INVESTIGATING THE EFFECT OF ANDROGRAPHOLIDE ON HMGCR PROTEIN EXPRESSION IN MDA-MB-231

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INVESTIGATING THE EFFECT OF ANDROGRAPHOLIDE ON HMGCR PROTEIN EXPRESSION IN MDA-MB-231

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LIST OF SYMBOLS AND ABREVIATIONS

Abbreviation	Expanded form
%	Percent
˚C	Degree Celsius
<	Less than
ABC	ATP-Binding Cassette
ANOVA	Analysis Of Variance
ASR	Age-Standardized Rate
BCS	Breast Conserving Surgery
BL1	Basal-Like 1
BL2	Basal-Like 2
BLIA	Basal-Like/Immune Activated
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
BSR	Breast Self-Examination
CIN	Chromosomal Instability
DCIS	Ductal Carcinoma In Situ
ddH_2O	Deionized distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribo Nucleic Acid
EGFR	The Epidermal Growth Factor Receptor

ER	Estrogen Receptor
ET	Endocrine Therapy
GLOBOCAN	Global Cancer Incidence, Mortality And Prevalence
H	Hour
HER 2	Human Epidermal Growth Factor Receptor
HMGCR	3-Hydroxy-Methylglutaryl Coenzyme-A
HR	Hormone- Receptor
HRT	Hormone Replacement Therapy
IDC	Infiltrating Ductal Carcinoma
IGF-1	Insulin-Like Growth Factor 1
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma
IM	Immunomodulatory
L	Liter
LAR	Luminal Androgen Receptor
LCIS	Lobular Carcinoma In Situ
LMIC	Low and Middle-Income Countries
M	Molar
MCF7	Michigan cancer foundation-7
MES	Mesenchymal
MIN	Microsatellite Instability
ml	Milliliter

mm	Millimeter
MRI	Magnetic Resonance Imaging
MSL	Mesenchymal Stem-Like
PAGE	Polyacrylamide gel electrophoresis
pH	Potential hydrogen
PR	Progesterone Receptor
RIPA	Radioimmunoprecipitation assay
TBS	Tris buffer saline
TBST	Tris buffer saline tween 20
TNBC	Triple-Negative Breast Cancer
WHO	World Health Organization
γ	gamma

MENYELESAIKAN KESAN ANDROGRAPHOLIDE PADA HMGCR PROTEIN EXPRESSION DALAM MDA-MB-231

ABSTRAK

Kanser payudara adalah pertumbuhan sel-sel malignan yang tidak terkawal dalam tisu payudara. Ia adalah jenis kanser kedua yang paling lazim di kalangan wanita dari segi kadar kematian dan insiden dengan anggaran tahunan kes didiagnosis melebihi 1.5 juta. Kanser payudara tiga kali ganda adalah subtipe kanser payudara yang tidak menyatakan mana-mana tiga reseptor PR, ER dan HER2 dan terdiri daripada 15% daripada semua kes kanser payudara. Andrographis paniculata tumbuhan asalnya dijumpai di Malaysia, India dan banyak negara Asia Tenggara, dan secara tradisinya digunakan penduduk tempatan untuk merawat penyakit seperti demam, disentri, diabetes, obesiti dan malaria. Komponen bioaktif utamanya dipanggil.

Andrographolide. Andrographolide mempunyai kesan anti-kanser pada pelbagai jenis kanser. Pada kanser payudara, kajian telah membuktikan bahawa ia mempunyai kesan anti-metastatik anti-proliferatif pada sel-sel kanser tetapi tidak mempunyai kesan pada sel-sel sel payudara biasa.

3-Hydroxy-MethylGlutaryl Coenzyme-A (HMGCR) adalah enzim (glikoprotein) mempunyai peranan penting dalam proses biosintesis kolesterol kolesterol melalui jalur mevalonate.

Blotting Barat digunakan untuk menganalisis tahap ekspresi protein HMGCR di MDA-MB-231 sel selepas rawatan dengan 100mM Andrographolide dalam masa bergantung (4 jam, 24 jam dan 48 jam).

Inhibisi proliferasi sel dan peraturan pergerakan protein HMGCR diperhatikan dalam sel yang dirawat dengan 100mM Andrographolide dalam masa yang bergantung

pada masa (4hrs, 24 jam dan 48hrs) apabila dibandingkan dengan sel yang dirawat dengan DMSO sahaja.

Andrographolide mempamerkan kesan antikanser pada sel-sel kanser payudara Triple-Negatif (MDA-MB-231). Ia juga mempunyai pengaruh terhadap ekspresi protein HMGCR.

INVESTIGATING THE EFFECT OF ANDROGRAPHOLIDE ON HMGCR PROTEIN EXPRESSION IN MDA-MB-231

ABSTRACT

Breast cancer is the uncontrolled malignant cellular growth in the tissues of the breast. It is the second most prevalent type of cancer among women in terms of mortality rates and incidents with annual approximation of diagnosed cases exceeds 1.5 million. Triple-Negative Breast Cancer is a subtype of breast cancer that does not express any of the three receptors PR, ER and HER2 and comprises 15% of all breast cancer cases. *Andrographis paniculata* a plant originally found in Malaysia, India and many Southeast Asia countries, and traditionally used by locals to treat diseases like fever, dysentery, diabetes, obesity and malaria. Its major bioactive compound is called Andrographolide. Andrographolide has anti-cancer effect on various types of cancer. On breast cancer, studies have proven that it has anti-proliferative anti-metastatic effects on cancerous cells but has no effect on normal breast cell lines. 3-Hydroxy-MethylGlutaryl Coenzyme-A (HMGCR) is an enzyme (glycoprotein) has a significant role in the process of cholesterol biosynthesis of cholesterol through the mevalonate pathway.

Western Blotting was used to analyze the HMGCR protein expression levels in MDA-MB-231 cells after treatment with 100mM of Andrographolide in time-dependent manner (4hrs, 24hrs and 48hrs).

Inhibition of cellular proliferation and down regulation of HMGCR protein expression was observed in cells treated with 100mM of Andrographolide in time-

dependent manner (4hrs, 24hrs and 48hrs) when compared with cells treated with only DMSO.

Andrographolide exhibited anticancer effect on Triple-Negative Breast Cancer cell line (MDA-MB-231). It also had an influence on HMGCR protein expression.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Breast cancer is the formation of malignant tumor within the normal tissues of the breast a shape of lumps. Statistically, it is the second type of cancer that cause high mortality rates among women worldwide and it is commonly diagnosed among them as well Siegel *et al.* (2017). In Malaysia, a women's odd of getting breast cancer are 1 in every 35 (Malay ethnicity), 1 in 22 (Chinese ethnicity) and 1 in 24 (Indians ethnicity) (Azizah, 2015). Not only in women, men are also at risk of developing breast cancer (about 1 in 1000 men will develop breast cancer in their life).

Histopathological classification divides breast cancer into four types: basal-like, human epidermal growth factor receptor 2 (HER2), luminal A and B (Cho, 2016). On the other hand classification based on immunohistochemistry (IHC) classify breast cancer into hormone receptor positive (PR+/ER+), HER+ and Triple-Negative Breast Cancer (TNBC) (Sun *et al.*, 2017).

Triple-Negative Breast Cancer (TNBC) is defined as a type of breast cancer in which the cancerous cells that lacks the expression of progesterone, estrogen and HER2 receptors when measured with IHC. It is the most aggressive type of breast cancer, due to its short overall survival, distant recurrences (after the diagnosis in 3 years) and the absence of hormone receptors and no overexpression of HER2 (Collignon *et al.*, 2016).

Andrographis paniculata also known as the king of bitters, a plant native in India, Malaysia, Thailand and China. In Malaysia it is known as Hempedu bumi (Jayakumar *et al.*, 2013). *A. paniculata* traditionally used to treat upper respiratory infection, flu

and sore throat. The major bioactive components of this plant known as Andrographolide. Andrographolide possesses anti-asthma, anti-stroke, anti-diabetic, anti-viral, anti-HIV and anti-inflammatory effects (Jayakumar *et al.*, 2013). Several studies suggest that andrographolide has shown to have anti-cancer activity against various types of cancer cell lines including lung cancer, leukemia, melanoma and breast cancer (Shi *et al.*, 2008a). A study reported that andrographolide inhibited the proliferation and migration of breast cancer cells and it induced apoptosis through caspase-independent pathway as well as the induction of cell cycle arrest at the G2/M (Kumar *et al.*, 2012b)

3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is an important enzyme which has a significant role in the process of cholesterol biosynthesis through the mevalonate pathway (Sharpe and Brown, 2013). HMGCR act as a rate limiting enzyme which responsible for formation of mevalonate from 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) (Hwang *et al.*, 2016).

1.2 Significance of the study

Current breast cancer treatment strategies are considered harsh and invasive. Thus, the urge for seeking alternative remedies such as medicinal plants is needed. The current study evaluates the anti-cancer effect of a plant called *Andrographis paniculate* on TNBC.

1.3 Objectives of the study

(a) Determine the anti-cancer activity of Andrographolide compound extracted from *Andrographis paniculata* on Triple-Negative Breast Cancer cell line (MDA-MB-231).

(b) Determining the effect of Andrographolide on the expression level of HMGCR by using Western Blot analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Genomic Instability and Cancer

Impaired and unstable genes are the causative agents of cancer. According to (Yao and Dai, 2014) the instability of the genes are considered the trade mark for most occurring cancer. Genomic instability is basically a spontaneous change in the genome of the cell obtained from the same ancestral precursors (Tubbs and Nussenzweig, 2017). In a normal healthy cell, the genomic stability is controlled by molecular mechanism. This particular mechanism in cancer cell is damaged beyond repair which leads to the mutation in the cell and these mutation are accumulated through time resulting in defected cellular mechanism (Tubbs and Nussenzweig, 2017). Genomic instabilities may occur due to microsatellite instability (MIN) with the mutator phenotype and chromosomal instability (CIN) with gross chromosomal changes (Raptis and Bapat, 2006) microsatellite instability (MIN) happens when there are alterations in the short repeat stretches of coding and non-coding DNA, thus the DNA damage repair genes are unable to inactivate the occurring mutations. Instability also may result from epigenetic mechanisms which may lead to hypomethylation or hyper methylation of promoter regions, thus increases gene expression in case of hypomethylation and gene silencing in case of hypermethylation. Other factors contribute to genetic instability are environmental and dietary factors (Yao and Dai, 2014)

CIN explains most of human cancers cases, these abnormality could be numerical and/or structural changes in the chromosomes (Aboalela *et al.*, 2015). According to

(Thompson and Compton, 2011) structural aberrations of the chromosomes are distinctive mark for human epithelial cancer and the end product of impaired genome stability mechanism. Most pathogen etic aberrations are related to deletion or amplification of tumor or oncogenes suppressor gene loci with imbalanced gene expression and loss of heterozygosity (Thompson and Compton, 2011). In epithelial cancers related to neoplasms originated in mesenchymal or hematopoietic lineages, high chromosomal aberrations are observed.

2.2 Breast cancer

Breast cancer is the abnormal and uncontrolled cellular growth in the tissues of the breast. It starts from various parts of the breast, but the majority starts from the ducts that carry the milk to the nipple, in this case it is called ductal cancers. It is estimated that 55% cases of breast cancer are caused by Invasive ductal carcinoma (IDC) (Eheman *et al.*, 2009) Fig (2.1). Another type is the one that starts in the glands that produces milk (lobular cancers) Fig (2.1). The growth of tumor is not related to the initiate site or the cellular origin of the cancer. However, it differs in term of the biology of the cells: whether it expresses E-cadherin or not (Makki, 2015).

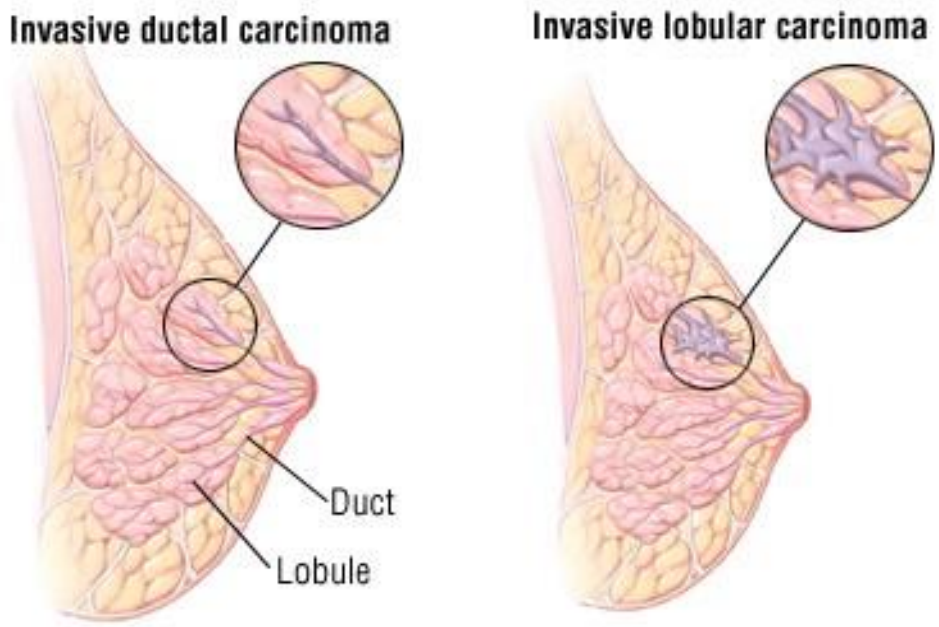

Figure 2.1 : The anatomy of invasive ductal carcinoma an invasive lobular carcinoma (Medium.com, 2018).

2.3 Burden of Breast Cancer

In 2012 the total number of diagnosed breast cancer cases was 1.7 million among females with total casualties of 521,900 deaths worldwide, 25% of deaths were cancer cases and 15% were related to cancer (Nounou *et al.*, 2015). High-income Countries such as Australia, America, New Zealand plus Western and Northern parts of Europe recorded the highest incidents of breast cancer (Torre *et al.*, 2017). In low and middle-income countries (LMIC) countries like sub-Saharan Africa has low incidents but high death rates because of the access to treatment is limited and the diagnosis stage is late (Jedy-Agba *et al.*, 2016) In men, of all breast carcinoma incidents it accounts about 0.8% to 1% (Gomez-Raposo *et al.*, 2010). However, majority of cases in men are positive hormone receptor which means it can show good treatment results (Nounou *et al.*, 2015). Male breast tumours are more likely to express progesterone

(PR) and estrogen (ER) receptors with overexpression of human epidermal growth hormone receptor (HER2) compared to females (Gomez-Raposo *et al.*, 2010). In terms of diagnosis, men are diagnosed at older age compared with women (Nounou *et al.*, 2015).

2.4 Prevalence of breast cancer in Asia

In Asia, breast cancer is considered the most common cancer. The rapid increase in cases of breast carcinoma are mainly localized in the undeveloped countries (Youlden *et al.*, 2014). Incidents in Asian countries are lower when compared with America and Europe, however, the mortality rate in Asia is higher than America and Europe (about 6-23 per 100,000) (Jemal *et al.*, 2011). Increased of incidence rate was reported in Asia among women under 40 years age (Ghoncheh *et al.*, 2016).

According to the world health organization (WHO), most Asian countries has no cancer registration system, thus the incidents rates are higher in reality (Ghoncheh *et al.*, 2016). Countries including Egypt, Brazil, Mauritius, Kuwait, Guatemala, Moldova and Mexico has increased in mortality rates due to the reduction in breast feeding, fertility, bad diet and lifestyle habits (low physical activity and obesity), and exposure to exogenous hormones (Ghoncheh *et al.*, 2016). Asian countries such as Japan, China, South Korea, Singapore, Philippines, Taiwan and Thailand has registered the highest breast cancer cases among Asian countries (Shin *et al.*, 2010).

Worth noting, in Asia the age of breast cancer diagnosis is between 45-55 years, however, in western countries is 55-60 years of age (Ghoncheh *et al.*, 2016). Thus,

among young women in Australia and New Zealand the rate of breast cancer is higher compared with Asian countries (Liu *et al.*, 2011).

Case studies and control studies reported that old age childbirth, early menstruation, late menopause and few full-term pregnancies are what causing high rates of breast cancer among Asian women (Yanhua *et al.*, 2012). Standardized death rates in Asia differs from country to another, as for countries with high standardized death rates including Lebanon, Armenia, Jordan, Pakistan and Syria. While Mongolia, China, Bhutan and Nepal has the lowest standardized death rates (Ghoncheh *et al.*, 2016). However, In many European countries and in US, the mortality rates has been reduced because of the introduction of mammography, early detection of the disease, progress in treatment and the early detection of small tumours (Ghoncheh *et al.*, 2016).

Reducing breast cancer mortality rate is improved through the implication of factors such as prognosis and early detection of the disease (Rahimzadeh *et al.*, 2014). The mortality rates around the world differs from region to another, it is low in the west (6 per 100,000) and high in the developing countries (20 per 100,000) (Ghoncheh *et al.*, 2016).

The reasons why breast cancer is poorly managed in Asia are related to cultural and economic status (lack of awareness, lack of appropriate diagnostic equipment, lack of treatment facilities, relying on traditional solution and competition in the healthcare (Yip, 2009).

2.4.1 Prevalence of Breast Cancer in Malaysia

In Malaysia 50% of women are diagnosed with breast cancer before the age of 50 compared with the western countries, the percentage of women diagnosed before the age of 50 is 20%, it because the Malay women present at early stages earlier than the women in the west (Yip *et al.*, 2014). According to the National Cancer Registry (NCR) the incidents of breast cancer between 2003-2005 were high among the Chinese about 59.9 per 100 000, as for Indians and Malays the numbers are 54.2 per 100 000 and 34.9 per 100 000 respectively (Yip *et al.*, 2014). Azizah (2015) reported that statistics of breast cancer between 2007-2012 in Malaysia, among Chinese, the cumulative risk of breast cancer was high and low among Malay. The lifetime risk among the Malay was 1 in 35, Chinese was 1 in 22 and among Indians it was 1 in 24. (Figure 2.4.1) shows the standard age rate risk of breast cancer in Malaysia.

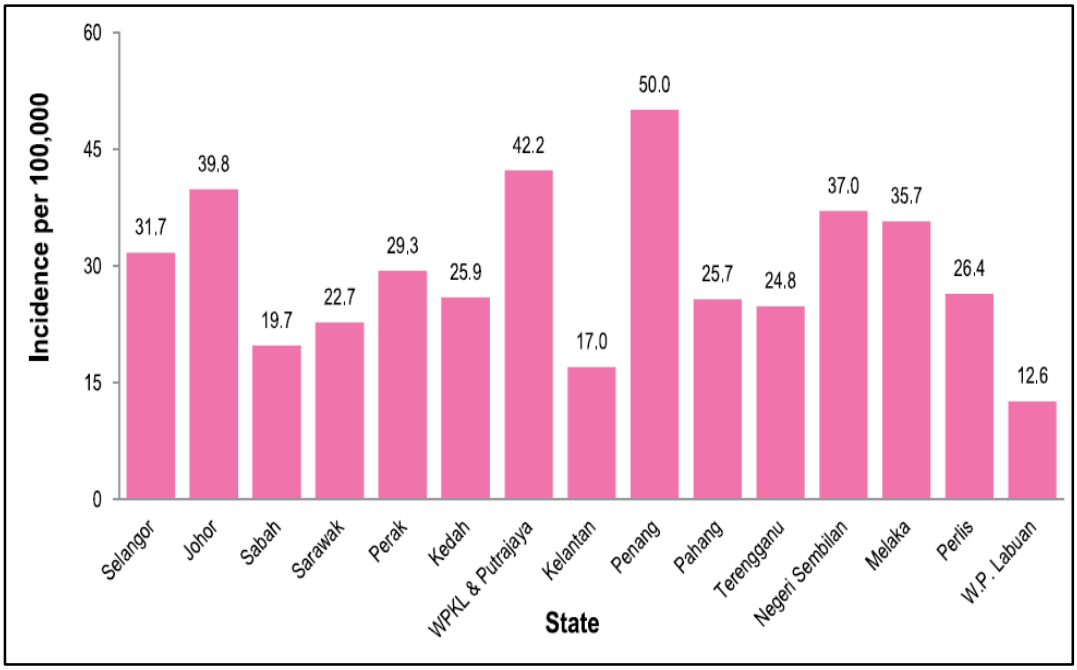

Figure 2.4.1: The Age-standardized rate (ASR) of breast cancer in the states of Malaysia (Azizah, 2015).

In 2012, The International Agency for Research in Cancer (GLOBOCAN) reported that the prevalence of breast cancer in Malaysia was 38.7 per 100,000 with 5410 new cases (Yip *et al.*, 2014).

Among the south-eastern Asia countries, Malaysia has the highest breast cancer mortality rate (18 per 100,000) (Nordin *et al.*). A population based study reported that Malaysia has a median of overall survival time of 68.1 months (Abdullah *et al.*, 2013).

2.5 Subtyping Breast Cancer by its receptor statues

2.5.1 Hormone Receptor-Positive Breast Cancer

Majority of breast cancer cases are hormone- receptor (HR) positive, which account about 80%. Hormone-receptor cancers tends to be slower course compared with TNBC and HER2 cancers, and it may express estrogen as well as progesterone (Peddi, 2018). ER/PR positive breast tumour means that breast cancer cell have estrogen and progesterone receptor, in which these receptors receive signals from estrogen and progesterone to start growing (Iqbal and Buch, 2016). ER/PR expression in patient's breast can be significant prediction and it benefits whether the patient will respond to endocrine therapy (ET) or not (Iqbal and Buch, 2016).

2.5.2 HER2-Positive Breast Cancer

Human epidermal growth factor receptor 2 (HER2) belongs to the family of human epidermal receptor proteins. HER2 is a transmembrane tyrosine kinase expressed in normal tissues of the breast, placenta, skin, urinary tract and the gastrointestinal (Zhu and Verma, 2015). HER2 is involved in the transduction of cell

signal, thus it interfere with proliferation, mortality and survival of the cells and it has no known ligands gastrointestinal (Zhu and Verma, 2015).

The percentage of breast cancer cases that shows positive amplification for HER2 is about 15%-20%, and has worse outcomes compared with the other breast cancer subtypes. The HER2 subtype is biologically aggressive and has short term reoccurrence and survival (Zhu and Verma 2015).

2.5.3 Triple-Negative Breast Cancer (TNBC)

A type of breast cancer that does not express estrogen, progesterone and HER2 receptors, hence the name Triple-Negative (Wahba and El-Hadaad, 2015). This type of cancer constitute approximately 10% to 20 % of total breast cancer cases (Brouckaert *et al.*, 2012). Profiling of gene expressions by microarray showed that approximately 75% to 80% of TNBCs are basal-like tumours (Badowska-Kozakiewicz and Budzik, 2016). TNBC is more severe than normal breast cancer because it has the worse prognosis with high risk of re-occurrence and death (Saraiva *et al.*, 2017). In fact, those who are treating from TNBC with standard taxane and/or anthracycline based chemotherapies have early relapse, and those who had been diagnosed with the disease within 1-3 years their condition may develop into visceral metastasis. The survival rate of patients with metastatic TNBC is low and it does not exceed 12 months even if they are responding to chemotherapy treatment (Diana *et al.*, 2018).

The standard treatment method for TNBC is chemotherapy despite the novel drug discoveries made twenty years ago (Diana *et al.*, 2018).

2.5.3.1 Classification of Triple-Negative Breast Cancer (TNBC)

The first classification of TNBC was reported by (Ahn *et al.*, 2016) using DNA microarray as a mean to detect the differences on gene expression. They grouped TNBC into five types: normal breast-like, basal-like, luminal A, luminal B and HER2-enriched. Some scientists classified TNBC into six subtypes: basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), immunomodulatory (IM), mesenchymal stem-like (MSL) and immunomodulatory (IM). Other scientist divided it into four subtypes: basal-like/immune activated (BLIA), basal-like/immune-suppressed (BLIS), mesenchymal (MES) and Luminal Androgen Receptor (LAR) (Lehmann *et al.*, 2011)

The terms TNBC and basal-like are used as synonymous in some occasions, although 75% to 80% of TNBCs are basal-like but it is important to note that not all TNBC cases are diagnosed as basal-like by gene expression profiling and not all basal-like cases are TNBC when diagnosed with IHC (Lehmann and Pietenpol, 2014).

2.5.3.1.1 Basal-Like Subtypes (BL1 and BL2)

According to Lehmann et al. basal-like 1 (BL 1) subtype enriched with pathways and has high content of cell-cycle genes which are important in the process of repairing DNA damages, leading to high expression levels of Ki-67 and cellular proliferation (Lehmann *et al.*, 2011). The second subtype (BL2) express high levels of signalling receptor tyrosine kinase, like insulin growth factor receptor (IGF1R), epithelial growth factor (EGF) and MET pathways plus cell signalling of gluconeogenesis and glycolysis (Diana *et al.*, 2018).

Recent sub-classification was done by (Burstein *et al.*, 2015) were he divided BL subtypes into two cluster BLIS 1 and BLIS 2. The BLIS 1 cluster displays downregulation in cellular immunity cells (T cells and B cells) as well as immune-regulating natural killer cells and cytokines pathways (Hubalek *et al.*, 2017). Due to its cellular immunity differentiation, cellular communication of innate and adoptive immunity and low expression of molecules that controls the antigen presentation, BLSI 1 has the worse prognosis. But its expression of transcription factors of the SOX family is uniquely present. The BLIS 2 cluster has characteristics similar to immunomodulatory subtype (IM) (Lehmann *et al.*, 2011)

Approximately all types of TNBC cell lines with known BRCA1 and BRCA 2 abnormalities have correlating patterns of gene expression with basal-like subtype (Stefansson *et al.*, 2009).

2.5.3.1.2 ImmunoModulatory (IM) Subtype

IM subtype has a high content of factors implicated in cellular immunity processes (cellular immunity signalling) (Hubalek *et al.*, 2017). IM immune signalling genes significantly correlates with a gene signature found in medullary breast cancer. IM subtype exhibits upregulation in genes that controls the cellular function of T, B, and natural killer cells, in contrast with BLIS. In terms of prognosis, IM has the best prognosis because it expresses high levels of STAT genes and displays STAT transcription factors activation (Hubalek *et al.*, 2017).

2.5.3.1.3 Mesenchymal Stem-Like (MSL) and Mesenchymal (M) subtypes

Both MSL and M subtypes have high content of pathways and components participates in cell motility, cellular differentiation and interaction of extracellular receptor. However, what makes MSL subtype uniquely different from M subtype are the processes and gene representing components which are linked to growth factor signalling pathways that include EGFR, inositol phosphate metabolism, PDGF, G-protein coupled receptor, calcium signaling, ERK1/2 signaling and adipocytokine signalling as well as ABC transporter (Hubalek *et al.*, 2017). Moreover (Burstein *et al.*, 2015) reported that MSL subtype express genes that are only found in adipocytes (ADIPOQ, PLIN1) and osteocytes (OGN), plus high expression of growth factors (IGF-1) is also expressed by this particular subtype.

2.5.3.1.4 Luminal Androgen Receptor (LAR) Subtype

LAR subtype differs from the rest of TNBC subtypes for being ER-negative despite the fact that its genes are enriched heavily with pathways regulated by hormones such as androgen/estrogen metabolism, steroid synthesis and porphyrin metabolism (Hubalek *et al.*, 2017). LAR tumours shows ER, AR, ErbB4 signalling and prolactin positive IHC staining but negative ERα. LAR expresses estrogen-regulated genes (GATA3, XBP1, PGR and FOXA) and ESR1 (ERα encoding gene) as shown by gene expression profiling report. Thus, ER activation is present in these ER-negative tumours (Burstein *et al.*, 2015). This is why these tumours are called ER-negative as defined by IHC staining because only 1% of these tumours express low levels of ER-protein (Burstein *et al.*, 2015). LAR subtype consist of AR-driven tumours because AR

is highly expressed at mRNA levels of LAR tumours, in fact, LAR tumours express AR 9 times greater than the other remaining subtypes of TNBC as shown by IHC staining. Plus LAR tumours express captivators and AR targets (Hubalek *et al.*, 2017).

2.6 Breast cancer risks and prediction

2.6.1 Age

With advancing in age the risk of developing breast cancer increases. Database acquired from the Surveillance, Epidemiology and End Result (SEER) shows that women in the united states developing cancer of the breast from birth to 39 years of age 1 in 8: 1 in 202, from 40-59 is 1 in 26, and 1 in 28 from 60-69 years (Siegel *et al.*, 2013)

2.6.2 Personal history

An important factor of risk getting breast cancer is personal history. Women with breast cancer has the chance of second reoccurrence of cancer in the same breast (ipsilateral or contralateral breast cancer (Geurts *et al.*, 2017). Methcronous contralateral breast cancer is considered a common cancer in amongst survivors (Curtis RE, 2006) other factors linked with reoccurrence of breast cancer such as young age, initial diagnosis of DCIS stage IIB, hormone receptor negative cancers (Buist *et al.*, 2010).

2.6.3 Family history

If there is a member in the family who has a history of breast cancer the chances of a women developing the disease are high. A study done by The Nurse Health, a mother diagnosed before she was 50 years of age her daughter had an adjusted risk of 1.69 and another mother diagnosed at age of 50 or older her daughter had a risk of 1.37 comparing with a woman with no family history of the disease. Between siblings an increase of 1.66 risk in women if she has a sister diagnosed with breast cancer before the age of 50 compared with patients who do not have family history (Colditz *et al.*, 2012). Increasing number of first-degree relatives with breast cancer diagnosed at a young age below 50 shows that women were associated with high risk of the disease. Those who have one, two or three affected relatives increases the chances by 1.80, 2.93 and 2.90 respectively compared with those who don't have affected relatives (Lancet, 2001).

2.6.4 Breast feeding

Breast feeding has protective effect against the development of breast cancer evidence suggests. It lowers the levels of endogenous sex hormones and delays the regular ovulatory cycles. The estimated reduction of breast cancer during breast feeding is 4.3% for each year (Lancet, 2002).

2.6.5 Breast pathology

Breast cancer is highly associated with proliferative breast disease. Proliferative diseases such as ductal hyperplasia, intraductal papillomas, sclerosing

adenosis and fibroadenomas without atypia confirm a small risk increase of developing breast cancer by approximately 1.5-2 times (Hartmann *et al.*, 2005). Ductal and lobular hyperplasia will increase the risk substantially. Proliferative disease with atypia will increase the risk by 4.3 (Dupont *et al.*, 1993).

2.6.6 Testosterone

High levels of sex hormone will increase the chances of breast cancer in both pre and postmenopausal women. In postmenopausal women high levels of circulating will increase the risk by 2.86-3.28 (Sieri *et al.*, 2009)

2.6.7 Age at menopause

Breast cancer is linked with delayed menopause. Every year of delay the risk increases by 3% and every 5 years the risk is 17% (Surakasula *et al.*, 2014).

2.6.8 Exposure to exogenous hormones

Studies have demonstrated the association of hormone replacement therapy (HRT) with breast cancer risk (Hou *et al.*, 2013). A meta-analysis international examination for breast cancer showed that women who are at menopause and did not use HRT, their risk factor increased by 1.028 each year as they grow older compared with HRT users (Lancet, 1997). A randomized control study done by the Woman's Health Initiative in which they used estrogen and progestin in postmenopausal women with healthy uterus increased the risk of the disease, delayed its detection a diagnosis plus increased the mortality. In difference, women with no uterus at postmenopausal

who used estrogen alone did not interfere with the detection of breast and significantly decreased the risk (Passarelli *et al.*, 2013). Other factors related to HRT such as duration and timing of the therapy are associated with breast cancer. Less than five years use of combined HRT is related to high risk whereas short term use of combined estrogen-progestin therapy has not shown any significant increase (RR = 1.023 per year) (Chlebowski *et al.*, 2013).

2.6.9 Genetics related to heredity

2.6.9.1 The BRCA genes and its mutations

The location of BRCA1 gene is on the chromosome number 17, while BRCA2 is located on the chromosome number 13 (Cavanagh and Rogers, 2015). The BRCA genes work by suppressing cellular growth and production of tumour suppresser gene proteins (TSG) (Mehrgou and Akouchekian, 2016). The number of amino acids found in BRCA1 is about 1863 with reported 300 mutations causing disease, while BRCA2 has 3418. (Mehrgou and Akouchekian, 2016). TSGs act as an anti-oncogene by repairing damaged DNA and ensure its genetic materials are well preserved, thus any problems with these proteins will eventually lead to unrepaired DNA strands leading to the occurrence of mutations (Mehrgou and Akouchekian 2016).

BRCA1/2 genes are autosomal dominant and highly expressed in breast and ovarian cancers (Ayub *et al.*, 2014). Any abnormalities or changes occur one or both BRCA genes increases the risk of developing prostate, ovarian and breast cancers (Mehrgou and Akouchekian, 2016). Mutations such as small deletion, small insertion, nonsense mutation, premature transcription mutation, missense mutation, and splicing troubles are considered to be the most common types of mutation that occur to the

BRCA genes (Mehrgou and Akouchekian, 2016). Splicing spots mutations can lead to the production of non-functional proteins, insertion and deletion mutations can produce frame shift (Neamatzadeh *et al.*, 2015).

2.6.9.2 The role of BRCA1/2 in the formation of cancer

Nearly 5-10% of breast cancer cases caused by hereditary and the reason behind it are the BRCA mutations in the western part of the world (Peshkin *et al.*, 2010). Meta-analysis study reported that BRAC1/2 mutations contribute to lifetime risk of devolving cancer of breast by a percentage of 40-57% and lifetime risk of developing cancer of ovary by a percentage of 18-40% (Chen and Parmigiani, 2007). Breast cancer patients who are diagnosed at the age of 25 years and carrying BRCA1/2 mutations have 50% risk of devolving contralateral breast cancer (Peshkin *et al.*, 2010). Approximately 80% of BRCA1 mutation are associated with TNBC (Turner and Tutt, 2012).

BRCA1 gene encodes a protein responsible for repairing damaged DNA (Godet and Gilkes, 2017). BRCA1 protein repairs damaged DNA through several cellular processes including the creation of heterochromatin on the chromosome X, regulating the transcription of genes associated with DNA repair, attaching ubiquitin with other proteins through a process called ubiquitination and repairing broken double stranded DNA (Godet and Gilkes, 2017). During DNA damage and cell cycle responses, BRCA1 binds with RAD51, TP53 and BRCA2 (Godet and Gilkes, 2017). Non-functional BRCA1 protein can force cells not to undergo cell cycle arrest at the G2 phase following damaged DNA and can cause deficit in transcription-coupled repair (Godet and Gilkes, 2017). Worth noting that BRCA1 allows proteins that repair

damaged DNA to the damage sites by interacting with γH2AX (Godet and Gilkes 2017).

BRCA2 gene responsible for encoding protein which has the same function as BRCA1 protein (Petrucelli *et al.*, 2010). Cells that lacks BRCA2 can cause unexpected abnormalities in the chromosomes (ri-radials, quadri-radials, and double-stranded) and shortfall in the separation of chromosomes (Godet and Gilkes 2017).

2.6.10 Radiation

Exposing radiation increase the chances of cancer in general. Various sources of radiation such as medical treatment or nuclear explosion elevates the risk of breast cancer. Radiation of chest wall to treat cancer in children increase the risk depending on the dose of radiation (Henderson *et al.*, 2010). Children who are receiving treatment for Hodgkin's disease develop the risk of getting breast cancer by (RR = 7) (Henderson *et al.*, 2010). Nuclear radiation incidents happened in japan during World War 2 led to the development of breast in Japanese women (Katayama and Narimatsu, 2016) at age younger than 35 years. In Belarus and Ukraine the Chernobyl reactor accidents led to the increased incidents of breast cancer especially in young women (Pukkala *et al.*, 2006).

2.7 Screening

2.7.1 Clinical breast examination and breast self-examination

Breast self-examination (BSE) is a questioned method because it did not demonstrate any benefits of mortality decreasing (Kosters and Gotzsche, 2003), but most clinician urge women to examine their breast monthly through breast self-

examination (BSE) so that they will be aware of their normal breast (McCready T, 2005). As for clinical breast examination (CBE), the NCCN recommend women who are younger than 40 years of age to perform both examinations (BSE and CBE) annually (Bevers *et al.*, 2009).

2.7.2 Mammography

In the 1960s the technique exhibited good results in terms of mortality decrease. The first randomized control trials comparing between clinical examination and mammography screening was done during that time. The results favored mammography screening and showed a decrease in mortality in one third of the experimented group. However, in breast cancer women aged between 40-49 years there is still debate concerning mortality in that particular subset (Shapiro *et al.*, 1985). In women aged 40 to 70 years, a contemporary randomized control trials have showed mammography screening benefits (Nelson *et al.*, 2009). A review done by the Cochrane in 2013 suggest that there is undue stress and uncertainty in the face of false positive due to routine mammography with increase in lumpectomies and mastectomies but no decrease in mortality (Gotzsche and Jorgensen, 2013). In screening for the disease, overestimation of survival time among screening-detected cases in comparison with clinically detected cases when the true survival time remains unchanged (Lead time) and overestimation of screening-detected cases which is cause by the slow progressed cases may never be clinically relevant (length time) are problems associated with mammography in which it raises controversial towards using this technique.

2.7.3 Magnetic resonance imaging

MRI plays a significant role in diagnosing, assessing and managing breast cancer in women. It has high sensitivity of 0.77-0.79 compared to sensitivity of mammography and less specific than mammography with 0.86-0.89 specificity while mammography has as specificity of 0.95 in cancer detection (Warner *et al.,* 2008). Systemic review to measure the sensitivity and specificity of both MRI and mammography, both showed a combined sensitivity and specificity of 0.94 and 0.77 respectively (Warner *et al.,* 2008). NCCN advice patients with increasing breast cancer developing risk (> 20%) to perform yearly MRI and mammography checkup starting at the age of 25. Patients who underwent breast augmentation and the mammography imaging is unclear, MRI is the best option for examination or for those who have equivocal findings on other imaging modalities.

2.7.4 Ultrasound

Several studies suggested that patients with high risk of disease and also have dense breast tissue, the use of ultrasound screening can give false positive results (Berg *et al.,* 2008). Clinicians who suspect in a certain case use ultrasound screening as a replacement to traditional method (mammography) especially in cases with dense breast tissue because the use of mammography in these cases gives low sensitivity (Kelly *et al.,* 2010). Studies have shown that the use of ultrasound followed by mammography to detect progressive breast cancer gives slight benefits in patients with average risk (Gartlehner *et al.,* 2013).

2.8 Diagnosis of breast cancer

2.8.1 Physical and history examination

The physical examination of the patient's breast like changes in the asymmetry of the nipples, the appearance of dimpling, erythema and peaud' orange and the formation of mass in the breast are taken into consideration as it indicates the formation of breast cancer

Clinical history focuses on the assessment of cancer risk and the absence and presence of breast cancer symptoms. The clinical history includes information about the suspected patients such as menopausal statues, previous pregnancies, age at menarche, oral contraceptive usage and family history of breast cancer

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2.8.2 Diagnosis through imaging

2.8.2.1 Mammography

In terms of breast cancer detection, mammography still the gold standard method in the diagnosis of breast cancer. Women who have palpable mass or breast cancer symptoms, diagnostic mammograms are used (Drukteinis *et al.*, 2013). According to The breast imaging reporting and database system (BI-RADS) malignant masses are described as oval, round, irregular or lobular while benign masses are different in terms of size (larger), lucent centre and round(Smetherman, 2013).

2.8.2.2 Magnetic resonance imaging (MRI)

MRI plays a critical role in diagnosis and management of breast cancer (Radhakrishna *et al.*, 2018). MRI is used when there are limitations in using

mammography especially in patients underwent breast augmentation (saline and silicone implants) or silicone injections (Ojeda-Fournier and Comstock, 2009). Other limitations such as knowing the disease extent from first diagnosis (muscular invasion by the disease), screening of asymptomatic patients with high risk of breast cancer and inclusive findings evaluation (Kilic *et al.*, 2012).

2.8.2.3 Ultrasound

Ultrasound examination for the breast could be done when there are certain cases like unavailability of MRI, axillary lymphadenopathy suspicion, clinical abnormality at the age of 40 (pregnant or lactating women), retraction of the skin newly inverted nipple or breast implants affecting MRI (Evans *et al.*, 2018).

2.8.3 Diagnosis through prognostic indicators

2.8.3.1 Statues of progesterone and estrogen receptors

ER and PR tests must be performed on all cases of invasive breast carcinoma (Duffy *et al.*, 2017). The assessment of ER and PR is achieved through staining of tissues using immunohistochemistry (IHC) technique (Sinn *et al.*, 2017). IHC identifies *in situ* or invasive cancer (Zaha, 2014). In order to achieve positive results, 1% of cancer cells are required. While negative results are interpreted when there is less than 1% of cancer cells in the tissue (Hammond *et al.*, 2010).

2.8.3.2 Assessment of HER2 protein expression and gene amplification

Generally, HER2 protein expression is evaluated using IHC staining (Hicks and Schiffhauer, 2011). However, if the result of IHC is ambiguous another method compensate IHC assay which is Fluorescence *in situ* hybridization (FISH) assay (Zoppoli *et al.*, 2017). HER2 is an indicator for both node-positive and node-negative