

SAHA: Suberoylanilide hydroxamic acid

established via EZH2 protein. This result was confirmed by immunocytochemistry on MDA-MB 231 treated with 17 β -estradiol and SAHA; a decrease in EZH2 staining has been shown in the cell nuclei (FIGURE 7B). A control was performed without the primary antibody and remained negative.

Discussion

The mutations in *BRCA1* or *BRCA2* tumor suppressor genes are important factors. The hormonal status changes in the female endocrine system that occur at different stages of life are also important. To deal with

that, in the present work we chose to study two different ER status breast cancer cell lines, MDA-MB-231 (negative ER α) and MCF7 (positive ER α). However, these two cell lines also exhibited positive ER β and further investigation, such as ER β gene knockdown, is necessary to demonstrate the role of ER β in phytoestrogen-mediated histone modifications.

In addition, environmental factors, such as diet, also affect the onset of breast cancer [34]. Breast cancer is a hormone-dependent cancer. In Asian countries, the risk of developing breast cancer remains lower than western countries. This could be explained by a diet

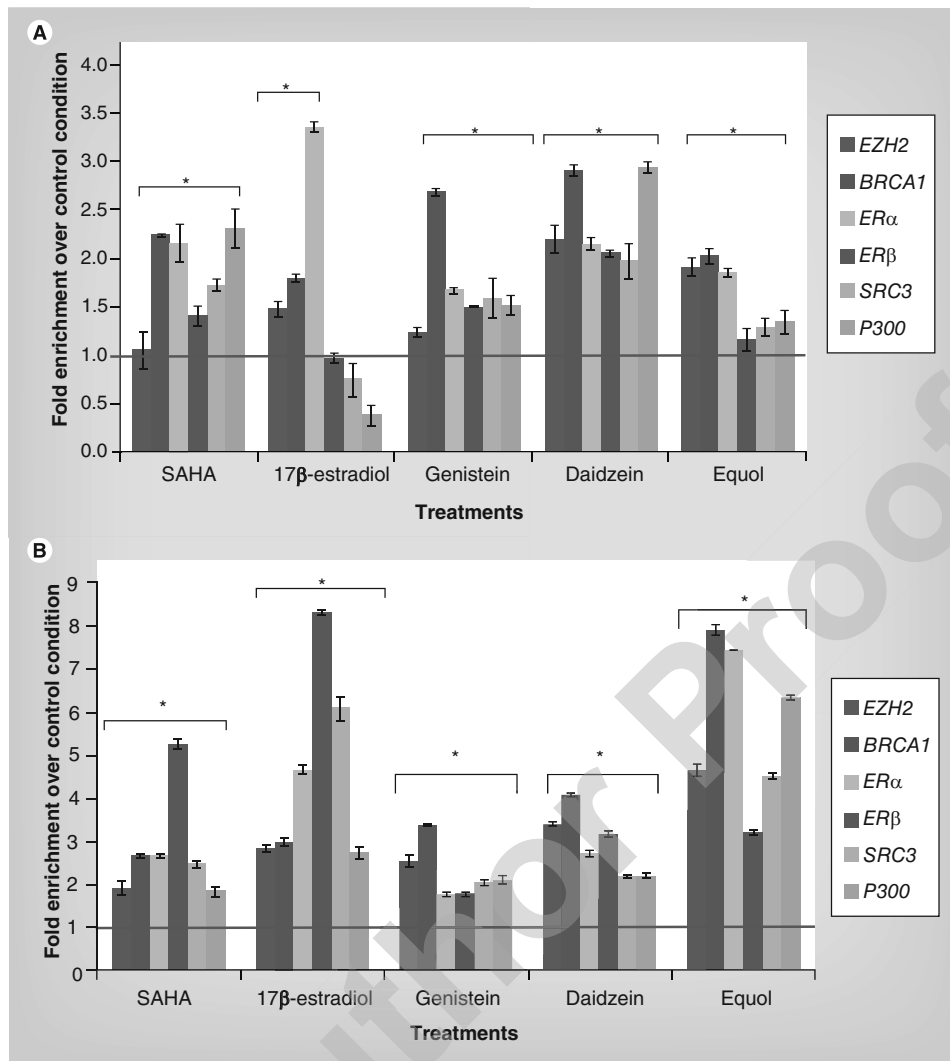


Figure 6. H3K4ac enrichment profiles in breast cancer cell lines, after different treatments.

Chromatin immunoprecipitation was performed with H3K4ac antibodies and analyzed by qPCR with selected genes (*EZH2*, *BRCA1*, *ER α* and *ER β* , *SRC3* and *P300*). (A) MCF7 cell line. (B) MDA-MB 231 line. The y-axis represented the fold enrichment of the studied mark over controls without any treatment, were normalized to 1 (red bar).

* $p < 0.05$.

SAHA: Suberoylanilide hydroxamic acid.

high in soy and its derivatives. The isoflavones from soy have been shown to exert an agonist or antagonist effect on breast tumor cell proliferation and have been determined as key in protecting against it [35]. Isoflavones belong to the phytoestrogen family and act as estrogen-like compounds, having various biological effects, with some data leading to caution [36]. Indeed in humans, phytoestrogens from soy have ambivalent effects on breast cancer [37].

A growing number of studies have revealed the importance of histone modifications in cancer, with a global hypermethylation at K27 of histone 3, leading to chromosomal instability

and loss of the expression of tumor suppressor genes [38]. In breast cancer, studies have shown variations of expression of *GSTP1*, *BRCA1* and *BRCA2* tumor suppressor genes, resulting from epigenetic changes such as hypermethylation of the promoters of these genes [9,39–41] by inhibiting DNMT [42], and acetylating action at histones via the recruitment of SRC2 and P300 after isoflavone treatments [43].

Following studies that have shown epigenetic post-translational modulation and DNA methylation play a key role in cancer. We decided to study the effects of phytoestrogens, estrogen and SAHA, as a positive control for

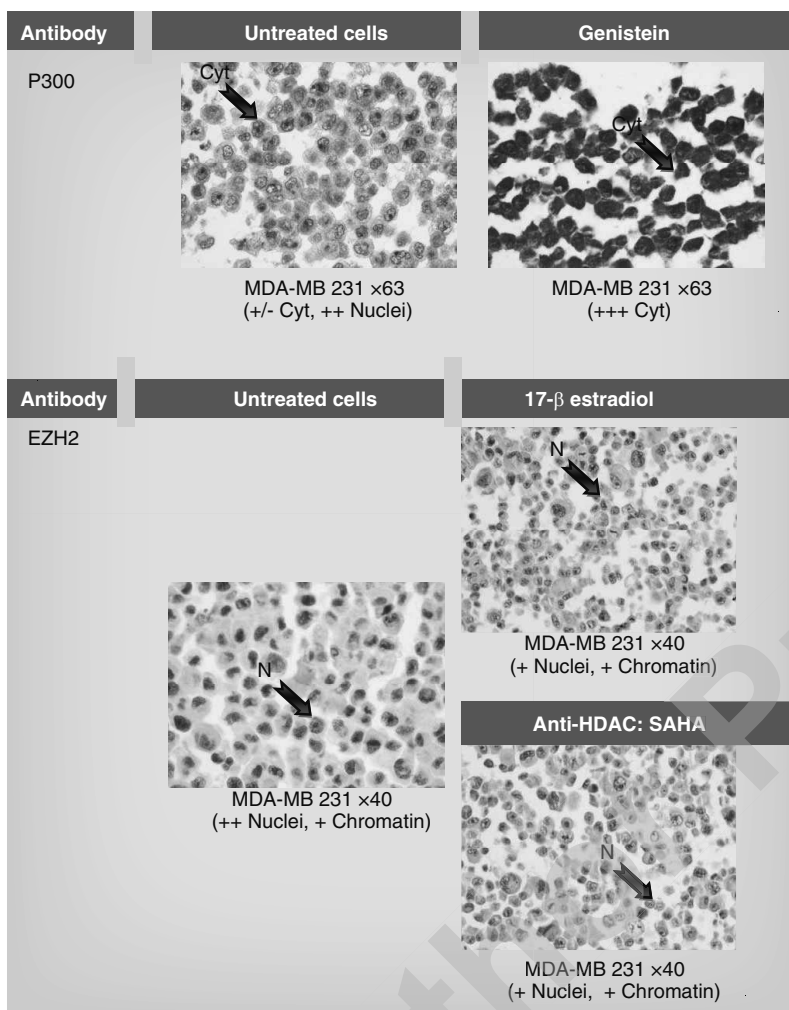


Figure 7. Immunohistochemistry staining of MDA-MB-231 human breast cancer cell lines. (A) Cytoplasmic staining was exhibited with 1:200 P300 polyclonal antibody (Santa-Cruz). The P300 staining after 18.5 μ M-Genistein treatment was considerably increased ($\times 63$). **(B)** Nuclear staining was exhibited with 1:100 EZH2 polyclonal antibody (anti-EZH2, Diagenode), shown by arrowheads and chromatin staining was exhibited. Nuclear staining was decreased in cells treated with 17 β -estradiol and SAHA ($\times 40$). Cyt: Cytoplasm; N: Nuclei; SAHA: Suberoylanilide hydroxamic acid.

acetylation, in breast cancer cell lines (MCF7 and MDA-MB 231) on selected genes (*EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3* and *P300*) involved in transcription. Previously, the *ER α* , *SRC3*, *P300* and *EZH2* genes were described as overexpressed and promoted the activation of transcription in breast cancer cells [18,44]. By contrast, the *BRCA1* gene is known to be repressed in sporadic breast cancers [22] and the *ER β* gene inhibits the proliferation induced by the hormone 17 β -estradiol in the breast cancer cells [45,46].

We studied the effects of treatments on the variation of post-translational modification of histones, such as lysine methylation and

acetylation of histone H3 and H4. We provided evidence that phytoestrogens demethylated and acetylated histones of these selected genes in MCF7 and MDA-MB 231 breast cancer cell lines, leads to unpacking of the chromatin and enhance transcription. SAHA acetylates histones in both cell lines to enhance transcription, and leads to cell apoptosis [27]. However, further exploration in the MCF7 and MDA-MB231 breast cancer cell lines might use DZNep, a histone methyltransferase inhibitor of histone H3K27, for other positive controls corresponding to the demethylation of histones.

In summary, this study showed that phytoestrogens have a demethylating and acetylating effects on histone modifications in *EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3* and *P300* genes. These results suggest a beneficial effect for the genes upregulated in cancer but an adverse effect for genes downregulated because the action did not seem to be specific for genes. With the support of previous studies, we can establish a hypothesis of the mechanism of action of phytoestrogens in breast cancer cells, at the establishment of epigenetic modifications. In cancer cells that were treated by soy phytoestrogens, the level of H3K27me3 decreased via Polycomb complex formation and increases the establishment of the H4K8ac mark via a transcriptional coactivator, such as P300, SRC3 and activates transcription (FIGURE 8A & 8B). Our data add a novel layer of complexity to epigenetic dysregulation in cancer and also established histone modification-mediated silencing or enhancing for a promising therapeutic target.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the

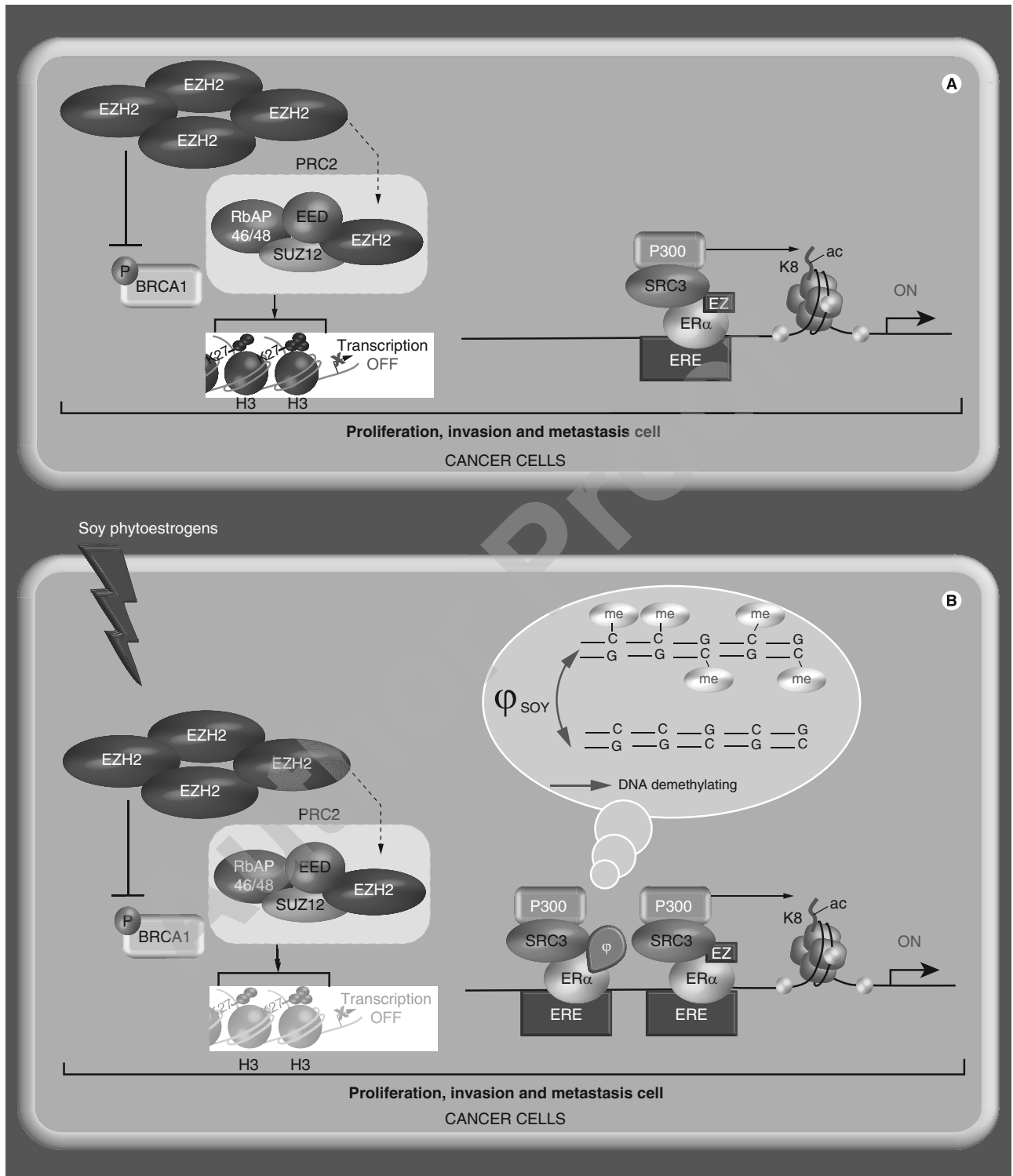


Figure 8. Hypothesis of the mechanism of action of phytoestrogens in H3K9me3, H3K27me3 and H4K8ac marks, in cancer cells. Model without any treatment (A); with soy phytoestrogens treatment (B).

principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects,

informed consent has been obtained from the participants involved.

Executive summary

- Histone methylation or acetylation, an epigenetic event observed in cancer, occurs at lysine or arginine on histones.
- This mechanism induces the opening or closing of chromatin and therefore gene enhancing or silencing.
- In this study, an investigation of isoflavones, 17 β -estradiol and anti-HDAC (suberoylanilide hydroxamic acid) on *EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3* and *P300* genes, was carried out by chromatin immunoprecipitation-qPCR.
- Two breast cancer cell lines, MCF7 and MDA-MB 231, were treated with 18.5 μ M genistein, 78.5 μ M daidzein, 12.8 μ M equol, 10 nM 17 β -estradiol and 1 μ M suberoylanilide hydroxamic acid (anti-HDAC) for 48 h.
- Decrease trimethylation and increase acetylating marks were observed in cells treated with soy phytoestrogens compared with untreated cells.
- Soy phytoestrogens tend to modify the transcription through the demethylation and acetylation of the histones in breast cancer cell lines.

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▪ of interest

▪▪ of considerable interest

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Conclusion of Publication 4

We assessed the effects of soy phytoestrogens, 17 β -estradiol and SAHA for selected histone modifications in breast cancer cell lines using chromatin immunoprecipitation and immunohistochemistry. We determined the relative levels of five modified histones, including H3K9me3, H3K27me3, H3K4me3, H4K8ac, and H3K4ac in MCF7 and MDA-MB 231 in breast cancer cell lines.

In cancer cells treated by soy phytoestrogens the level of H3K27me3 decreased through polycomb complex formation and the treatment increased the establishment of the H4K8ac mark via transcriptional co-activator such as *P300*, *SRC3* and activates transcription.

B. Epigenetic Therapy in Breast Cancer Cell Lines

Presentation of Publication 5

Dagdemir A, Karsli-Ceppioglu S, Judes G, Lebert A, Echegut M, Ngollo M, Penault-Llorca F, Bignon YJ, Bernard-Gallon D. What are the Effects of Histone Methylation Inhibitor and/or Histone Deacetylase Inhibitor on Histone Lysine Trimethylation or Acetylation in Breast Cancer Cell Lines? (2014, submitted).

The aim of this study was an understanding of the latest ‘*epi-drug*’ discoveries focusing on the chemical characterization and use of epigenetic modulators in pre-clinical and clinical settings against breast cancer. We know that histone modifications are major epigenetic modifications and several enzymes are responsible in this mechanism. For example, the dynamic process of histone acetylation has been linked to gene transcription, and histone deacetylation has been related to inactive chromatin. Under physiological conditions, chromatin acetylation is regulated by the balanced action of histone acetyltransferases (HATs) and deacetylases (HDACs).

For this reason, we investigated two HDAC inhibitors which are NaBu and SAHA; and a HMT inhibitor which is DZNep. We focused on the chemical aspects of such molecules, joined to their effective (or potential) application in breast cancer therapy.

What are the Effects of the Histone Methylation Inhibitor and/or the Histone Deacetylase Inhibitor on Histone Lysine Trimethylation or Acetylation in Breast Cancer Cell Lines?

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Abstract

The Histone Deacetylase Inhibitors (HDACi) and Histone Methylation Inhibitors (HMTi) are known to interact with epigenetic modifications. In our study we used two breast cancer cell lines MCF-7 and MDA-MB-231, treated with 3-Deazaneplanocin A hydrochloride (DZNep) [5 μ M], Sodium Butyrate (NaBu) [2 mM] and Suberoylanilide Hydroxamic acid (SAHA) [1 μ M] for 48 hours. We used a culture control with untreated cells. Firstly, we applied chromatin immunoprecipitation (ChIP) coupled with QPCR. This was to follow the impact of HDACis and HMTi on H3K27me3, H3K9ac and H3K4ac marks, and we selected *EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3*, *P300* genes for analysis. Secondly, we examined Western Blot with correspondent proteins including PGR and RNA analyses also.

HDACi induced a decrease of trimethylated marks and activates of acetylating marks studied with selected genes. SAHA and NaBu increased acetylation at lysine 4 of histone H3 in MCF7 and DZNep decreased on H3K27me3 marks a slight difference in MDA-MB-231. Moreover we found that by Western blot analysis; DZNep acted as a global inhibitor of histone methylation and it was not selective to BRCA1 for each cell line. Finally, when we investigated mRNA expression levels, DZNep acted globally and it could inhibit both activating and repressive histone marks, conversely NaBu and SAHA were increasing of all mRNA expression especially in MCF 7.

These results suggested of HDACi and HMTi have beneficial effects on the genes, which are upregulated in cancer; however an adverse effects for downregulated genes, so the action did not seem to be specific for genes. Our results add a new outlook of complication to epigenetic dysregulation in cancer and also established histone modifications-mediated silencing or enhancing for up-and-coming therapeutic target. Our data demonstrate that HDACi disposed to modify the transcription in the demethylation and acetylation of the histones in breast cancer cell lines.

Key words

Breast cancer, HMTi, HDACi, Histone Methylation, Histone Acetylation.

Introduction

Breast cancer is a heterogeneous disease and it can be impressionable by way of multi-factorial processes and it is also a hormone-dependent cancer. Previously, many studies have shown that breast cancer and epigenetic mechanisms have a very powerful interactive relation. In recent times, epigenetic modifications like an abnormal methylation and acetylation of genes and histones have been shown to play a critical role in breast cancer development. With this information, our study focuses our investigation on this issue.

Histone methylation or acetylation is an epigenetic event observed in cancer. It occurs at lysine or arginine on histones. This mechanism induces the opening or closing of chromatin, thus gene enhancing or silencing. Breast cancer is a heterogeneous disease organizing from premalignant hyperproliferation to incurable and metastatic carcinoma [1]. Chromatin-modifying enzymes are very important for in gene regulation by catalyzing reversible post-translational modifications of histones like lysine acetylation and methylation [2]. These histone modifications and other modifications generate a synthesis of histone code that separate chromatin regions for transcription activation or repression [3].

Histone deacetylases (HDAC) can catalyze the hydrolysis of acetyl groups on lysine residues of histones, cause the wrapped and packing of chromosomal DNA around histones, and then regulate gene expression. Deacetylation of histones, catalyzed by HDACs, is also reported to be associated with gene silencing. In large group of HDAC there are 18 members classified into four groups. Class I includes HDAC1, HDAC2, HDAC 3 and HDAC 8. Class II HDACs occurs two subtype HDACs group; Class IIa comprises HDAC 4, HDAC 5, HDAC 7, HDAC 9 and Class IIb HDACs composed HDAC 6 and HDAC 10. Class III uses a different mechanism of action as a call like NAD⁺-dependent proteins; SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7. The last group of HDAC is Class IV and it involved HDAC11 [4,5]. Inhibitors of histone deacetylase (HDACi) de-repress genes that afterwards developed in evolution the defense mechanism, differentiation and

apoptosis of cancer cells. This is especially true for HDAC1, HDAC2, HDAC3, HDAC4 and HDAC6 associated with cancer cell proliferation. For instance, the role of HDAC3 is inducing G2/M arrest and also HDAC1 and HDAC2 downregulation leads to *p21^{Waf1/Cip1}* upregulation that induce apoptosis. HDACi is a significant group of anti-tumor agents as a future function in the treatment of breast cancer. According to a recent study which was carried out in Estrogen (ER)-positive cells, treatment with HDACi has been identified with a transcriptional down-regulation of ER and its response genes [6]. In ER-negative cell lines, HDACi demonstrated the reconstruction of ER expression. Furthermore, HDACi has been reported to adjust the progesterone receptor [7]. To date, seven groups of HDACis have been reported; short-chain fatty acids, hydroxamic acids, benzamides, cyclic tetrapeptides containing a 2-amino-8-oxo-9, 10-epoxy-decanoyl (AOE) moiety, cyclic peptides without the AOE moiety, epoxides and psammaplins [8].

Histone methylation is mediated through histone methyltransferases (HMTs) and it appears mono-, di- or tri- methylated at lysine or arginine [9]. Many HMTs (including histone lysine methyltransferase (HKMT) and histone arginine methyltransferase (HRMT)) effected with estrogen coactivators are involved with breast cancer [10]. Recently, there are a lot of studies concerning HKMTs because it can be an activator or repressor of gene expression, depending on the position of the modified residue. The Polycombgroup (PcG) proteins are in charge of maintaining the inactive expression of many genes. In the last days, three different polycomb repressive complexes have been identified: polycomb repressive complex 1 (PRC1), polycomb repressive complex 2 (PRC2), and pleiohomeotic-repressive complex (PhoRC) [11].

PRC1 and PRC2 are related to chromatin contraction. This is especially true for PRC1 which catalyzes the monoubiquitylation of H2A. On the other hand, PRC2 provides the methylation of H3K27. PRC2 is certainly important in the midst of PcG proteins because it has connected with stem cell biology and cancer. PRC2 multi-protein complexes include 4 core proteins. Enhancer of zeste homolog 2 (EZH2) is the most important subunit of the PRC2 and also PRC2 comprises Suppressor

of Zeste 12 Homolog (SUZ12), Embryonic Ectoderm Development (EED) and Retinoblastoma binding protein 4 (RbAp46/48). EZH2 acts as an HKMTase [12].

There are a lot of modifications that occur on the histone N-terminal region in a manner like acetylation, methylation, ubiquitination or like a phosphorylation. Primarily, the targets of HMTs are K4, K9, K27 and K36 on the H3. Recent studies have shown that overexpression of EZH2 is associated with breast cancer [13]. We have chosen that gene for its histone methylase acts, specifically at lysine 27 of histone 3. There are 3 transcription factors that assist in the regulation of EZH2. The first factor is E2F; the target of the retinoblastoma protein (pRb). It has a crucial role in cell cycle process, especially activating genes that control for S phase in cell cycle. The other transcription factor is the HIF response elements (HRE) which are controlled EZH2 expression levels in gene promoter. Once and for all, MEK/ERk/Elk pathway provides to EZH2 overexpression in ERBB2-overexpressing breast cancer cell line [14].

Previous study has shown that mutation in BRCA1 causes of increase EZH2 concentration particularly in the basal like breast carcinomas [15]. Generally EZH2 is overexpressed in ER-negative breast cancer and inhibits BRCA1 phosphorylation. For this reason, transmission from G2 to M is accelerated in cell cycle and increased cell proliferation.

DZNep expend the cellular levels of EZH2 and inhibits the associated histone H3 lysine 27 trimethylation. Recent studies have shown that DZNep inhibits cell proliferation by inducing G1 arrest and apoptosis. DZNep repressed S-adenosylhomocysteine hydrolase; a factor of metionin cycle, resulting in increasing of the S-adenosylhomocysteine hydrolase repression with a knock-on interruption of methylation of substrates by EZH2 [16,17] (Figure1).

Suberoylanilide hydroxamic acid (SAHA) and Sodium Butyrate (NaBu) are commonly used as a HDACi [18]. SAHA shows strong anti-proliferative effects on various cancer cell lines and is currently in clinical trial for the treatment of solid and hematological tumors [19]. However, the

mechanism by which SAHA induces autophagy cell death in acquired endocrine-therapy is not clearly understood.

NaBu is sodium salt of butyric acid. It is short-chain fatty acid that aims at the activated region of zinc in HDAC. It has a very short half-life [20]. It has many effects like an inhibition of proliferation, induction or repression of gene expression and induction of differentiation [21]. Particularly, NaBu has been a necessary instrument for identifying the role of histone acetylation in chromatin structure [22]. Previous study has shown that it affected the expression of only 2% of mammalian genes by inhibiting the HDAC activity [23]. Inhibition of HDAC1 and HDAC2 with NaBu, *P300* activities increase because HAT continued, therefore hyperacetylation of the histones and induction of *P21^{Waf/Cip1}* gene expression. This gene plays key role in G1/S phase of the cell cycle (Figure 1).

P300 is a member of the mammalian histone acetyl transferase (HAT) family. HAT acts are activating the induce of apoptosis in a breast cancer cell line [24]. *EZH2* and *P300* genes, chromatin –modifying enzymes, implicated in gene regulation by catalyzing reversible post-translational modifications of histones [14].

Steroid receptor coactivator-3 (*SRC-3*), a transcription coactivator, is frequently overexpressed in breast cancer [25]. We chose that gene due to function of the coactivator for HAT [26]. *BRCA1* is a tumor suppressor gene and transcription factor. Also, it has been shown that before BRCA1 inhibits ligand-induced ER α signaling and blocks subsequent ER α transcriptional activation [27]. In many breast cancers, estrogen-mediated signaling is a very important function of adjusting to evolution and endurance signaling pathways and can directly drive tumorigenesis by leading the expression of genes. Some authors have reported the ER β is down-regulated in breast tumorigenesis and another recent study has shown the regulation of ER β expression by promoter methylation [28]. In vitro studies point out ER β plays a role in the modulator of proliferation and invasion of breast cancer cells, so the loss of ER β expression could be one of the reasons for breast cancer development [29]. In addition to these 6 genes, we decided to add Progesterone Growth Factor gene (PGR) in

accordance with previous study which has shown that; progesterone uses its effects mainly via estrogen-dependent PGR, the expression of progesterone effects may be dominated by the primer effect of estrogen [30].

It becomes necessary to investigate epigenetic alterations in breast cancers to describe new prognostic markers and therapeutic targets. For these reasons, we can determine the effects of HMTi (DZNep) and HDACi (SAHA and NaBu) in cell lines on selected histone modifications.

Materials and Methods

Cell culture and cell treatment

We chose two breast cancer cell lines MCF7 and MDA-MB 231 derived from a pleural effusion breast adenocarcinoma. The MCF-7 cell line an estrogen receptor (ER) positive control cell line that we selected for this study [31]. On the other hand, we used MDA-MB-231 cells as a contrast to MCF7 because MDA-MB-231 human breast cancer cell line is a model of ER-negative breast cancers for both ER isoforms [32].

All cell lines supplied by the American Type Culture Collection. MCF7 were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 20 µg/ml of L-Glutamine (Invitrogen), Gentamycin (20 µg/ml; Panpharma), 10% fetal bovine serum (Invitrogen) and Insulin (1-4 mg/ml; Novo Nordisk) in a humidified atmosphere at 37°C containing 5% CO₂. MDA-MB-231 cells were cultured in Leibovitz L-15 medium with 15% fetal bovine serum (Invitrogen), gentamycin (20 µg/ml; Panpharma) and 20 µg/ml of L-glutamine in a 37°C humidified atmosphere without CO₂. Cells (1x10⁶ per T75 flask) were cultured in the medium and treated with 5 µM DZNep, 2 mM NaBu and 1µM SAHA provided by the Sigma-Aldrich and dissolved them successively in water, ethanol and dimethyl sulfoxide; all the concentrations have already been determined by our previous

studies [33], [34] . For controls, each cell line was conditioned in the medium without any treatment, during 48 hours.

ChIP Assay

All the cell lines were trypsinized and counted by Millipore Scepter 2.0 Cell (Fisher Scientific) in the event of 80% confluence. Before the cross linking, we used 1×10^6 cells for each treatment. A 36.5% concentration of formaldehyde (Sigma-Aldrich) was diluted to 1% in the culture media after that cells were incubated 8 minutes at room temperature for fixation. To finish the cross-linked, we added 1.25 M Glycine and incubated it for 5 minutes at room temperature. Afterwards, all chromatin preparations and ChIP reactions were handled at 4°C [33].

After the cross-linked, cells were washed with a PBS-Inhibitor and cell membranes were lysed by HighCell ChIP Kit (Diagenode). Finally, the cross-linked cells were ready for chromatin shearing.

The chromatin was prepared in special tubes provided by the Diagenode (TPX tubes) with shearing buffer (S1) and 1X volume protease inhibitor (Diagenode). The samples were sonicated by Bioruptor® Standard, which was provided by the Diagenode, for 5 runs of 5 cycles. Each cycle contained all 30 seconds “ON” and 30 seconds “OFF” at 200 Watt, and were held at 4°C at all times during the sonication process. Between each run, samples were vortexed after a short spin. The sheared chromatin could be frozen at -80°C for later use [33].

ChIP reactions were performed on SX-8G IP-Star® Compact Automated System supplied by Diagenode. ChIP experiments were applied with anti-H3K27me3 (pAb-069-050, Diagenode), anti-H3K9ac (pAb-103-050, Diagenode), anti-H3K4ac (pAb-165-050, Diagenode)] and non-immune rabbit IgG (Kch-504-250, Diagenode) (*e.g.* negative control). ChIP reactions were created 200 µl volumes each samples. All Auto-ChIP samples were exerted Auto Histone ChIP-seq kit reagents and included 1µg of input chromatin. Reaction incubated for 2h for Antibodies-coating with protein A-coated magnetic beads, and 10h for IP reactions for 8 strips, at 4°C [33].

Quantitative Real-Time PCR (Q-PCR)

After the ChIP reactions, a Quantitative Real-Time PCR (Q-PCR) was performed in triplicate. We used a ninety-six-well optical tray with optical adhesive film. Two control genes: *C-FOS* (pp-1004-500, Diagenode), which was the positive control for the acetylation marks, and *TSH2B* (pp-1041-500, Diagenode), which was positive control for the methylation marks, was used. Final reaction volume was 25 μ l and it contained 5 μ l DNA IP (e.g immunoprecipitated DNA) or DNA Input (e.g Total DNA), 1X SYBR Green Supermix (Applied Biosystem) and 200 nM each primer of control genes (*C-FOS- TSH2B*). For the other genes (*EZH2, BRCA1, ER α , ER β , SRC3, P300*), we used Taqman Universal PCR Master Mix at 1X, 400 nM each with forward and reverse primers and 250 nM of the probe for each gene. Initial denaturation was at 95°C for 10 min to activate DNA polymerase. This was followed by 45 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. Q-PCR was performed using 7900HT, Real-Time PCR System from Applied Biosystems. Primer and probe sequences were selected by Primer Express software (ABI) (Table 1).

The efficiency of chromatin immunoprecipitation of particular genomic locus was calculated by qPCR data and reported as a percentage of starting material:

$$\%(\text{ChIP/Total Input}) = 2^{[(\text{Ct}(\text{x}\%\text{input}) - \log(\text{x}\%)/\log 2) - \text{Ct}(\text{ChIP})]} \times 100\%.$$

Western Blot

After the Q-PCR we examined the proteins for each cell line by Western Blot. MCF-7 and MDA-MB-231 cells treated with DZNep, NaBu and SAHA were collected after 48 hours. All the protein was extracted after cell lysis. Lysis buffer contained 20 mM Tris (pH 8), 50 mM EDTA, 0.8% NaCl, 0.1% Triton X-100 and 1% Glycerol. Protease Inhibitor Cocktail (Sigma) (1% Protease inhibitors) and Phosphatase Inhibitor Cocktail 2 (Sigma) (1% phosphatase inhibitors) were added to the basic

buffer. Protein was quantified using the Bradford Method. Twenty five microgram proteins were loaded onto 10% gels (for Anti BRCA1 and Anti P300 we used 4% gels, which are heavier than the others) for SDS-PAGE and electrophoresed. When proteins were separated, they were transferred to a polyvinylidene difluoride (PVDF) membrane. After one-hour, blocking in Tris Buffered Saline Tween 0.1% (TBST) containing 5% milk membranes were incubated overnight at 4°C respectively with Anti BRCA1 (1:50 [Ab-1] monoclonal Calbiochem), Anti P300 (1 :100 [N-15] polyclonal Santa Cruz Biotechnology), Anti SRC-3 (1:500 NCOA3 [N1N2] polyclonal Gene Tex), Anti PGR (1:250 monoclonal ABNOVA), Anti EZH2 (1:1000 monoclonal MILLIPORE), Anti ER- α (1:500 [F-10] polyclonal Santa Cruz Biotechnology, 1:500 monoclonal [HC-20] Santa Cruz Biotechnologie), Anti ER- β (1:500 [H-150] polyclonal Santa Cruz Biotechnology, 1:500 polyclonal ZYMED) and Anti-actin (1:120,000 Mouse [Ab-1], Calbiochem) antibodies. Membranes were washed three times with TBST and incubated for one hour with alkaline phosphatase-conjugated secondary antibody (1:2000 anti-mouse IgG (H&L) AP conjugate or 1:2000 anti-rabbit IgG (Fc) AP conjugate, Promega). Detection was performed with Western Blue® Stabilized Substrate for Alkaline Phosphatase produced by Promega. Relative quantification of immunoblotted proteins was achieved using the Bio-Rad Quantity One software with local background subtraction method. Membranes were re-probed with actin antibody as a loading control.

Real-time quantitative RT-PCR

Finally, we applied Reverse Transcription and RT-QPCR on the RNA. To begin, we did an RNA extraction. For this, after 48 hours of treatment, each of the cell lines were washed three times with phosphate buffered saline (PBS). Afterwards, the cells were lysed in 5 mL of RNA B™, according to manufacturer's protocol of BioProbe Systems. All the RNA samples were verified using a NanoDrop ND-8000 Spectrophotometer. The samples were then kept in liquid nitrogen.

Thereafter, 25µg of total RNA were reverse transcribed in a total volume of 15 µl using the First-Strand DNA Synthesis Kit according to the manufacturer's protocol (GE Healthcare Life Science). Reverse transcriptase was inactivated for 10 minutes at 95°C. The resulting cDNA was then quantified with the TaqMan® method supplied by ABI Prism® 7900 HT Applied Biosystems, USA. Multiplex PCR was performed in 96-well plates: 5 ng of cDNA and 20 µl of reaction mix containing 12.5 µl TaqMan universal PCR Master Mix (dATP, dCTP, dGTP and dUTP, MgCl₂, AmpliTaqGold, Amperase uracil-N-glycosylase), 200 nM probe of each gene (*EZH2*, *BRCA1*, *ERα*, *ERβ*, *SRC3*, *P300*, *PGR*) assay-on-demand, 10 µM of *18S* rRNA forward and reverse primers and 5 µM of *18S* rRNA TaqMan probe. For all genes, assay-on-demand, primers and Taqman® probes were purchased from Applied Biosystems as follows: *BRCA1*:Hs01556193_m1, *ERα*: Hs00174860_m1, *ERβ*: Hs01003531_m1, *P300*: Hs00914223_m1, *SRC3*: Hs01105251_m1, *EZH2*: Hs01016789_m1, *PGR*: Hs01556702_m1 ; *18S*, forward: 5'-CGG CTA CCA CAT CCA AGG AA-3', reverse: 5'-GCT GGA ATT ACC GCG GCT-3', probe: 5'-TGC TGG CAC CAG ACT TGC CCT C-3' (VIC). Data were collected using an ABI Prism® 7900 Sequence Detector System by Applied Biosystems. Relative quantitation of mRNA level was done using the $\Delta\Delta C_T$ method [35], which is the quantitative normalization of cDNA in each sample to an internal control (i.e., 18S rRNA) to normalize quantity and quality of cDNA samples. All data were generated in triplicate and expressed as mean +/- SD.

Statistical Analysis

All statistical analyses were performed using R 3.0.1 software and the statistical packages agricolae, HH and multcomp [36]. All the data obtained were statistically analyzed by three-ways ANOVA to test the level of statistical significance of cell lines, treatments (and their interactions) and marks on methylation and acetylation status of the six genes. Post-hoc procedures were used when the F-test

was significant ($p < 0.05$). Multiple comparisons among means were examined by a Tukey test for cell lines and treatments. The level of statistical significance was set at $p < 0.05$.

Results

Controls of activator and repressive marks for MCF7 and MDA-MB 231 cells

We verified expression levels of *C-FOS* and *TSH2B* genes by qPCR, and polyclonal non immune rabbit. IgG was used as a control antibody for ChIP analyses. We examined the enrichment of acetylation modification on *C-FOS* (positive control) over *TSH2B* (negative control). *C-FOS* expression levels demonstrated that H3K4ac and H3K9ac marks were an activator in MCF7 and MDA-MB 231 cell lines treated with anti-HDAC or HMTi and cell lines without any treatment (Figure 2A). Likewise, for methylation mark of H3K27me3, we used over *C-FOS* gene as a negative control and we investigated that *TSH2B* expression levels were increased associated with in both cell lines (Figure 2B). We started to work with other genes by the way of these control results.

The Global results of ChIP experiments

The results were expressed for the 2 cell lines (MCF7 and MDA-MB-231) as a function of the three studied marks H3Kac, H3K4ac and H3K27me3; the treatments, DZNep, NaBu and SAHA compared to the untreated control; the 6 genes (*EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3*, *P300*) and for each, the efficiency of ChIP was calculated by qPCR and reported as a percentage of starting material $\%(\text{ChIP}/\text{Total Input})$ on the Y-axis. When we examined the overall figure 3; for H3K9ac and H3K4ac marks, we had demonstrated that the HMTi and HDACi treatments are more effective in MCF7 than in MDA-MB-231 cell lines. Conversely, we demonstrated that for H3K27me3 mark, the treatments are more effective in MDA-MB-231 than in the MCF7 cell line. After the examination of this figure we explained in detail for each gene.

Consequently, this analysis allowed determination of which genes seem to be more specific in the 2 cell lines with different treatments and inside the tested marks.

DZNep reduce EZH2 on H3K27me3 marks in MDA-MB-231

Then, the enlightened genes were statistically analyzed by three-ways ANOVA to test the level of statistical significance. Post-hoc procedures were used when the F-test was significant ($p < 0.05$). Multiple comparisons among means were examined by a Tukey test. The level of statistical significance was set at $p < 0.05$.

When we evaluated the global results of ChIP experiments, there is not a lot of distinct differences at the expression levels of EZH2 gene between three treatments in all cell lines for H3K9ac mark (Figure 3).

Conversely; EZH2 expression level showed diversity and more effective on H3K27me3 in MDA-MB-231 cell line. Furthermore, when we estimated the results of EZH2 on H3K4ac mark, we discerned that H3K4ac mark was very effective in MCF7 cell line (Figure 3).

We then examined in the cells in more details to understand the effects of treatments; especially EZH2 level reduced on H3K27me3 in all cell line with DZNep treatment (Figure 4). This result was supported by Western blot results (Figure 5). Western blot results have shown that; there is a significant difference between control and treatment with DZNep for two cell lines.

After the western blot, we performed RT-PCR to clarify the DZNep effects on two cell lines (Figure 6). For level of DZNep, there was no significant difference between MCF7 and MDA-MB-231 cell lines, but the rate of DZNep was lower than the other treatments (NaBu and SAHA).

Although it has clearly shown that DZNep acts globally and can inhibit both active and repressive histone marks[37], DZNep may still be clinically useful [38]. We therefore demonstrated that further investigation of the potential of DZNep as an epigenetic therapeutic was warranted, in addition with using DZNep to further elucidate how chromatin structure affects gene expression [39].

HDACi allows the HAT activity of *P300* to increase the histone acetylation levels

When we evaluated the *P300* ChIP experiment results and there was no difference between three treatments on H3K27me3 and H3K9ac marks (Figure 3). Furthermore, the expression level of *P300* significantly increased with treatments of NaBu and SAHA on H3K4ac in MCF7 cell line when they were statistically analyzed ($p < 0.05$) (Figure 7A). When we investigated the difference in treatment between m-RNA levels, *P300* was increased when treated with NaBu and SAHA in all cell lines (Figure 6). Also we found that in the Western blot results, there was an increase in P300 protein expression following treatment with NaBu and SAHA for all cell lines. These results enforce our knowledge that, P300 belongs to the HAT family.

High-throughput screening of *SRC-3* treatments with HDACi

Similarly, *SRC3* has shown that the like features as *P300* (Figure 3). The Expression level of *SRC3* increased after treatment with NaBu and SAHA in MCF7 cell line. Moreover, expression was decreased in the MDA-MB-231 cell line on the H3K4ac mark associated with DZNep treatment (Figure 7B). In addition, we found that after Tukey test, the difference between treatment was statistically significant ($p < 0.05$). In addition, the SRC3 protein expression increased in following treatment with NaBu and SAHA (Figure 5). Likewise, when we examined western blot results, it was observed that DZNep levels were very low for each cell line and they were supported by the RT-PCR results (Figure 6).

HDACi induce *BRCA1* expression in MCF7 cell line on H3K4ac

ChIP experiments demonstrated that there were no difference at expression levels of *BRCA1* between H3K27me3 and H3K9ac for all treatments in two cell line (Figure 3). In spite of this, the expression level of *BRCA1* increased on H3K4ac in the MCF7 cell line treated with NaBu and SAHA and these

results were statically significant in accordance with Tukey test, ($p < 0.05$) (Figure 7 C). We observed that there was no distinction in the western blot results for each cell line (Figure 5). To support these results, we indicated RT-PCR results; DZNep reduced BRCA1 mRNA level as distinct from other treatments in all cell lines (Figure 6).

Effect of estrogen on HDACi and HMTi

According to the results from two cells lines, the expression level of ER α was not activated with all treatments at acetylated marks H3K9ac and H3K4ac in MCF7 cell line (Figure 3). On the other hand, the expression level of ER α increased dramatically on H3K27me3 in MDA-MB-231 treated with HDACi and the Tukey test supported this data statistically ($p < 0.05$) (Fig 8A).

When we investigated the results of ER β ; although not to the same degree with the ER α , the results closely aligned to each other (Figure 3).

Expression levels of ER α and ER β increased on the H3K27me3 mark that was associated especially with the SAHA treatment in MDA-MB-231. Nevertheless, with the treatment of SAHA, rate of ER α and ER β increased on the two activator marks H3K4ac and H3K9ac in MCF7 cell line and the differences derived from the Tukey test were significant ($p < 0.05$) (Figure 8B).

This situation was supported by analyses with Western blot and m-RNA levels (Figure 6). m-RNA levels of ER α and ER β improved with the treatment of SAHA in MCF7, which is the ER α receptor positive cell line (Figure 5). Also, these results could be associated with MCF7 ER status.

Our results have shown that; related by treatment with NaBu, the MDA-MB-231 cell line has a better survival rate than the MCF cell line. Furthermore, when we contrasted with PGR expression levels on two cell lines, we observed that at all treatment groups PGR levels were decreased in MDA-MB-231 cell line. This is due to the property of progesterone negative (Figure 6).

Discussion

The following studies have shown that epigenetic post-translational modulation and DNA methylation played a key role in cancer, so we decided to study the effect of HMTi (DZNep) and HDACi (SAHA, NaBu) in breast cancer cell lines (MCF7 and MDA-MB 231) on selected genes (*EZH2*, *BRCA1*, *ER α* , *ER β* , *PGR*, *SRC3* and *P300*) involved in transcription.

Breast cancer is a hormone-dependent cancer. The hormonal status changes in the female endocrine system that occurred at different stages of life are also important. ER expression in breast cancer is one of the most important factors to predict response to therapy in breast cancer patients. To deal with that, we chose to study two different ER status breast cancer cell lines, MDA-MB-231 (negative ER α) and MCF7 (positive ER α). Previously, study has shown that ER α negative breast cancers are more aggressive than ER α positive ones. Moreover, ER α -negative forms are unresponsive to endocrine therapy and resistant to anoikis [40]. Our results make evident that DZNep, SAHA and NaBu were effective in both ER α -positive MCF-7 and ER α -negative MDA-MB231 breast cancer cells by the effectiveness of the different mechanisms. We investigated our global results from the point of the status- ER; MDA-MB-231 cell line has an effect on the methylation mark H3K27me3. Also, the MCF7 cell line is efficient on acetylation marks.

In this field, authors have reported many studies which have revealed the importance of histone modifications in cancer, with a global hypermethylation at lysine 27 of histone 3, leading to chromosomal instability and loss of the expression of tumor suppressor genes [13,14,37,41].

Recent studies have shown that DZNep, a well-known histone methyltransferase inhibitor, disrupts polycomb-repressive complex 2 (PRC2), and induces apoptosis, while inhibiting proliferation and metastasis, in cancer cells, including acute myeloid leukemia, breast cancer and glioblastoma. DZNep was previously reported to be a selective inhibitor of H3K27 trimethylation [37] and also in this study we found similar results [42].

Recent studies have shown that *ERα*, *SRC3*, *P300* and *EZH2* genes were described as overexpressed and promoted the activation of transcription in breast cancer cells [43,44]. In breast cancer, studies have shown variations of expression of *GSTP1*, *BRCA1* and *BRCA2* tumor suppressor genes resulting from epigenetic changes such as hypermethylation of the promoters of these genes [45,46,47] by inhibiting DNA methyltransferase (DNMT) [48] and acetylating action at histone via the recruitment of SRC3 and P300 protein after treatments [49]. In contrast, the *BRCA1* gene is known to be repressed in sporadic breast cancers [50], and also there are some data that the *BRCA1* inhibits the transcriptional activity of the estrogen receptor (*ERα*) in human breast cancer cell lines [51]. Further study has shown that the cofactors p300 and CBP regulate the capacity of the BRCA1 protein to inhibit the transcriptional activity of *ERα* [27]. This mechanism seems to include protein interactions mediated primarily by their conserved cysteine-histidine rich domain, CH3. Further Fan *et al.* reported that the BRCA1 inhibition of *ERα* activity is deliverable to the down-regulation of the expression of p300, which may reduce the level of p300 + CBP too low to induce the *ERα* transcriptional pathway[27]. On the basis of the overall studies, we understood that these proteins have all gotten an influential interaction.

Also, in a report by Puppe *et al.*, DZNep is about 20-fold more effective in killing BRCA1-deficient cells compared to BRCA1-proficient mammary tumor cells [15]. We thought that this report could be associated with results of BRCA1 m-RNA levels, because when we examined the results, the BRCA1 m-RNA levels with the DZNep treatment was lower than with the HDACi treatments.

There are a lot of studies have shown that; DZNep effectively consumes cellular levels of PRC2, particularly EZH2 [52,53,54]. In addition, DZNep strongly stimulates apoptotic cell death in breast cancer cells, but not in normal cells [53]. In addition, researches have explained that the EZH2 protein levels are strongly associated with tumor aggressiveness. This data could be supported by our EZH2 western blot results (Figure 4) which reflects that when cells are treated with DZNep,

differences occur between the two cell lines and also supported by ChIP experiments which relates of EZH2 levels treated with DZNep on H3K27me3 mark in all cell lines (Figure3) [13].

Two enzymes are consorted with in histone deacetylation; histone acetyltransferase (HAT) and histone deacetyltransferase (HDAC). These enzymes catalyse the transfer of an acetyl group from acetyl-CoA molecules to the lysine-amino groups on the N-terminal tails of histones. The currently available HDACi are thought to inhibit class I and class II HDACs which we used as HDACi for this study [55]. HDACi can be converted into heterochromatin to euchromatin [20]. HDACi induces G1-phase cell cycle arrest with downregulation of cyclin D1 and upregulation of *p21* in breast cancer cells. When HDAC activity is inhibited, expressions of only 2% of the mammalian genes are affected [55].

NaBu plays a strategic role in the regulation of gene expression; inducing growth arrest, apoptosis or differentiation on different cancer cell line. NaBu inhibits HDAC, excluding class III HDAC and class II HDAC 6 and 10. However, HAT activity continues during the inhibition of HDAC activity. Briefly, NaBu induces *p21* gene expression and cell cycle arrests in breast cancer cells. Inhibition of HDAC activity with Nabu enables the HAT activity of *P300* to increase the histone acetylation levels at the promoter [23]. These data support our results about with *P300*. Our western blot and mRNA results shown that, *P300* levels always increase when treated with NaBu (Fig4-Fig5).

Furthermore, NaBu may also alter histone methylation [56]. Also Cho *et al.* demonstrated that HDACi and demethylating agents have some effects, but NaBu has strong effect in MCF7 cell lines [20]. In another study about NaBu has shown that MDA-MB-231 cells were more resistance to apoptosis effects after NaBu treatment as compared to MCF7 cells [57]. When considering these points of view in conjunction with our work, the NaBu treatment on H3K27me3 mark is more effective than H3K9ac and H3K4ac marks (Fig2).

SAHA increased acetylated histone H3 and H4, and also significantly reduced the expression of HDAC1, 2, 3, 4 and 7. In addition, SAHA arrest the cell cycle by inducing G2/M phase [40].

Chiu *et al.* investigated of cell viability in MDA-MB-231 and MCF7 cell lines treated with SAHA and they explained there were no differences between two cell lines [58]. But when we consider the results of SAHA, there was always a slight difference between two cell lines.

SAHA and NaBu, HDACi, acetylate histones in both cell lines enhance transcription and lead to cell apoptosis [59]. Recent studies indicate that the HDACi (SAHA and NaBu) induced DNA damage in normal and cancer cells, but cancer cells cannot be repaired. Thus, the selectivity of HDACi in causing cancer cell death may be associated with impaired DNA repair mechanism in cancer cells [60].

These results suggested a beneficial effect for the genes upregulated in cancer, but an adverse effect for downregulated genes because the action did not seem to be specific for genes.

Conclusion

We found out that HDACi is prone to modulate the transcription via the demethylation and acetylation of the histones in breast cancer cell lines. Our data add a new layer of complexity to epigenetic dysregulation in cancer and also established histone modifications-mediated silencing or enhancing for a promising therapeutic target.

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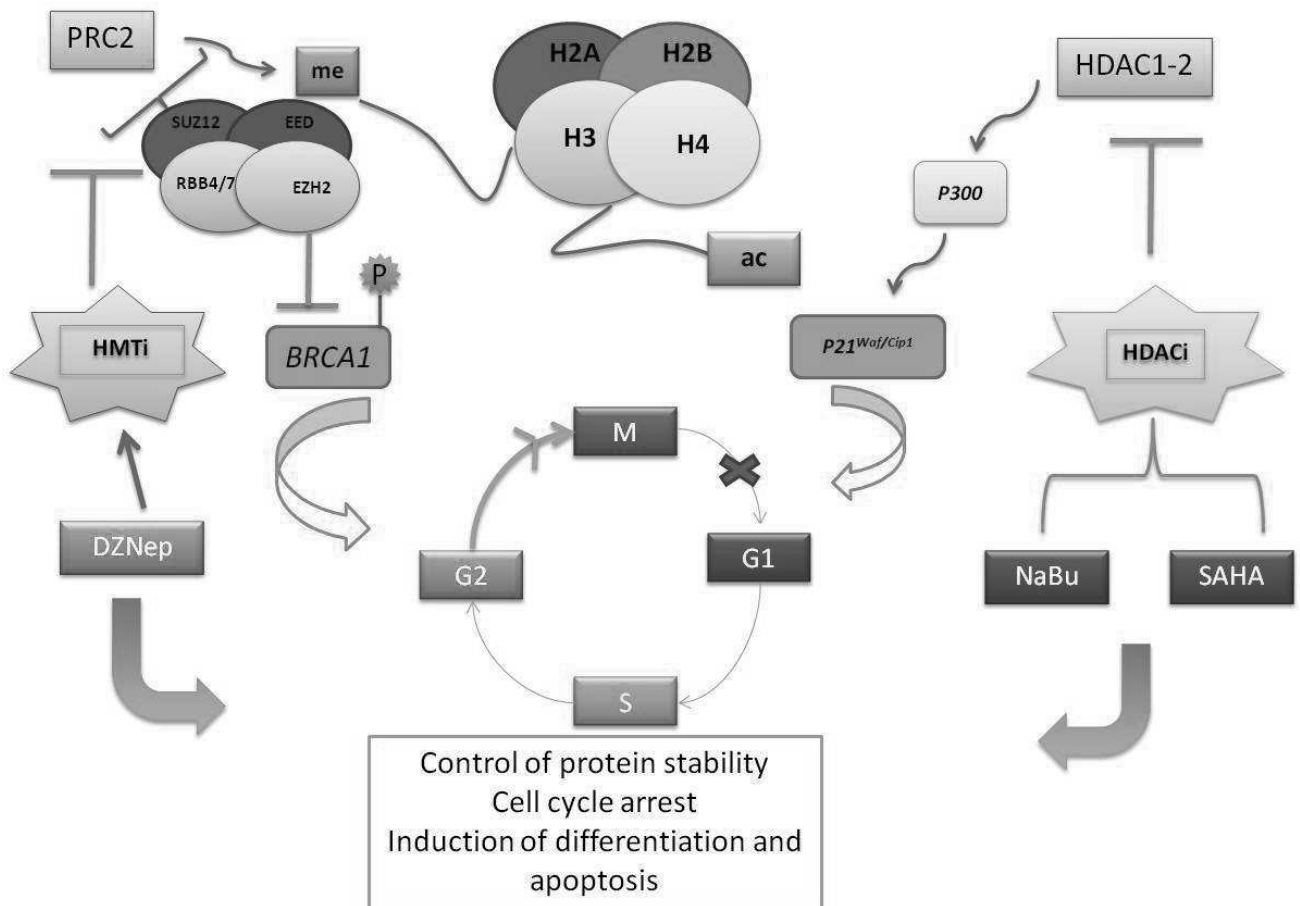
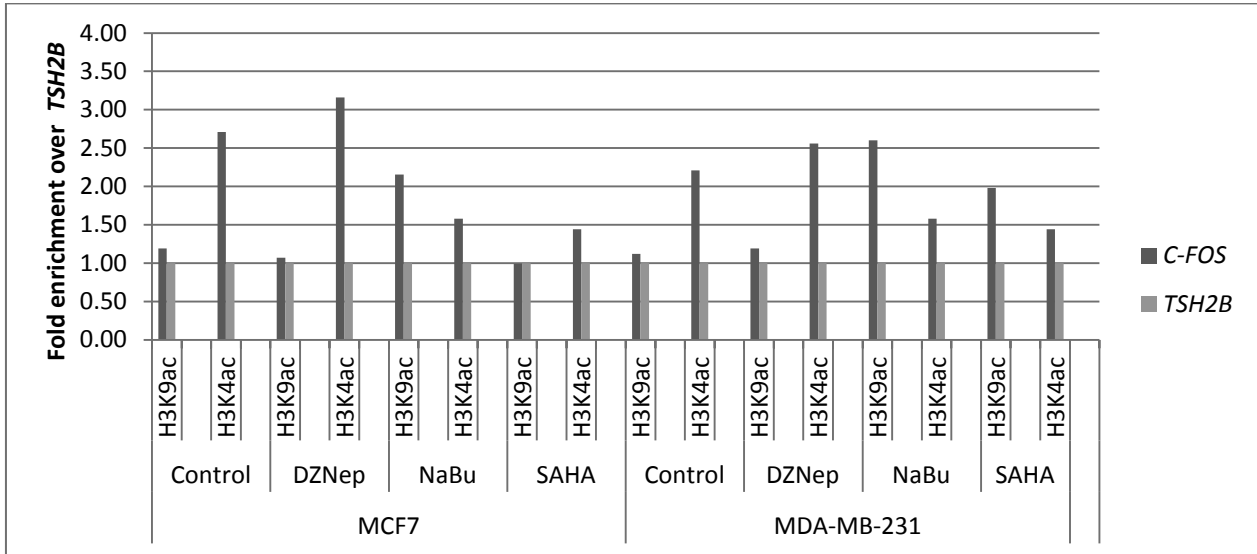


Figure1. Mechanism of HDACi and HMTi. DZNep effectively consume cellular levels of PRC2, particularly EZH2 and induces apoptosis while inhibiting proliferation and metastasis in breast cancer. Overexpression of EZH2 inhibited BRCA1 phosphorylation, for this reason transmission from G2 to M accelerated in cell cycle and cell proliferation was increased. Also, DZNep is a selective inhibitor of H3K27 trimethylation. HAT activity continues when HDACi inhibits HDAC 1 and HDAC2, due to *P300* activity increase and hyperacetylation of the histones occurs. As a consequence of that, *P21^{Waf/Cip1}* gene expression induces G1-phase cell cycle arrest in breast cancer cells.

A



B

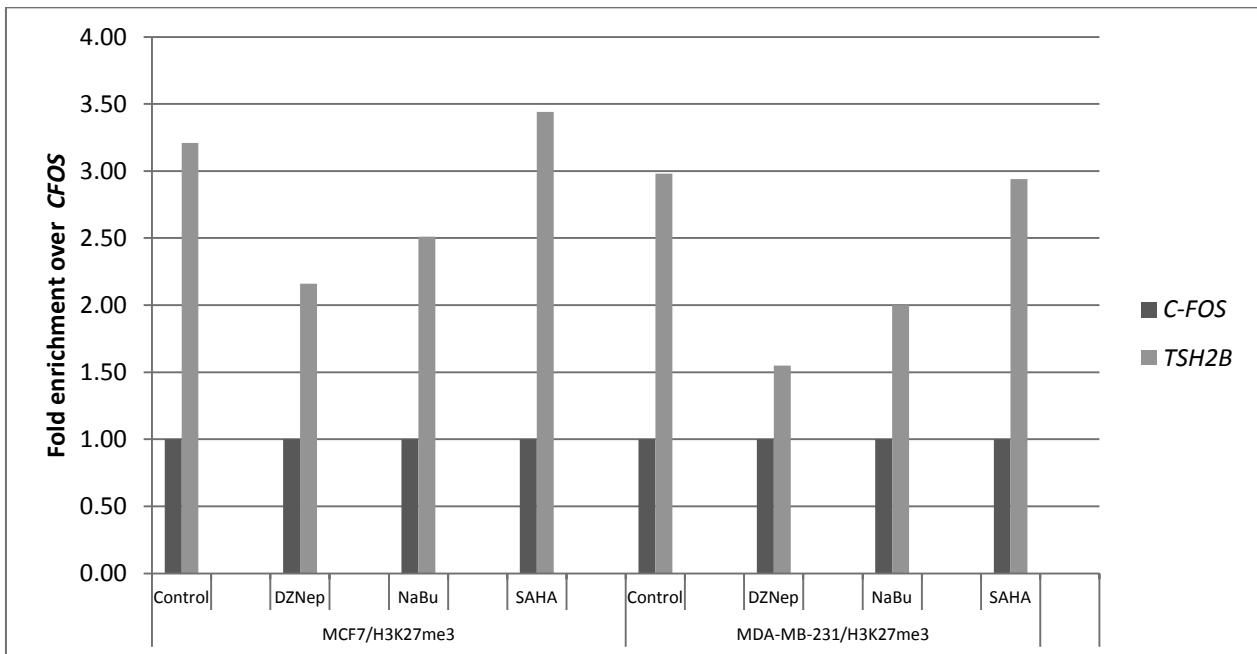


Figure2 Control genes for activator and repressive marks in MCF7 and MDA-MB 231 cells. A:

We examined the enrichment over *TSH2B* for acetylation marks (H3K9ac and H3K4ac). *C-FOS* level has to be greater than *TSH2B* when we used *TSH2B* for fold enrichment over on acetylation marks which are H3K4ac and H3K9ac. B: We applied the enrichment over *CFOS* for methylation mark, H3K27me3. Also *TSH2B* level higher than *C-FOS* when *C-FOS* employed for fold enrichment.

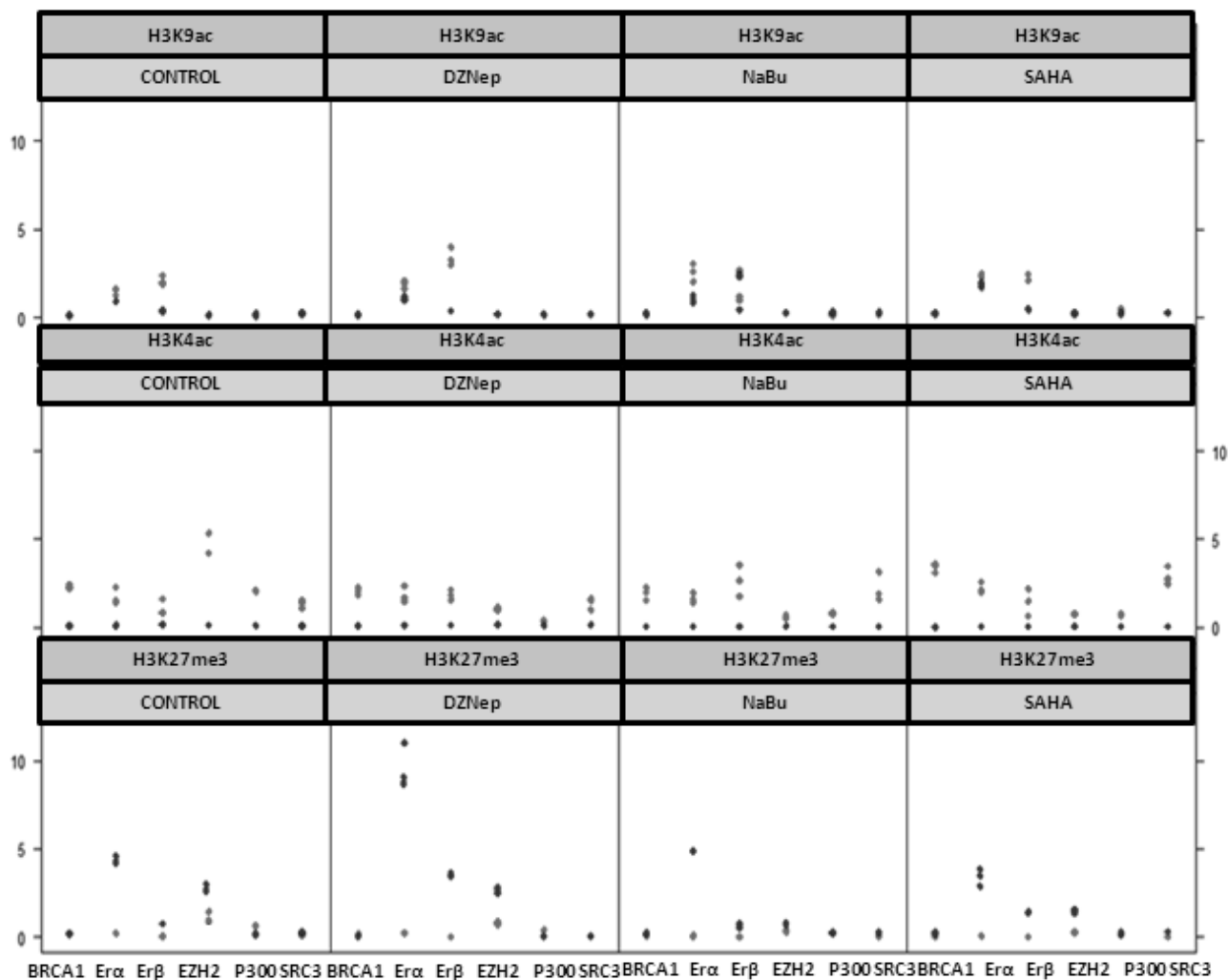


Figure3. Global results of effects HMTi and HDACi . In this figure, reflects the effect of HMTi and HDACi on H3K27me3, H3K9ac and H3K4ac marks for *EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3*, *P300* genes in 2 Breast Cancer cell lines. The general aspect; in acetylation marks which are H3K9ac and H3K4ac prevalent in MCF7 treated with HMTi and HDACi. Conversely, for H3K27me3 mark, with the all treatment in MDA-MB-231 is more effective than in MCF7 cell line. Red: MCF7, Blue: MDA-MB-231 (n=3).

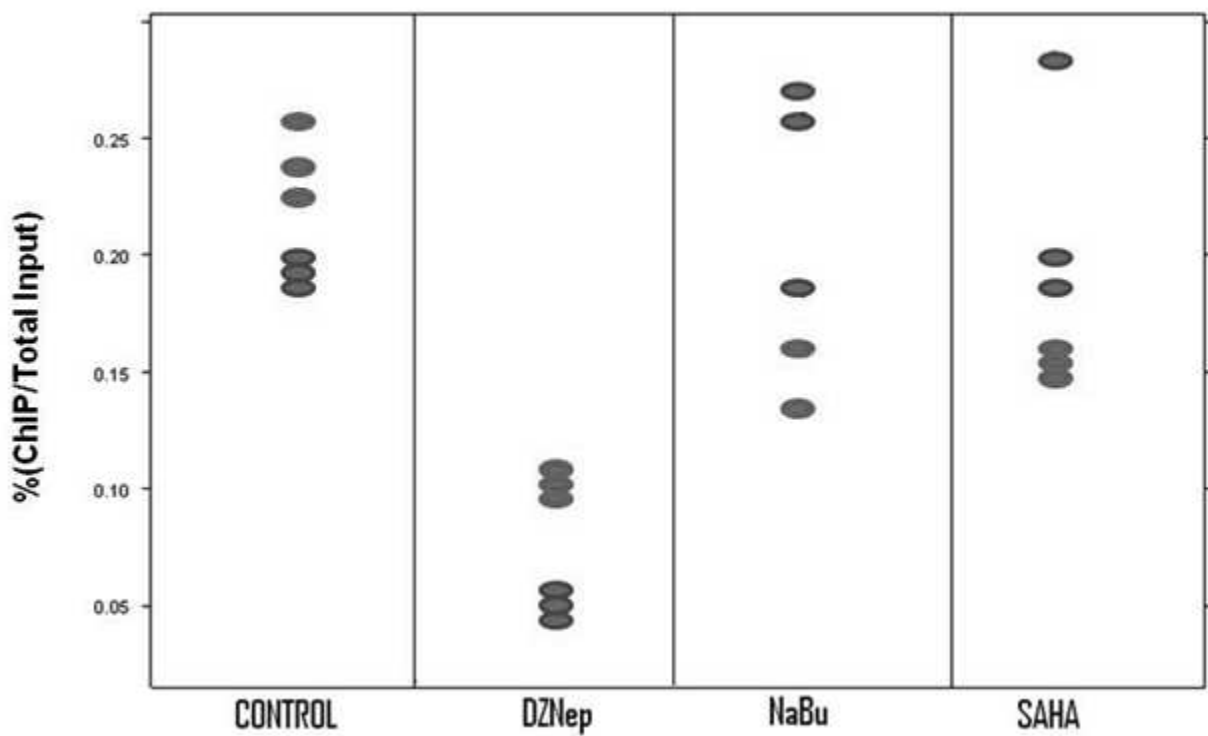


Figure 4. *EZH2* results on H3K27me3 mark. *EZH2* results were statistically significant on H3K27me3 in two breast cancer cell lines with respect to Tukey Test ($p < 0.005$) ($n=3$). *EZH2* reduces expression level treated with DZNep on H3K27me3 in MCF7 and MDA-MB-231 cell line.

Red: MCF7, Blue: MDA-MB-231

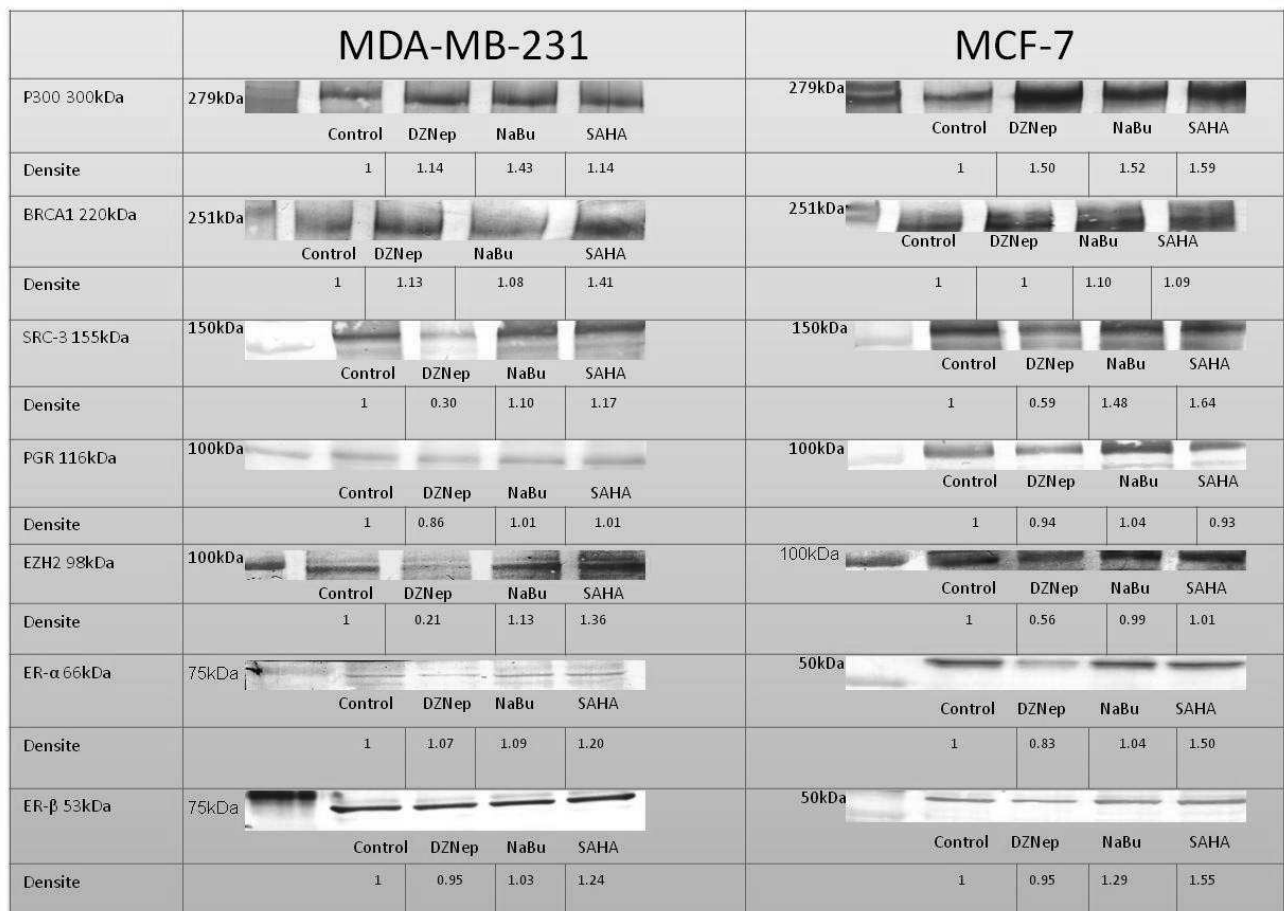


Figure 5. Western Blot analyses. Western blot analyses were performed using anti Anti BRCA1 (1:50), Anti P300 (1 :100), Anti SRC-3 (1:500) Anti PGR (1:250), Anti EZH2 (1:1000), Anti ER- α (1:500) and Anti ER- β (1:500). Relative quantification of immunoblotted proteins was achieved using the Bio-Rad Quantity One software. Untreated cells were considered control and control was used as 1 for compare with other treatments.

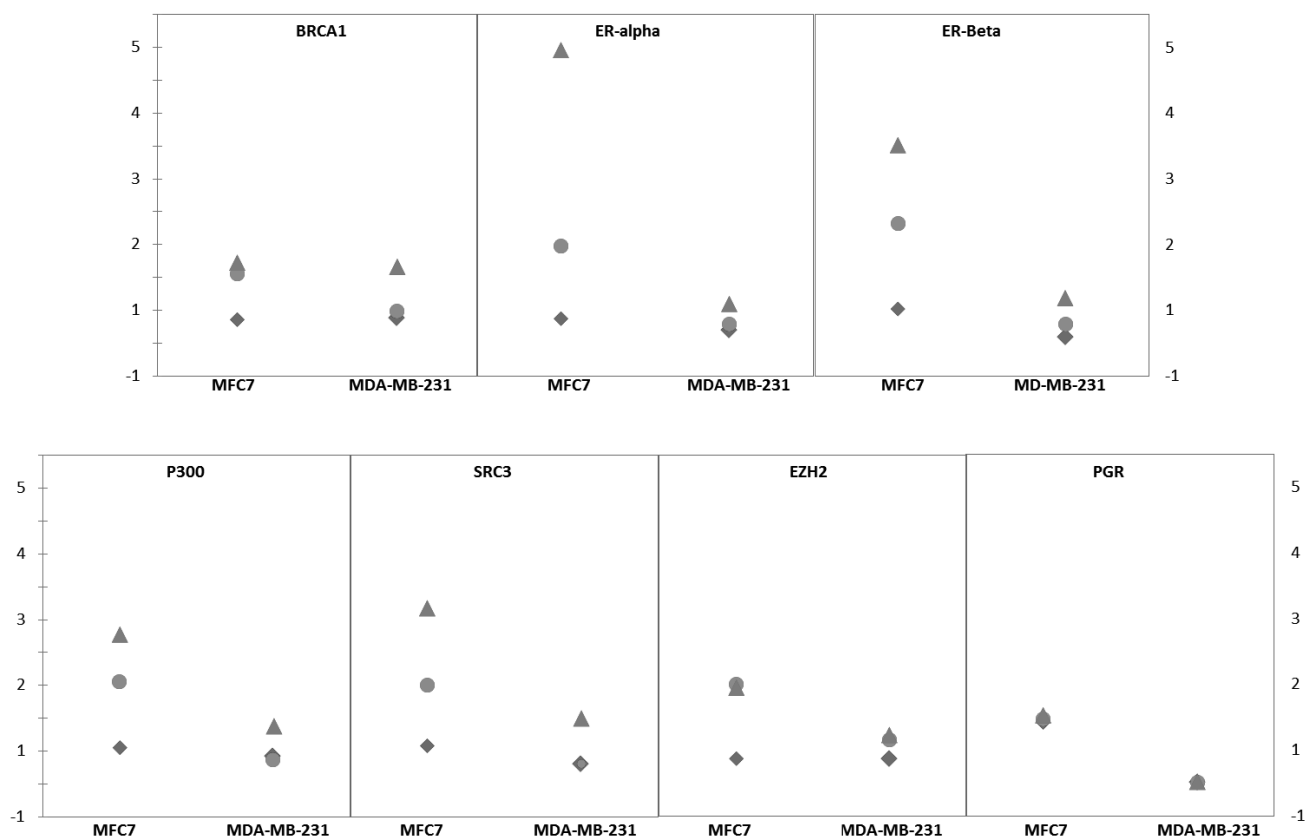
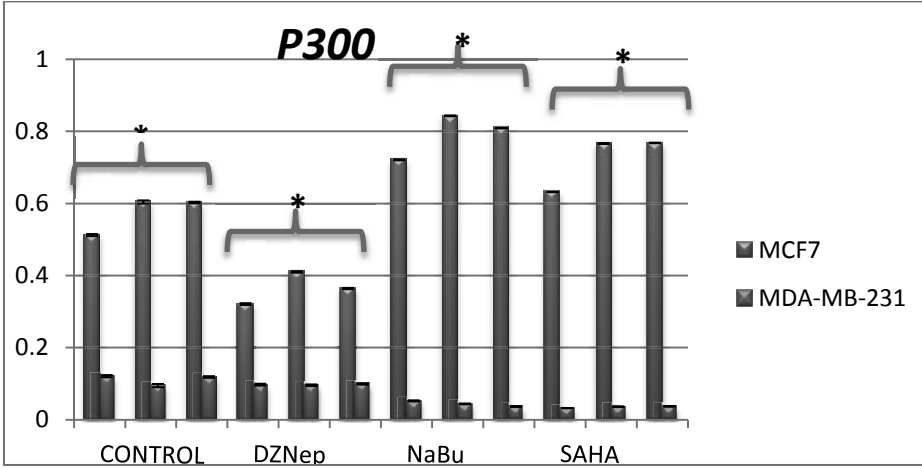


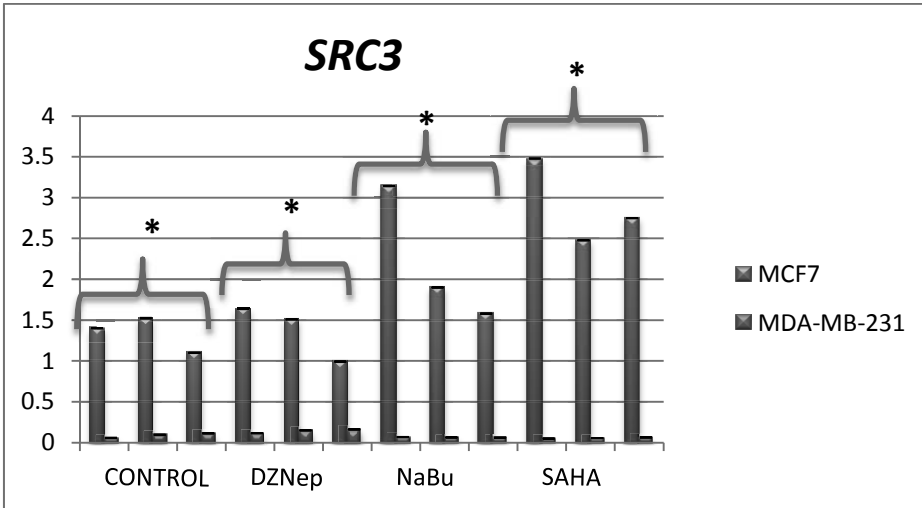
Figure 6. Effects of DZNep, NaBu and SAHA treatments to m-RNA levels of selected genes. m-RNA levels decreased in all cell lines with the treatment of DZNep, however with the treatment of SAHA and NaBu m-RNA, levels decreased only in the MDA-MB-231 cell line. PGR does not have a different level within itself for each treatment in MCF7 and MDA-MB-231 cell lines ,but MDA-MB-231 and MCF7 levels are completely different. We think that it is due to the PGR status.

(DZNep: ◆ NaBu: ● SAHA: ▲)

A



B



C

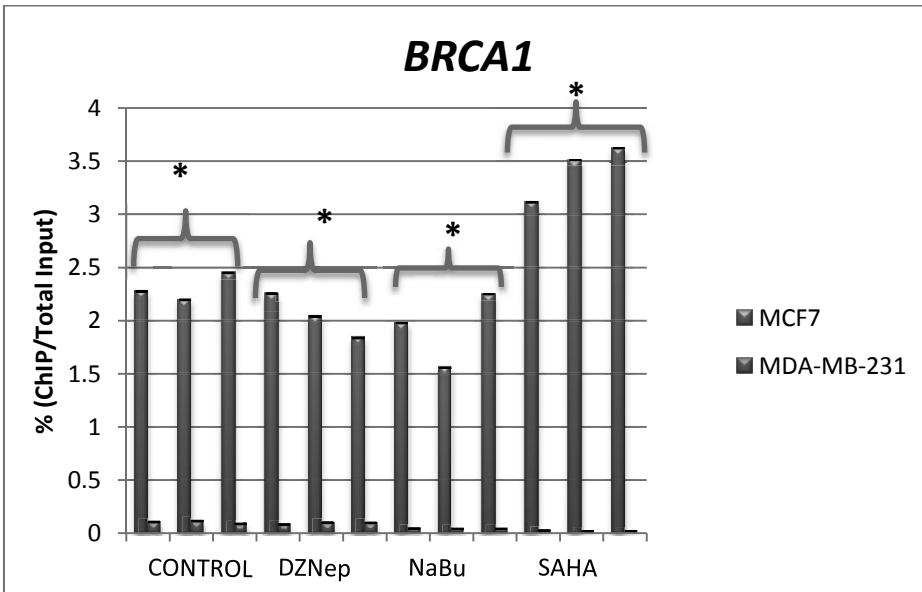


Figure 7. ChIP experiments results for *P300*, *SRC3* and *BRCA1* on H3K4ac. Results of ChIP experiments levels for *P300* (A), *SRC3* (B) and *BRCA1* on H3K4ac histone mark in breast cancer cell lines and all the data obtained were statistically analyzed by three-ways ANOVA to test the level of statistical significance of cell lines, treatments (and their interactions) and H3K4ac mark. Post-hoc procedures were used when the F-test was significant ($p < 0.05$). Multiple comparisons among means were examined by a Tukey test for cell lines and treatments and these data statistical significant ($p < 0.05$) ($n=3$). Considering these results especially treatment with HDACis are more effective than DZNep in the MCF7 cell line. Red: MCF7, Blue: MDA-MB-231

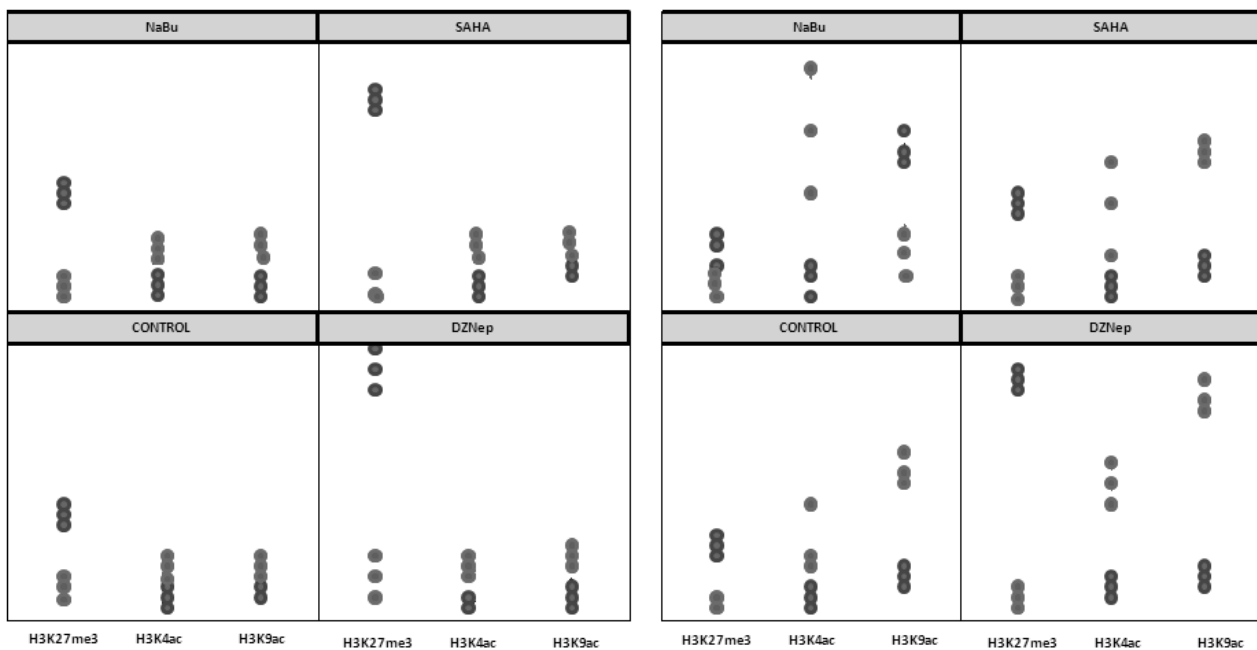


Figure 8. ChIP experiments results for *ERα* and *Er β*. In this figure, we showed that results of ChIP experiments levels for *ERα* (A) and *Er β* (B) on different histone marks in breast cancer cell lines. This graphic is prepared by three ways ANOVA to test the level of statistical significance of cell lines, treatments and all marks. Post-hoc procedures were used when the F-test was significant ($p < 0.05$). Tukey test and results were significant ($p < 0.005$) ($n=3$) Red: MCF7, Blue: MDA-MB-231

Table1: Primer and probe sequences

Genes	Forward	Reverse	Probes
<i>ERA</i>	CCCTGACATTGGCTTAAACATCA	TCTTTGGATCGCTCCAAAT	6FAM-TCCAGGCACAACCTC-MGBNFQ
<i>ERB</i>	GAGAGGCTTTGGGTTTGTCAAAT	CCTCTAGTCCACGGCTTTGC	6FAM-CAGCAAACGTAACCTCGGGCCCTG-TAMRA
<i>P300</i>	CGATGGCACAGGTTAGTTTCG	GCGCACCGAGTAGAAAAGATTAA	6FAM-CAGCCCCGGCCTTCCACGTT-TAMRA
<i>SRC3</i>	AAAATTAAGGGCAGGGCTAGGA	GTGCGGCCGCTTTCG	6FAM-TCCGGATCCCGAGGGAGCTCC-TAMRA
<i>EZH2</i>	CCCTCCAGAAACACAATCAATAGA	CCGCCTGGTCTGGCTTTAT	6FAM-CAGAGCAGCTCGACTCTTCCCTCAAACCTT-TAMRA
<i>BRCA1</i>	CCCCGTCCAGGAAGTCTCA	GCGCGGGAATTACAGATAAATT	6FAM-CGAGCTCACGCCGCGCAG-TAMRA
<i>PGR</i>	GAGCCGCGTGTCACTAAATTG	TCACAAGTCCGGCACTTGAG	6FAM-CGTCGCAGCCGCA-MGBNFQ

Conclusion of Publication 5

We chose two different breast cancer cell lines which are MCF7 (ER positive) and MDA-MB-231 (ER negative) and we investigated HDACi and HMTi effects in these cell lines (Table2).

We found it clear that HDACi is leaning to one side to adjust the transcription via the demethylation and acetylation of the histones in breast cancer cell lines. Our results supply a new landscape of convolution to epigenetic dysregulation in breast cancer and also established histone modifications-mediated silencing or enhancing for a promising therapeutic target.

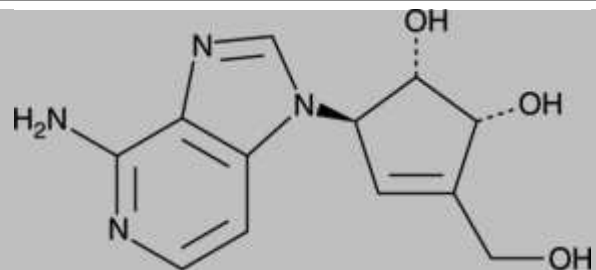
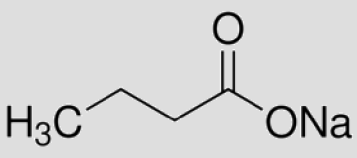
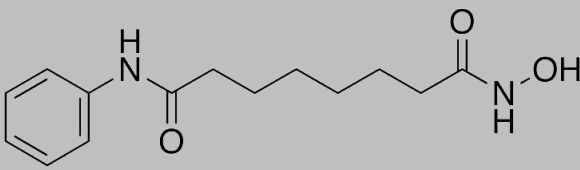
TREATMENT	STRUCTURE	DESCRIPTION
DZNep		Histone Methyltransferase Inhibitor (HMTi)
NaBu		Histone Deacetylase Inhibitor (HDACi)
SAHA		Histone Deacetylase Inhibitor (HDACi)

Table 2. Different treatments with epigenetic descriptions

C. Epigenetic Modifications between Tumor and Normal Tissue in Sporadic Breast Cancer According to Clinicopathological Parameters

Despite the fact that breast cancer progression is not well understood, it is likely that it occurs due to a great amount of genetic mutations leading to widespread changes in gene expression profiles and especially the expression of tumor suppressors and oncogenes. In addition to the the genetic mutations, we must now further investigate epigenetic modifications in breast cancer like histone marks. Many post-translational modifications of histones, lysine acetylation, lysine methylation, and arginine methylation seem to be implicated in gene regulation. So a combinatorial histone code is able to recognize these and other modifications on chromatin regions and create transcription activation or repression of genes. Though the “epigenetic” code is not completely elucidated, lysine acetylation (H3K4ac, H3K9ac, and H4K8ac), and lysine trimethylation (H3K4me3) modifications are able to generate transcriptionally active gene promoters, whereas other marks such as lysine methylation (H3K9me3 and H3K27me3) are associated with repressed chromatin.

Therefore, in the team with Gaëlle Judes and Seher Karsli-Ceppioglu; we aimed to assess chromatin immunoprecipitation (ChIP) of selected histone modifications in breast tumors. The relative levels of three modified histones, including H3K27me3, H3K9ac, and H3K4ac will be determined in breast tumors compared to normal tissue. In order to identify the variations in global levels of histone marks in 200 tumors and normal tissues which are classified by St Gallen, we proposed to follow ChIP with Q-PCR of 7 genes implicated in breast cancer and for which the decrease or increase in their expression can result from bulk histone modifications. We have chosen *EZH2* and *P300* genes that code respectively for a histone methyl transferase (HMT) and a histone acetyl transferase (HAT) which are chromatin-modifying enzymes implicated in gene regulation by catalyzing reversible post-translational modifications of histones. *SRC3*, coded by the *ncoa* gene, is a steroid receptor co-activator belonging to the p160 family (*SRC1*, *SRC2* and *SRC3*), constituting a platform for other co-activators such as *P300* and *BRCA1* tumor suppressor genes that are transcription factors, *Era* genes that are nuclear receptors activated by 17 β -estradiol that stimulate the proliferation and *ER β* that bind phytoestrogens and induce the cellular proliferation and invasion. Also, we will study *PGR* gene which is important in triple negative breast cancer. Likewise, we will follow the expression of the 7 selected genes with RTQ-PCR and western blotting.

Until now, the function of H3K4c has received little attention. This acetylation mark is particularly interesting, because it occurs on lysine residues; its methylation is associated with transcriptional activation. The difference between methylation and acetylation of the same residue results in a functional “switch” between transcriptional activation or silencing, as has been described for H3K9 and H3K27 [79].

To perform this study, we used tumors and matched normal tissues of five different classes belonging to the classification of breast cancer with Saint Gallen criteria (Table 3). We have shown our results with boxplot graphics of gene expression levels. They are presented to demonstrate the differences between tumor and matched-normal tissue samples. Expression levels of *ERα*, *EZH2*, *BRCA1*, *SRC3*, *P300*, *ERβ* and *PGR* genes on H3K27me3, H3K9ac and H3K4ac histone marks are shown in Figure 8.

Our results demonstrated that H3K27me3 histone marks have an important role in controlling the gene expression. Gene expression levels of studied H3K27me3-enriched genes were significantly low in breast cancer tumor samples when compared with matched-control normal tissue ($p < 0.05$). In addition, we have evaluated the effects of acetylated marks H3K9ac and H3K4ac on gene expression, which are supposed to be transcriptionally active. We found that expression levels of *ERβ* and *PGR* genes were decreased on H3K4ac mark-enriched sites in tumor samples ($p < 0.05$). *ERβ* and *PGR* genes play a crucial role in determining breast cancer aggressiveness, thus the modified H3K4ac mark would have a substantial transcriptional expression of these genes in breast cancer. However, we did not find significant differences in gene expression levels related with H3K9ac mark modification.

Moreover, transcriptional effects of histone marks on gene expression levels based upon different sub-types of breast cancer were investigated. The correlation between transcriptional effects of histone marks on gene expression and breast cancer sub-types is shown in Figure 9. Our results showed that over-expression of *ERα*, *ERβ* and *EZH2* genes were found on H3K27me3-enriched sites in Luminal B-like (HER2 negative) breast tumors. On the other hand, expression levels of *ERα* and *ERβ* genes were decreased in basal-like tumors. The expression level of the *ERβ* gene on the H3K9ac mark was increased in HER2 positive tumors. In addition, *PGR* was over-expressed on the H3K4ac mark in HER2 positive tumors. As a result, we have found that modified histone marks effected transcriptional expression of genes that code hormonal receptors.

CATEGORIES	CHARACTERISTICS	NUMBER of PATIENTS
Luminal A	<ul style="list-style-type: none"> ER and PgR positive HER2 negative Ki-67 low Recurrence risk 'low' based on Multi-gene-expression assay (if available) 	40
Luminal B	<p>HER2 negative</p> <ul style="list-style-type: none"> ER positive HER2 negative <p>and at least one of:</p> <ul style="list-style-type: none"> Ki-67 'high' PgR 'negative or low' <p>HER2 positive</p> <ul style="list-style-type: none"> ER positive HER2 over-expressed or amplified Any Ki-67 Any PgR 	40
HER2 Positive Non-Luminal	<ul style="list-style-type: none"> HER2 over-expressed or amplified ER and PgR absent 	39
Basal like	<ul style="list-style-type: none"> ER and PgR absent HER2 negative 	40

Table 3. Check list of patients according to Saint Gallen classification.

The initiation and progression of breast cancer is apparently linked with epigenetic alterations. In future studies, it is likely that the addition of multiple genes and the inclusion of histone alterations to predictive panels may be determined as having an improving effect in sensitivity and specificity. To increase the ability to remove all “pre-cancerous” tissues and decrease local recurrences analysis of histologically normal tumor margins for epigenetic alterations and field cancerisation can be used. Targeted therapies may someday be used as preventive measures since epigenetic modifications can also be used as biomarkers.

Also we determined the protein levels of ER α , EZH2, SRC3, P300, ER β and PGR in classified breast cancer tumors (Figure 10-11). According to our western blot results, ER α level in luminal-like tumors was higher than in control tissue samples. We found that EZH2 levels in basal-like tumors were increased when compared with other subtypes. We know that EZH2 is a catalytic sub-unit of PRC2 and its over-expression is associated with various cancers, especially breast cancer. Our findings are compatible with current knowledge. In addition, mRNA levels of EZH2 were higher in tumor samples than in normal tissue samples. Protein levels of P300, a histone acetyltransferase, were increased in tumor samples in all subtypes of tumors. Function of *P300* is critical for regulating gene expression. P300 undergoes a variety of covalent modifications like methylation, sumoylation and phosphorylation; therefore it participates in different cancer types. Protein levels of PGR were also increased in tumor tissues and these results were consisted with mRNA levels of PGR. mRNA levels of ER α , EZH2, BRCA1, SRC3, P300, ER β and PGR were shown in Figure 12.

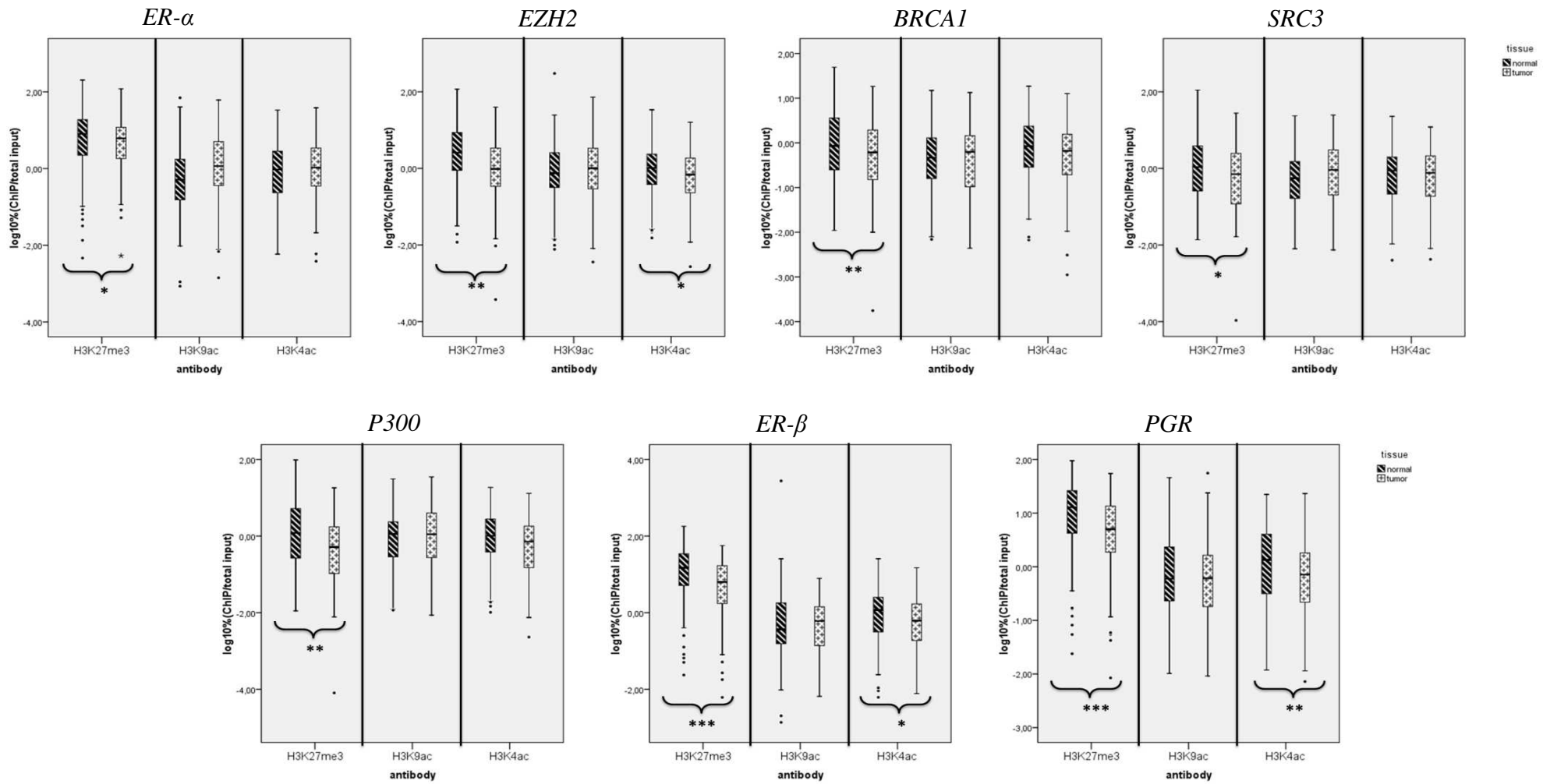
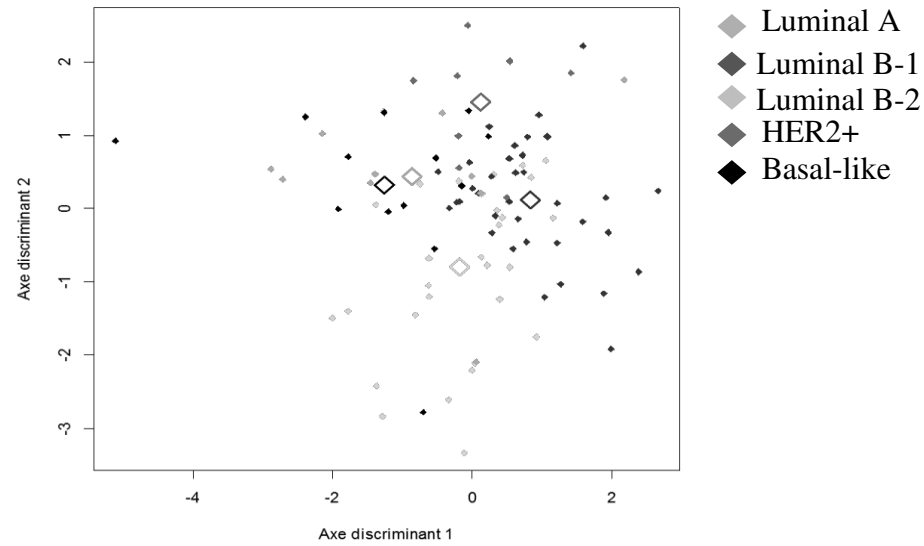
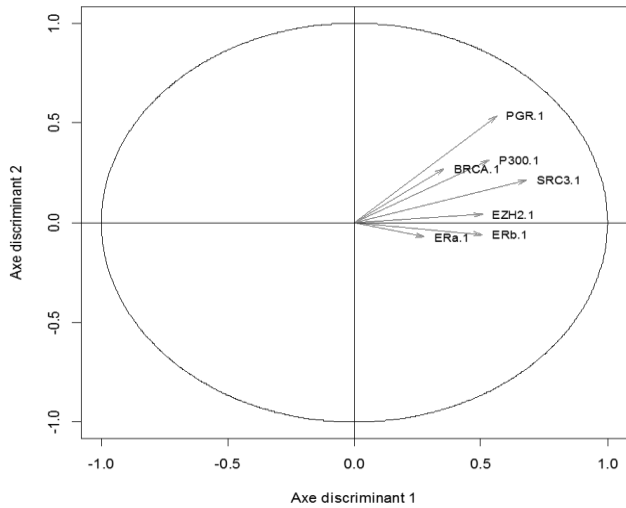


Figure 8. Gene expression levels of *ERα*, *EZH2*, *BRCA1*, *SRC3*, *P300*, *ERβ* and *PGR* genes on H3K27me3, H3K9ac and H3K4ac histone marks (n=114).

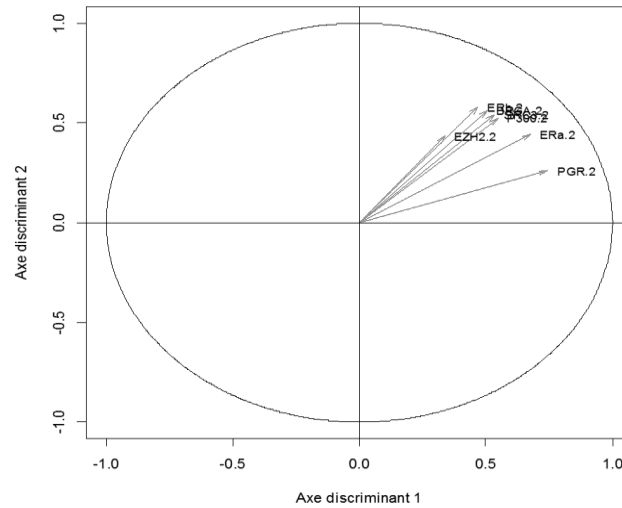
Breast Cancer Subtypes



H3K27me3



H3K9ac



H3K4ac

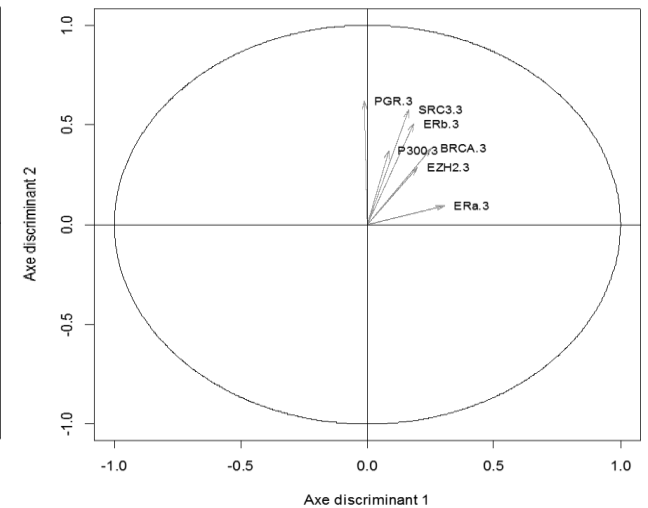
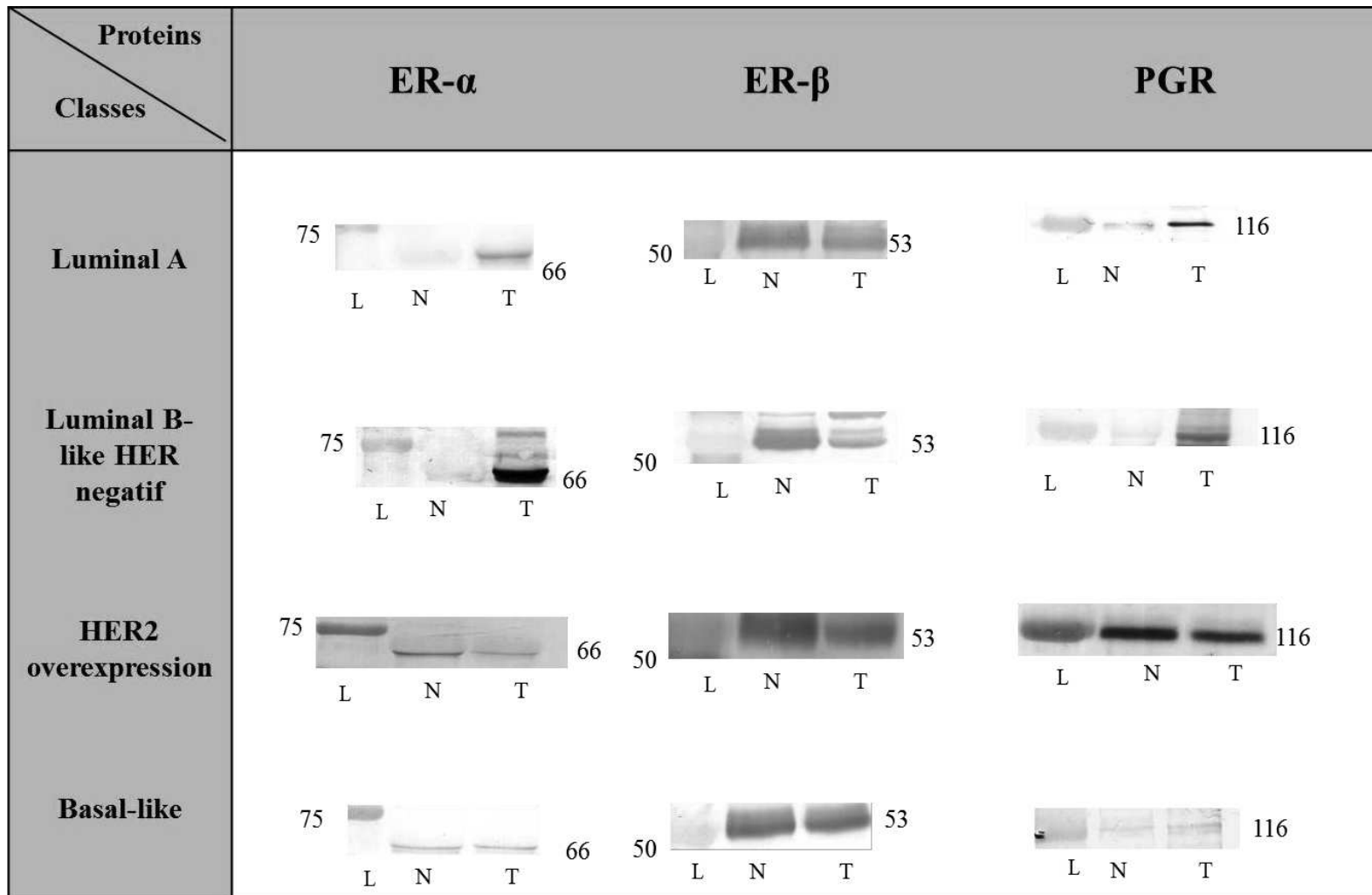
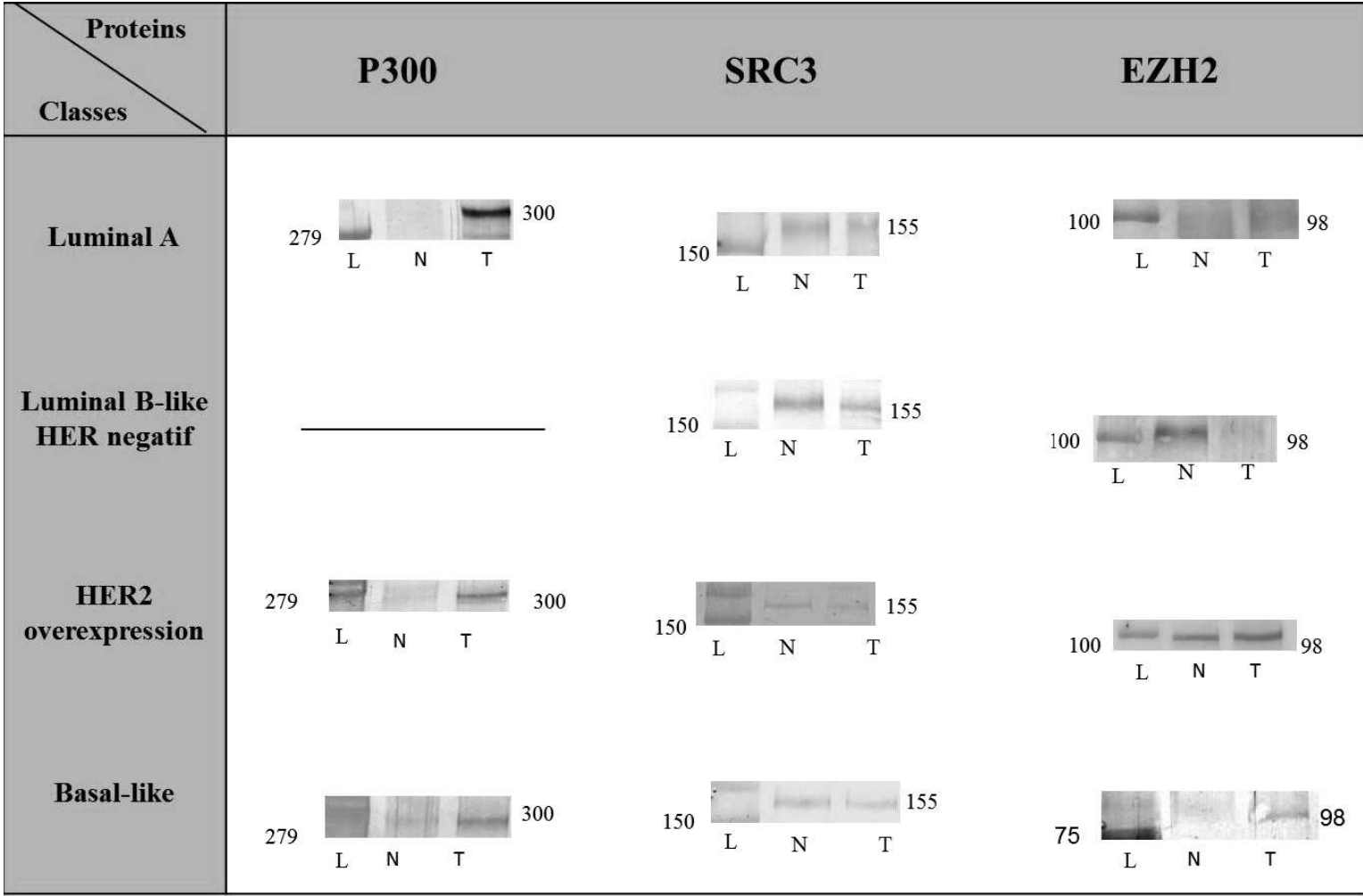


Figure 9. The correlation between transcriptional effects of histone marks on gene expression and breast cancer subtypes



N= Normal T= Tumor L= Ladder

Figure 10. Western blots ER α , ER β and PGR in classified breast cancer tumors.



N= Normal T= Tumor L= Ladder

Figure 11. Western blots P300, SRC3 and EZH2 in classified breast cancer tumors.

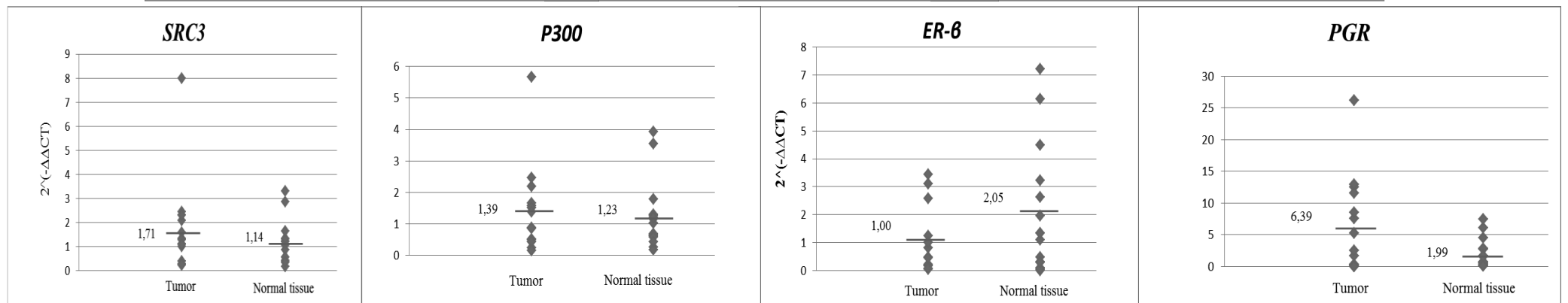
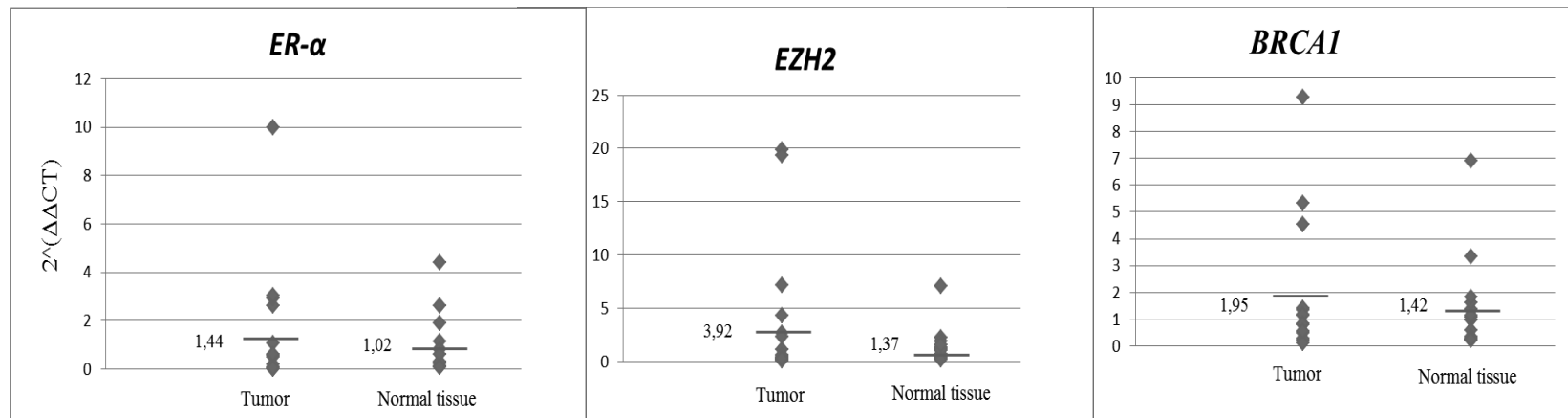


Figure 12. mRNA levels of ER α , EZH2, BRCA1, SRC3, P300, ER β and PGR (n=15)

D. Promoter Genome-Wide Analysis Tumor and Normal Tissue in Sporadic Breast Cancer

Nowadays, ChIP has been coupled with promoter DNA microarrays to evaluate the mechanisms of human gene regulation on a genome-wide scale. ChIP-on-chip technology could be used to investigate the alterations of global gene expression in tumorigenesis. New methodological improvements in epigenetic researches such as ChIP-on-chip methods, would lead to a better understanding of underlying mechanisms of breast tumorigenesis and the approaches to prevention and treatment with clarifying the role of additional mechanisms and complex epigenetic regulations.

In this study, along with Seher Karsli-Ceppioglu, we aimed to investigate differentially expressed genes associated with modified histones H3K27me3 and H3K9ac in breast cancer tumors by ChIP-on-chip method. For this purpose, Agilent SurePrint G3 400k microarrays containing approximately 21,000 of human transcripts were used to scan the enriched region at each gene promoters in fifteen breast tumors with their matched normal tissue samples.

For this study, we used 15 breast tumors with their matched normal tissue samples and two histone marks where one had acetylation marks of H3K9ac and other one had methylation marks of H3K27me3. It means that, we used three tumors and normal tissues for each group belonging to the classification of Saint Gallen. So at the end 30 promoter microarrays were used (Figure 13).

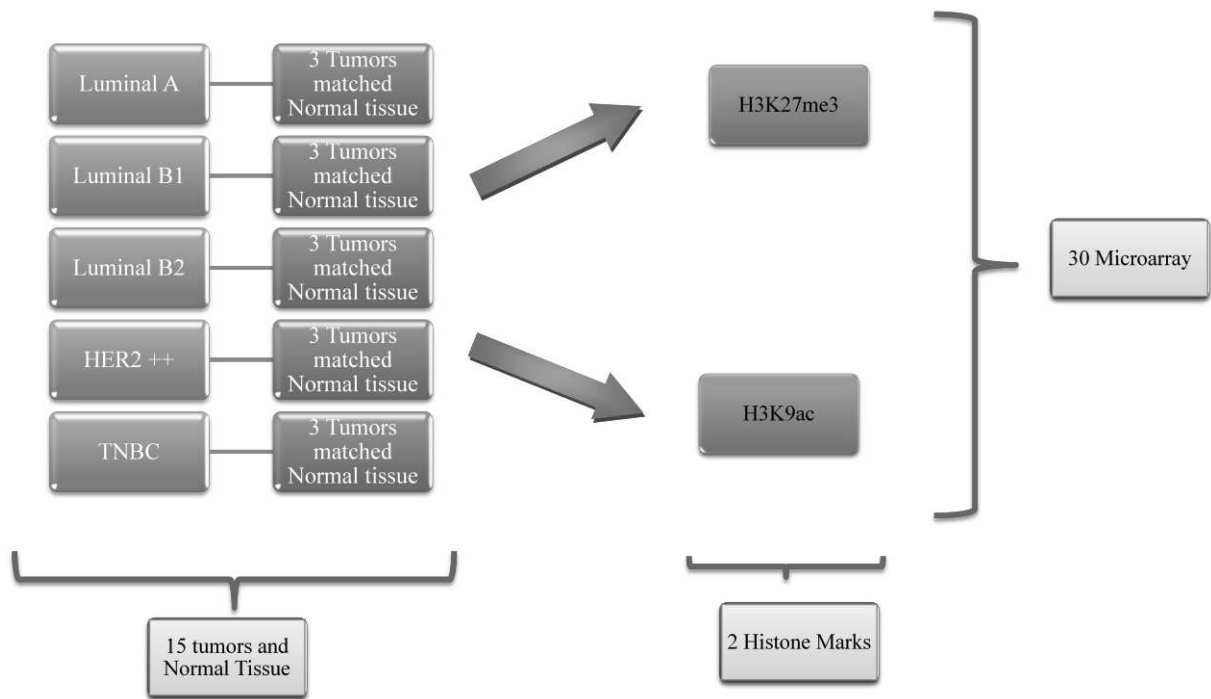
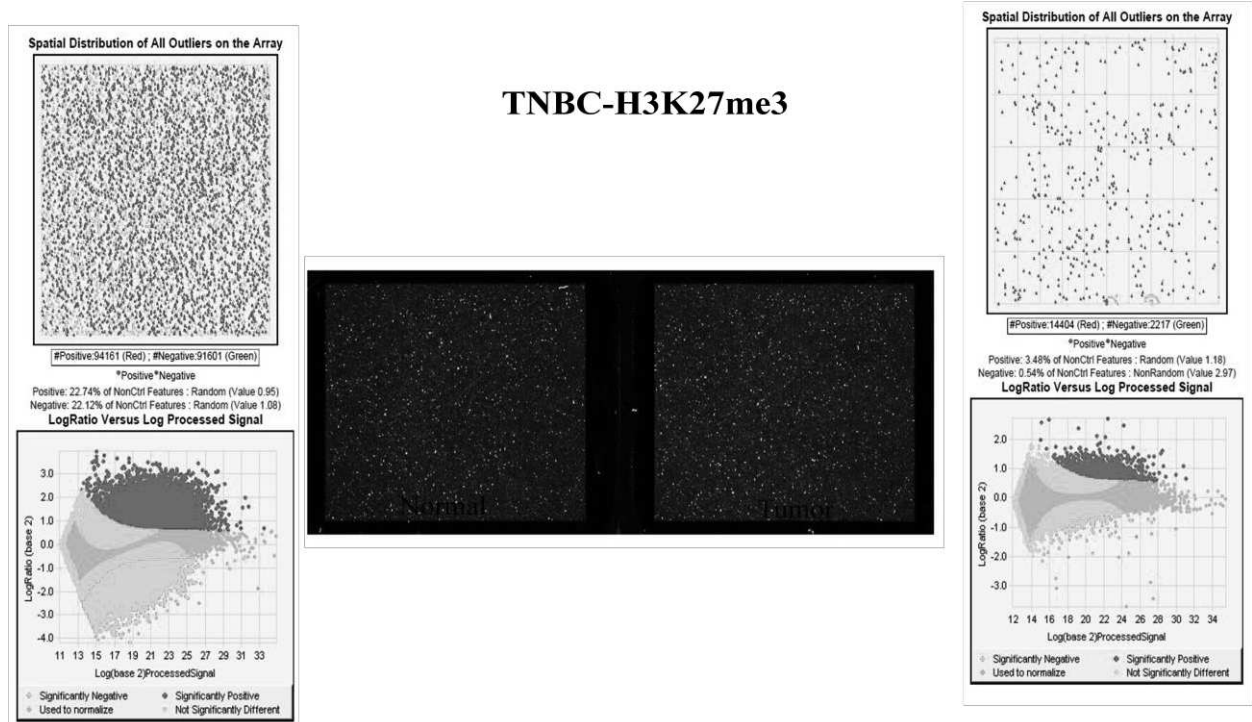


Figure13. Shedule of ChIP on chip

After the manipulation we sent all of the data to ViroScan 3D Profile expert for data analysis. We have not received the results yet but we have added an image of the array and some of the information generated with The Agilent Genomic Workbench software and each Agilent microarray in the form of a QC (Quality Control) report and how to interpret in figure 14. Net signal statistics are an indication of the dynamic range of the signal on a microarray for both non-control probes and spike-in probes. The QC report uses the range from the 1st percentile to the 99th percentile as an indicator of dynamic range for that microarray. The Negative Control Stats table includes the average and standard deviation of the net signals (mean signal minus scanner offset) and the background-subtracted signals for both the red and green channels in the negative controls. These statistics filter out saturated and feature non-uniform and population outliers and give a rough estimate of the background noise on the microarray.

A



B

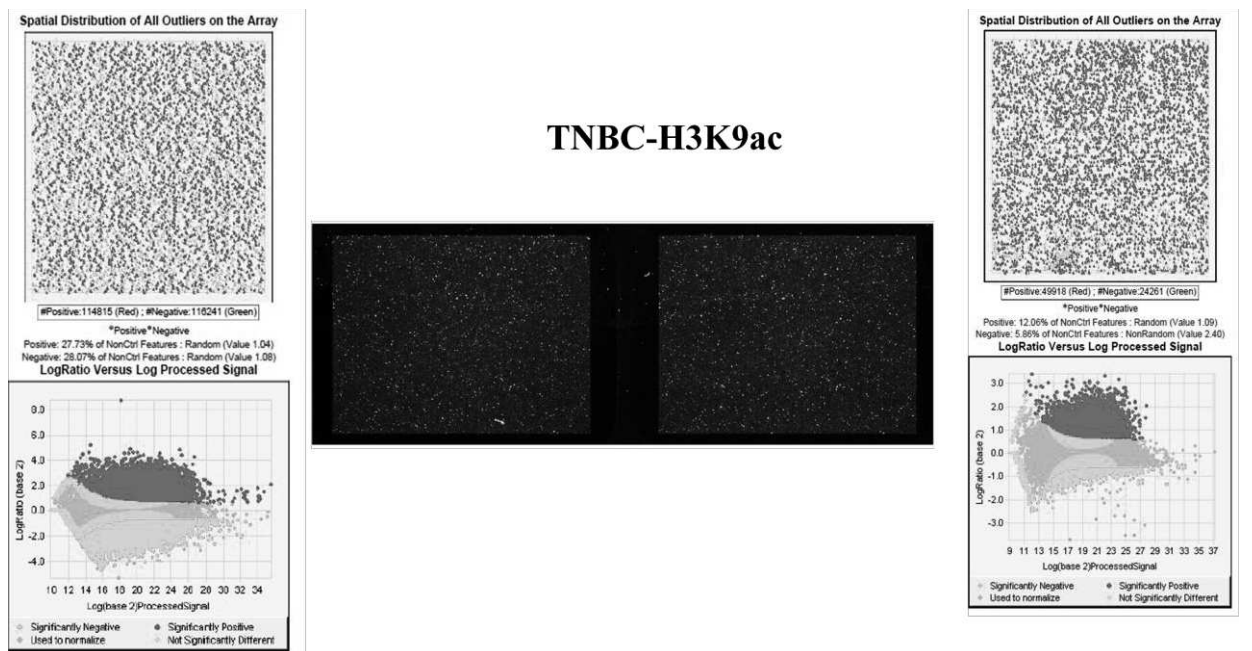


Figure14. An example image and QC report of the Agilent Genomic Workbench software for TNBC group on H3K27me3(A) and H3K9ac (B) histone marks.

5. DISCUSSION

In 2012, 1.7 million of women were diagnosed with breast cancer and there were 6.3 million women alive who had been diagnosed with breast cancer in the previous five years. Since the 2008 estimates, breast cancer incidence has increased by more than 20%, while mortality has increased by 14%. Breast cancer is also the most common cause of cancer death among women (522 000 deaths in 2012) and the most frequently diagnosed cancer among women in 140 of 184 countries worldwide. It now represents one in four of all cancers in women [80]. This study includes the epigenetic modifications with different treatments in breast cancer cell lines and breast tumors.

In East and Southeast Asia, the average daily intake of phytoestrogens is estimated to be between 20 and 50 mg [81]. In contrast, the typical diet of an adult in the United States contains only 0.15–3 mg phytoestrogens per day, and in Europe the average daily phytoestrogen consumption is estimated to be even lower, falling between 0.49 and 1 mg. According to various epidemiologic studies, plasma isoflavone concentrations range from 2 μ M (Japanese men) to 5 nM (Finnish study subjects); however, local tissue phytoestrogen concentrations are suggested to be 2–3 times higher than plasma levels [82, 83].

For this reason, our first study was about the effects of phytoestrogens on breast cancer cell line with different histone marks. We chose three phytoestrogens which are genistein, daidzein and equol; because these phytoestrogens are biologically active phenolic compounds of plant origin that structurally mimic the principal mammalian estrogen 17 β -estradiol.

Epidemiologic evidence suggests that diet and nutrition can influence cancer development and women living in Asia, where diets have traditionally included soybean products, report fewer postmenopausal symptoms and experience fewer breast cancers than women in Western countries. There are lot of mounting of evidence for interactions between phytoestrogens and breast cancer. In recent years, investigations of their potential as anticancer agents have increased dramatically.

Li *et al.* showed that histone modifications may play a more important role in regulating genistein, modulate ER α restoration as compared to DNA methylation. Histone modifications affect the basic structure of the chromatin unit, the nucleosome, and histone

acetylation or deacetylation changes are considered to be the most prevalent mechanisms of histone modifications. Histone acetylation results in an open chromatin structure leading to active gene transcription. They found that treatment with genistein increased the histone acetylation level in the *ERα* promoter region, which could be considered as an important contributor for *ERα* reactivation. Although they did not find any methylation status changes in the *ERα* promoter region by genistein treatment, *ERα* can be regulated by numerous cis-regulatory elements located upstream of the coding sequence of *ERα* and DNA methylation may influence these elements leading to *ERα* expression change. Hence Magee *et al.* have demonstrated that daidzein and equol inhibit the invasive capacity of MDA-MB-231 human breast cancer cells [84]. Also Charalambous *et al.* presented in their study related to equol and MCF7; equol is significantly potent to induce gene expression [85].

No studies combining equol and histone modifications were found, so we thought that our study would light the way for effects of equol on histone modifications in breast cancer cell lines.

We studied the effects of phyto treatments on the variation of post-translational modification of histones such as lysine methylation and acetylation of histone H3 and H4. We provided evidence that phytoestrogens demethylated and acetylated histones of these selected genes in MCF7 and MDA-MB 231 breast cancer cell lines, leads to unpack the chromatin and enhance transcription.

When we looked our results of phytoestrogens; the fold enrichment of H3K9me3 and H3K27me3 over control conditions decreased significantly such as 17 β -estradiol treatment in MCF7 and MDA-MB 231 cell lines. Also, when we controlled the results of acetylation marks like H3Kac and H4K8ac, we saw that phytoestrogens and 17 β -estradiol increased acetylation significantly in selected genes.

These results suggested that phytoestrogens, histone methyltransferase inhibitors and histone deacetylase inhibitors have beneficial effects on the genes which are up-regulated in cancer. However, an adverse effect for down-regulated genes was observed. So the action did not seem to be gene specific.

When we finished the study of phytoestrogens we start to work with other therapeutic agents that are histone methylation inhibitors and histone deacetylase inhibitors on breast cancer cell lines.

Previous studies have shown that, DZNep selectively inhibits H3K27me3 and H4K20me3 in cancer cells [86]. This makes DZNep a possible candidate as an epigenetic therapeutic for the treatment of cancer.

However, DZNep is a known *S*-adenosylhomocysteine (AdoHcy) hydrolase inhibitor, which leads to the indirect inhibition of *S*-adenosyl-methionine (AdoMet)-dependent reactions, including those carried out by many methyltransferases. In a study by Miranda *et al.* they focused only on the methylation of H3K27, H3K9, and H4K20, then they expanded the study to include other histone methylation modifications and to show that DZNep is not a selective inhibitor of H3K27me3 and H4K20me3 as previously reported [86]. Instead DZNep was found to globally inhibit both repressive and active histone methylation marks. In addition, they tested other AdoHcy hydrolase (*S*-Adenosyl-L-homocysteine hydrolase which is a potent feedback inhibitor of AdoMet-dependent methyltransferase enzymes) and global methyltransferase inhibitors and found that *EZH2* inhibition is not specific to DZNep. However, DZNep may have more clinical potential than many of these other inhibitors due to the known limitations of other AdoHcy hydrolase inhibitors [87]. Also they have shown that although repressive histone marks can be reversed by DZNep treatment, a transcript cannot be re-expressed if it contains a methylated CpG island. As a matter of fact there are many studies accede to DZNep global methyltransferase inhibitors [87-89].

All these studies supported our results as we found that H3K27me3 level was reduced on *EZH2* in all cell line with DZNep treatment and also to strengthen our case, we did a western blot analysis. The Western blot results have shown that there is a significant difference between control and treatment with DZNep for the two studied cell lines.

Although it has clearly shown that DZNep acts globally and can inhibit both active and repressive histone marks, DZNep may still be clinically useful. Therefore, we decided that further investigations of the potential of DZNep as an epigenetic therapeutic were warranted, in addition with using DZNep to further elucidate how chromatin structure affects gene expression [90].

A previous study has shown that mutation in *BRCA1* causes of increase *EZH2* concentration particularly in the basal like breast carcinomas [91]. Generally *EZH2* is overexpressed in ER-negative breast cancer and inhibits *BRCA1* phosphorylation. For this reason, transition from G2 to M is accelerated in cell cycle and increased cell proliferation. Hence, we found an *EZH2* depletion by treatment with DZNep, indicating that an epigenetic therapy that pharmacologically targets *EZH2* via DZNep may constitute a novel approach to treat breast cancer (Figure 15).

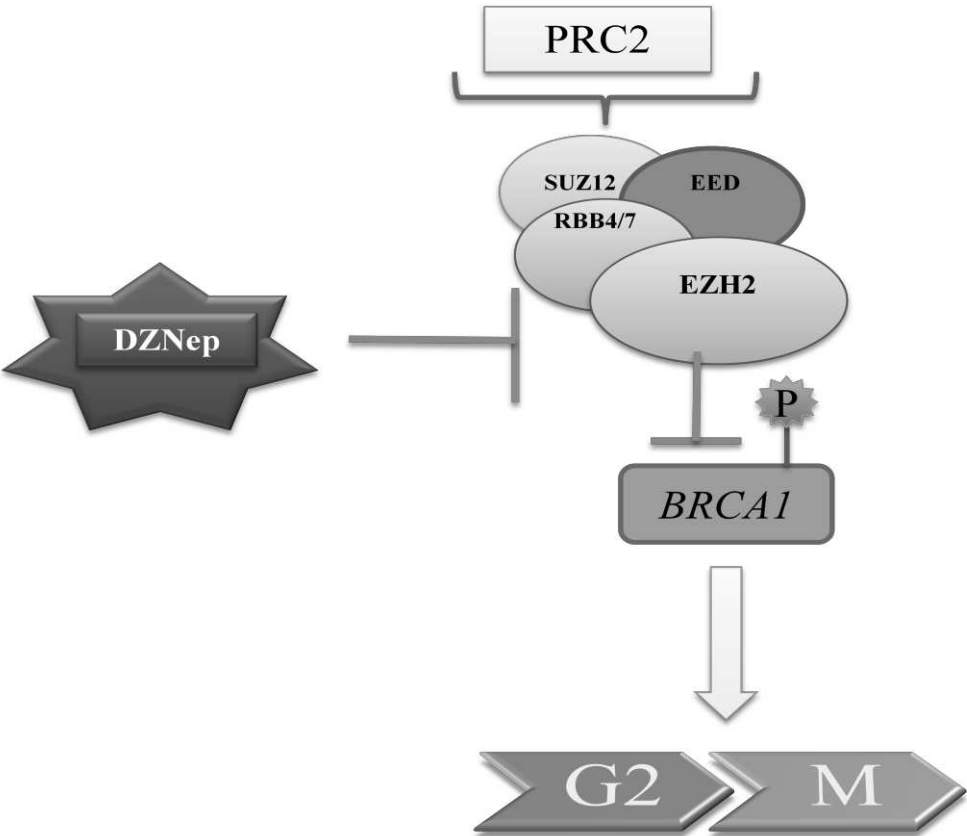


Figure15. Effects of DZNep in the cell.

We then applied another therapeutic, NaBu. Several research groups have reported that NaBu halts DNA synthesis, arrests cell proliferation, alters cell morphology and increases or decreases gene expression. As a potent HDACi *in vivo*, NaBu has been reported to regulate a large number of genes in breast cell lines [92].

In a report by Davie *et al.*, inhibition of HDAC activity with NaBu allows the HAT activity of *P300* to increase the histone acetylation levels at the promoter and nearby regions. They also say that NaBu induces the expression of *p21^{Waf1/Cip1}* and thereby inhibits cyclin E–Cdk2 activity and halts the subsequent events that are required for cells to enter S phase. The cell cycle–arrested cells may differentiate or undergo cell death by apoptosis [93].

Furthermore Shin *et al.* utilized ChIP technology combined with next generation sequencing technology (ChIP-seq) to analyze histone modification (acetylation) induced by butyrate and to map the epigenomic landscape of normal histone H3 and acetylated H3K9 and H3K27 on a large scale. Their data revealed that NaBu induced acetylation of H3K9 and H3K27 played a role in gene expression regulation and in the complicated and dynamic epigenomic landscape that is formed by a variety of histone modifications [94].

We have demonstrated that; NaBu treatment, H3K9ac and H3K4ac marks were more effective than H3K27me3 mark in MCF7 cell line. This means that NaBu induced acetylation of histone H3K9 and H3K4.

HDACs are enzymes that regulate histone acetylation on core nucleosomal histones by catalyzing the removal of acetyl groups on their amino-terminal lysine residue. Decreased histones H3 and H4 acetylation compact the chromatin structure resulting in disruption of the access of transcriptional factors and repressing the transcription of certain genes [95]. To date, the most convincing evidence that HDACs behave differently in cancer cells than in normal cells is derived from the pharmacological manipulation of HDACs through HDAC inhibitors [96]. However, the molecular mechanism of the tumor selective action of HDAC inhibitors is unclear. Recent studies indicated that the HDAC inhibitors induced DNA damage in normal and cancer cells, but cancer cells cannot repair themselves. Thus, the selectivity of HDAC inhibitors in causing cancer cell death may be associated with impaired DNA repair mechanism in cancer cells [97, 98].

Chiu *et al.* studied SAHA in MCF7 and MDA-MB-231. Their results are the first demonstrating that SAHA enhances the radiation response. Furthermore, the combined treatment induced stronger cytotoxicity in breast cancer cells. One of the mechanisms whereby SAHA inhibited the cell capacity to repair IR-induced DNA damage by affecting the DNA repair pathways could contribute to this combined effect. Induction of autophagy and ER stress could also be involved in the underlying mechanisms. In addition, they further found that SAHA inhibited the invasion and migration of breast cancer cells [99].

Moreover, Stark *et al.* reported that HDAC inhibitors can alter the gene expression profile of mesenchymal-like triple negative breast cancer cells such as MDA-MB-231 to become less aggressive and to have a more favorable prognostic profile [100]. Yi *et al.* reported that SAHA effectively depletes ER α in breast cancer MCF7 cells [101].

This result also supported our study because when we investigated the levels of ER α and ER β , they increased on the H3K27me3 mark especially with the SAHA treatment in MDA-MB-231. Nevertheless, with the treatment of SAHA, rate of ER α and ER β increased on the two activator marks H3K4ac and H3K9ac in MCF7 cell line.

These results suggested that phytoestrogens, histone methyltransferase inhibitors and histone deacetylase inhibitors have beneficial effects on the genes which are up-regulated in cancer. However, an adverse effect for down-regulated genes was observed. Thus, the action did not seem to be gene specific.

Waby *et al.* assessed the effects of multiple members of the HDACi family on cell cycle progression and on acetylation of Sp1. Their data indicated that a G2/M arrest is consistently observed with several of the HDACi used and they thought that Sp1 is acetylated in response to multiple HDACi (Figure16) [102].

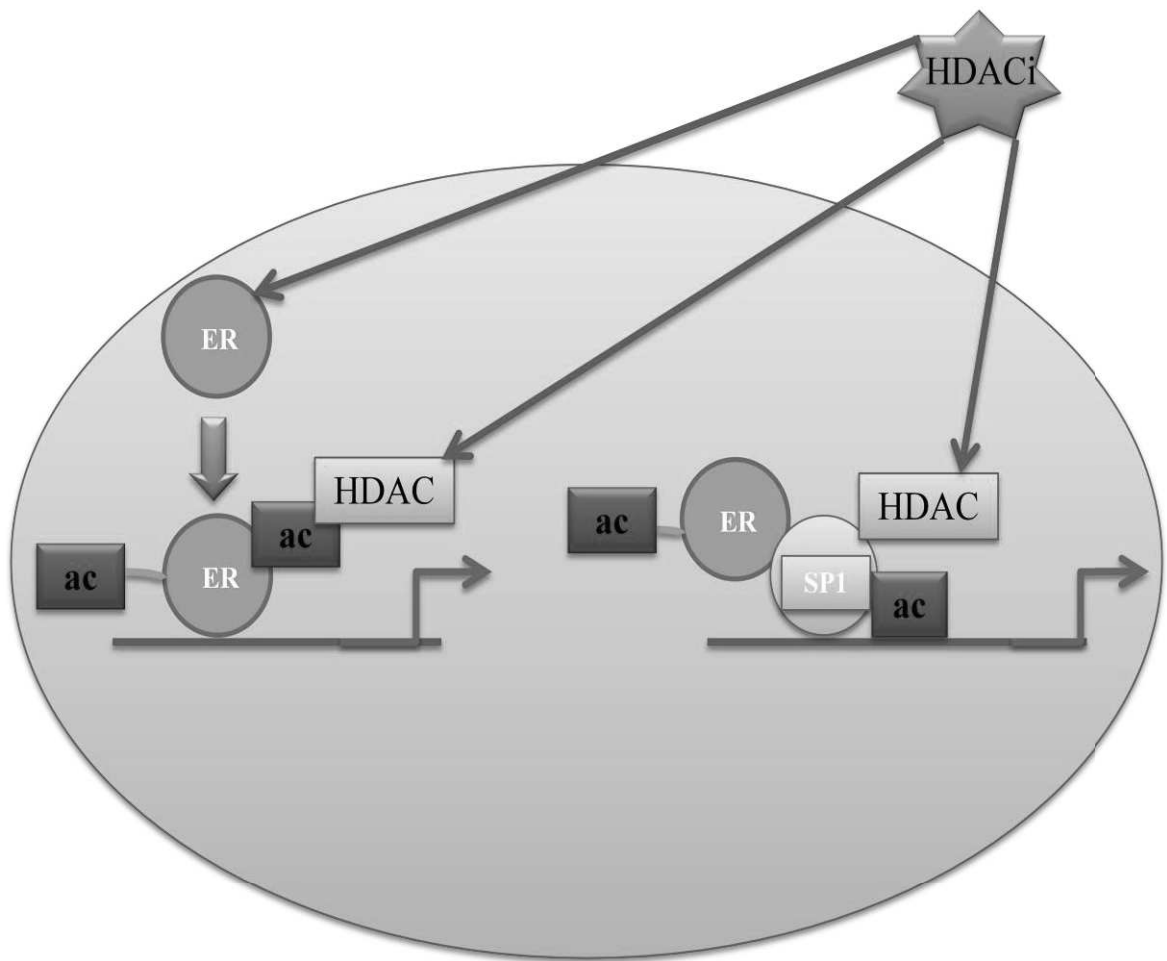


Figure16. Effects of HDACi in the cell

First ChIP experiments directed at the ER, focused on a limited number of known binding sites and investigated binding of ER and cofactors. For example, Shang *et al.* found that the ER and a number of coactivators are associated with chromatin at the c-Myc, pS2 and CATD estrogen responsive promoters following estrogen treatment in a cyclic fashion [103]. More recently, a high throughput ChIP approach, ChIP cloning, was described by Laganier *et al* [104].

We chose the method of ChIP to understand the interactions between specific proteins or modified forms of proteins and a genomic DNA region. ChIP can be used to monitor the presence of histones with post-translational modifications at specific genomic locations and is used to determine whether a transcription factor interacts with a candidate target gene. There was a highly significant correlation between histone modification status, tumor biomarker phenotype, and clinical outcome. High relative levels of global histone acetylation and

methylation were associated with a prognosis and detected almost exclusively in luminal-like breast tumors (93%).

Elseikh *et al.* reported that basal like and HER2++ tumors were associated with low levels of all seven histone marks. Low detection levels of H4R3me2, H3K9ac, and H4K16ac were significantly associated with large tumor size and high detection levels of H4R3me2 and H3K9ac were associated with low lymph node stage. These results may indicate that reduced detection of selected histone modifications correlates with poor prognostic characteristics. High-level detection of histone modifications was significantly correlated with steroid receptor (ER, PR, AR)-positive tumors [105].

For our third project, we studied breast tumors and normal tissue comparing inside the classification St Gallen. A number of genes have been investigated for changes in expression level during progression of breast cancer. In all of the studies we used same selected genes like *EZH2*, *BRCA1*, *ER α* , *ER β* , *SRC3*, *P300* and *PGR*. This is because we know that *ER α* , *EZH2*, *SRC3* and *P300* are overexpressed genes and promoted the activation of transcription in breast cancer and also that *BRCA1* is a human tumor suppressor gene. *ER α* , *ER β* and *PGR* and their ligands play important roles in the development and function of the mammary gland in order to identify gene expression changes that may be linked to clinical outcomes such as relapse-free survival and overall survival.

Several experimental studies have established that elevated *EZH2* levels in human breast carcinomas are associated with the aggressive ER-negative basal-like phenotype characterized by lack of *ER* expression, nuclear polymorphism and lack of BRCA1 protein [106], [107].

Some studies have used a gene *PRC2* target gene set and identified 391 (59.8%) and 336 (51.4%) genes that harbor epi-modification alterations in luminal and basal subtypes in study of 2014. In particular, of these *PRC2* targets like a *EZH2*, there were more genes having reduced H3K27me3 signal in luminal subtype than that in basal subtype (228 and 146 genes in luminal and basal, respectively). This finding is in line with the fact that basal breast cancer has more undifferentiated features than luminal subtype [108].

When we investigated our results of *EZH2* for each subtype of breast cancer, this data supported our *EZH2* results on H3K27me3 marks and in addition, we demonstrated that *EZH2* reduced H3K27me3 in luminal B1 subtype than that in luminal A.

Hudelist *et al.* found that overexpression of *SRC3* in breast tumors correlates with poor disease-free survival. Higher *SRC3* levels are also preferentially present in higher grade invasive tumors. In addition Mc Ilroy *et al.* study results indicate an association between high *SRC3* levels and breast cancer [109, 110]. Furthermore Bouras *et al.* revealed that *SRC3* overexpression also correlated with the absence of ER and PR in breast cancer [111]. Our findings suggest that, overexpression of *SRC3* accessible in Luminal A and Luminal B1 in repressive marks in H3K27me3 besides subclass of HER2++ which is absence of ER and PR groups, have a high level of *SRC3* in activatrice marks like H3K4ac and H3K9ac.

Some studies indicate that *P300* plays a role as tumor suppression, yet other studies suggest it plays a role as tumor promotion. It has been reported that *P300* behaves as a classical tumor suppressor gene [112-114]. Xiao *et al.* reported that in 2012, high expression of *P300* was more frequently observed in breast cancer tissues when compared to that in the non-malignant breast tissues. If we analyzed those results in comparison to our classification of St Gallen; non-malignant breast tissues are combined with a class of Luminal. The expression of *P300* in non-malignant breast tissues was either absent or at low levels. In contrast, high expression of *P300* was frequently observed in large number of breast cancer tissues [115].

We did not find any association between three histone marks H3K27me3, H3K9ac and H3K4ac on expression level of *P300*. However, when we investigated the difference between in subclass, we found that Luminal B1 has *P300* high expression level for all modification of histones.

Our results demonstrated that expression levels of *ERβ* and *PGR* genes were decreased on H3K4ac mark-enriched sites in tumor samples. *ERβ* and *PGR* genes play a crucial role in determining breast cancer aggressiveness, thus the modified H3K4ac mark would be significant transcriptional expression of these genes in breast cancer. However, we did not find significant differences in gene expression levels related with H3K9ac mark modification.

According to our results, over-expression of *ERα*, *ERβ* and *EZH2* genes were found on H3K27me3-enriched sites in Luminal B-like (HER2 negative) breast tumors. On the other

hand, expression levels of *ER α* and *ER β* genes were decreased in basal-like tumors. The expression level of *ER β* gene on H3K9ac mark was increased in HER2 positive tumors. In addition, *PGR* was over-expressed on H3K4ac mark in HER2 positive tumors. As a result, we have found that modified histone marks affect transcriptional expression of genes that code hormonal receptors.

After all these studies, we used promoter microarrays on breast cancer tumor and matched normal tissue with previously selected histone marks to work with a wider spectrum of genes. The overall goal of this project was to study breast cancer with reference to gene expression analysis using clinical specimens and to advance the understanding of the heterogeneity of breast cancer using gene expression analysis, and to identify gene expression differences among the normal and cancer breast tissue by comparing various inside classifications of St Gallen.

On the other hand, Li *et al.*, compared the alterations of six types of histone modifications H3K4me1, H3K4me3, H3K27ac, H3K36me3, H3K9me3 and H3K27me3 and DNA methylation between two cell lines (MCF7, HCC1954) that had been shown to represent two different breast cancer subtypes-luminal and basal. They used ChIP-seq and microarray as in their previously study [116].

In all cell lines, they found thousands of differential histone modification sites were identified in both luminal and basal subtypes, compared with control. They also explained that different patterns of alterations of epi-modifications between luminal and basal subtypes were observed. In MCF7 cell line with the up-regulation of *ER* and *PR* in luminal subtype, they found that H3K4me3 and H3K27ac were up-regulated and H3K27me3 was down-regulated. However, the epigenetic patterns of *ER* and *PR* in this subtype were different from those in basal subtype (HCC 1954 cell line).

Consistent with ERBB2 high expression in basal subtype, epigenetic alterations were found within all the six types of histone modifications [117].

The advent of microarray technology has revolutionized the molecular profiling of disease tissues and tumors. It is one of the most promising and powerful methodologies in molecular oncology. Microarrays have a number of advantages over TaqMan assays, especially when many clinical samples are to be screened. In addition, microarrays can incorporate multiple positive and negative controls as well as multiples of the same samples at

different locations of the array to ensure reproducibility and statistical significance. Ideally, initial screening of biological samples could be done by microarray analysis and individual positive samples could be confirmed by additional tests using TaqMan assays.

6. CONCLUSION

This PhD research first focused on the biological effects of phytoestrogens and some epidrugs *in vitro* and characterization of bioactive compounds from breast cancer.

These results suggested that phytoestrogens, histone methyltransferase inhibitors and histone deacetylase inhibitors have beneficial effects on the genes which are up-regulated in cancer. However, an adverse effect for down-regulated genes was observed. Thus, action did not seem to be gene specific. Our results add a new outlook of complication to epigenetic deregulation in breast cancer and also established histone modifications-mediated silencing or enhancing for up-and-coming therapeutic target.

Subsequently, study with normal tissue and sporadic breast cancer have allowed us to present effective differences on Saint Gallen classifications of breast tumors with ChIP. It also allowed us to gain more insight into how epigenetic modification patterns are regulated in detail especially how the patterns are established. However, future studies focusing on the clinical relevance and mechanism of epigenetic modification of bio-active dietary factors are needed to further assess the applicability of dietary factors as cancer preventive and chemo preventive agents.

ChIP can be used to identify regions that are associated with proteins, or conversely, it can be used to identify proteins that are associated with a particular region of the genome.

The combination of ChIP and microarray (ChIP on chip) allows genome-wide identification of binding sites for the chromatin-associated proteins. Alternatively, high throughput sequencing of the immunoprecipitated DNA (ChIP-Seq) offers a more precise way of mapping protein-DNA interactions across the genome.

We have not yet received our results, but we hope that the transition from normal tissue to tumor is an important aspect in understanding the biology of breast cancer. Gene expression profiling can help identify the differences among the normal and tumor tissues and can help better design drugs to target the disease. The normal and cancer specimens were compared in order to identify genes and pathways that contribute to the transition from normal breast tissue to a cancerous state.

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ABSTRACT

Breast cancer is the leading cause of cancer mortality in women. Female death caused by female breast cancers are consist of 6.1% of the total women deaths. Breast cancer is a significantly heterogeneous disease in histology, genetics and prognosis, and it is clear that epigenetic alterations have crucial role in breast cancer tumorigenesis.

Epigenetic alterations, which are heritable changes in gene expression that exist without changes in DNA sequence, are important key factors for cancer development and prognosis. In recent years, we have determined that epigenetic alterations are involved in the mechanisms of carcinogenesis, especially DNA methylation or histone modifications. Epigenetic factors can regulate the activation or repression of genes in the cell.

Therefore, we aimed to assess chromatin immunoprecipitation (ChIP) of selected histone modifications (H3K27me3, H3K9me3, H3K4me3, H3K4ac, H3K9ac and H4K8ac) in breast cell lines and their associations with phytoestrogens, histone methyltransferase inhibitors (HMTi) and histone deacetylase inhibitors (HDACi). After that, we strived to understand the relative levels of three modified histones, including H3K27me3, H3K9ac and H3K4ac that will be determined in breast tumors compared with the matched normal tissue. In order to identify the presence of variations in global levels of histone marks in tumors and the matched normal tissues, we proposed to follow ChIP with Q-PCR of 7 genes implicated in breast cancer and for which the decrease or increase in their expression can be resulted on histone modifications. Finally, we applied ChIP on chip, to investigate differentially expressed genes associated with modified histones H3K27me3, H3K9ac and H3K4ac in breast tumors.

Our results suggested that phytoestrogens, histone methyltransferase inhibitors and histone deacetylase inhibitors have beneficial effects on the genes, which are up-regulated in cancer. However an adverse effect for down-regulated genes was observed. So the action did not seem to be specific for genes. Our results add a new outlook on the complication with epigenetic deregulation in breast cancer and also established histone modifications-mediated silencing or enhancing as an up-and-coming therapeutic target.