MODULATION OF TAMOXIFEN EFFECTS ON ERα-POSITIVE AND ERα-NEGATIVE BREAST CANCER CELLS BY TUALANG HONEY

by

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cm^2	centimeter squared
Gy	gray
h	hour
kDa	kilodalton
kg	kilogram
L	litre
MV	megavoltage
mA	miliamphere
mg	miligram
min	minute
ml	mililitre
nm	nanometre
ng	nanogram
°C	degree celcius
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre
V	voltage
$\Delta\psi m$	mitochondrial membrane potential
AIF	apoptosis-inducing factor
ATCC	American Type Cell Culture
ATP	adenosine triphosphate
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia Rad3-related protein
BCT	breast conservation therapy
BCS+RT	breast conservation surgery and radiotherapy
CDC	cell division cycle
CDK	cyclin-dependent kinases
CEA	carcinoembryonic antigen
CIP/KIP	CDK interacting protein/Kinase inhibitory protein
CKI	cyclin-dependent kinases inhibitors
CMF	cyclophosphamide, methotrexate and 5-fluorouracil
CO_2	carbon dioxide
CT	computerised tomography
Ct	threshold cycle
Ca^{2+}	ion calcium
cDNA	complementary dioxyribonucleic acid
cyto c	cytochrome c
DCIS	ductal carcinoma in situ
DISC	death-inducing signalling complex
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DR5	death receptor 5
EC_{50}	half maximal effective concentration
EGFR	epidermal growth factor receptor
ER	estrogen receptor

ERE	estrogen response elements
FITC	fluorescein isothiocyanate
FLICA	fluorescent labeled inhibitor of caspases
HSP90	heat shock protein 90
H_2O_2	hydrogen peroxide
IHC	immunohistochemical
IAP	the inhibitor of apoptotic protein
ICAD	inhibitor of the caspase-activated DNase
IC_{50}	half maximal inhibitory concentration
IFN-γ	interferon-gamma
IL-18	interleukin-18
IgG	immunoglobulin G
LFS	Li-Fraumeni syndrome
LCIS	lobular carcinoma in situ
LDH	lactate dehydrogenase
MADD	MAP-kinase activating death domain
MAPK	mitogen-activated protein kinase
MRI	magnetic resonance imaging
MRP-1	multidrug resistance-associated protein 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	messenger ribonucliec acid
mdm-2	mouse double minute 2 homolog
NAD^+	nicotinamide adenine dinucleotide
NCE	new chemical entities
NF-κβ	nuclear factor kappa light chain enhancer of activated B cells
NSCLC	non-small cell lung cancer
PAK2	p21-activated kinase 2
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
PR	progesterone receptor
PS	phosphatidylserine
PSA	prostate specific antigen
Pgp	P-glycoprotein
RAIDD	RIP-associated ICH-1/CED-3-homologous protein with death domain
RIP	receptor-interacting serine/threonine-protein kinase 1
ROS	reactive oxygen species
Rb	retinablastoma
rpm	revolutions per minute
SERMs	selective ER modulators
Smac/ DIABLO	second mitochondria-derived activator of caspases
TAM	tamoxifen
TH	*Tualang* honey
TRAIL	TNF-related apoptosis-inducing ligand
WHO	World Health Organization

MODULASI KESAN TAMOKSIFEN TERHADAP SEL-SEL ERα-POSITIF DAN ERα-NEGATIF KANSER PAYUDARA OLEH MADU TUALANG

ABSTRAK

Tamoksifen (TAM) adalah drug anti-estrogen yang biasa digunakan untuk merawat kanser payudara hormon reseptor-positif. Walau bagaimanapun, kesan sampingan dan kerintangan sel kanser terhadap TAM mengehadkan keberkesanan klinikalnya. Oleh itu, banyak kajian telah dijalankan terhadap kombinasi rawatan TAM dengan ejen atau sebatian baru untuk mencapai objektif rawatan dengan kesan sampingan yang minimum. Untuk itu dan berdasarkan peningkatan laporan mengenai kegunaan perubatannya, madu Tualang (TH) telah dipilih untuk kajian ini. Kajian terkini bertujuan untuk menilai peranan TH dalam memodulasi kesan TAM and laluan pengisyaratan kematian di dalam sel-sel kanser payudara, MCF-7 (ERα-positif) dan MDA-MB-231 (ERα-negatif) beserta sel-sel epitelial payudara bukan malignan, MCF10A. Pada awalnya, kesemua sel dirawat dengan TH untuk menentukan kesan sitotoksik dan mengenalpasti mekanisme jenis kematian sel. Bagi analisis seterusnya, sel-sel dirawat dengan TH, TAM dan kombinasi TH+TAM untuk menentukan aktiviti apoptotik, perubahan dalam potensi membran mitokondria (Δμm), pengaktifan *caspase* dan pengawalaturan kitaran sel. Kesan TH, TAM dan TH+TAM di dalam sel-sel MCF-7 dan MDA-MB-231 juga ditentukan pada peringkat gen dan protein masing-masing menggunakan sistem PCR Array dan pemblotan Western. Keputusan menunjukkan bahawa TH adalah sitotoksik dan mengaruh apoptosis di dalam sel-sel MCF-7 dan MDA-MB-231 tetapi bukan pada sel-sel MCF10A. TH memodulasi aktiviti TAM dengan menggalakkan apoptosis peringkat akhir dan

menyebabkan kesan pemberhentian kitaran sel aruhan TAM yang berbeza di dalam sel-sel MCF-7 dan MDA-MB-231. Data PCR Array menunjukkan peningkatan dan penurunan pengawalaturan beberapa gen apoptotik yang jelas di dalam sel-sel MCF-7 termasuk p53 (~1300-kali ganda), GADD45A (~120-kali ganda), LTBR (~790-kali ganda) dan BCL-2 (~-180-kali ganda), tetapi bukan di dalam sel-sel MDA-MB-231. Kenaikan ekspresi protein FADD, p21 dan p53 di dalam sel-sel MCF-7 menunjukkan aktiviti apoptotik aruhan TH diperantara oleh laluan pengisyaratan reseptor kematian dan p21 bersandarkan p53. Kenaikan ekspresi protein p21 tanpa peningkatan paras p53 di dalam sel-sel MDA-MB-231 mencadangkan aktiviti apoptotik aruhan TH adalah tidak bersandarkan p53. Kebolehan TH untuk memodulasi aktiviti apoptotik TAM di dalam sel-sel kanser payudara ERα-positif (MCF-7) dan ERα-negatif (MDA-MB-231) adalah indikasi terhadap potentsi TH sebagai ejen/adjuvan antikanser di dalam rawatan kanser payudara.

MODULATION OF TAMOXIFEN EFFECTS ON ERα-POSITIVE AND ERα-NEGATIVE BREAST CANCER CELLS BY TUALANG HONEY

ABSTRACT

Tamoxifen (TAM) is an anti-estrogen drug commonly used for treatment of hormone receptor-positive breast cancer. However, the side effects and cancer cell resistance to TAM limits its clinical efficacy. Therefore, many studies have been conducted to focus on the combination treatment of TAM with new agents or compounds to achieve the treatment objective with minimal side effects. For these reasons and based on the increasing reports of its medicinal use, *Tualang* honey (TH) was selected in this study. The current study aimed to evaluate the role of TH in modulating TAM effects and its death signalling pathways in human breast cancer cells, MCF-7 (ERα-positive) and MDA-MB-231 (ERα-negative) as well as in non-malignant breast epithelial cells, MCF10A. Initially, all cells were treated with TH to determine its cytotoxic effects and to identify the type of cell death mechanism. For further analyses, the cells were treated with TH, TAM and the combination of TH+TAM for determination of apoptotic activity, alteration in mitochondrial membrane potential ($\Delta\psi m$), caspase activation and cell cycle regulation. The effects of TH, TAM and TH+TAM in MCF-7 and MDA-MB-231 cells were also determined at the gene and protein levels by PCR Array system and Western blotting, respectively. The results showed that TH was cytotoxic and induced apoptosis of MCF-7 and MDA-MB-231 cells but not MCF10A cells. TH modulates TAM activities by promoting late apoptosis and differentially affects TAM-induced cell cycle arrest in MCF-7 and MDA-MB-231 cells. PCR Array data showed remarkable up- and down-regulation of several apoptotic genes in MCF-7 cells

including p53 (~1300-fold), GADD45A (~120-fold), LTBR (~790-fold) and BCL-2 (~-180-fold), but not in MDA-MB-231 cells. The induction of FADD, p53 and p21 protein expression in MCF-7 cells showed that TH-induced apoptotic activities are mediated by death receptor and p53-dependent p21 signalling pathways. Increased expression of p21 without elevated level of p53 in MDA-MB-231 cells suggests that the TH induced-apoptotic activity is p53-independent. The ability of TH to modulate TAM apoptotic activities in ERα-positive (MCF-7) and ERα-negative (MDA-MB-231) cells indicates the potential of TH as anticancer agent/adjuvant in breast cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer cells or malignant neoplasms can be defined as abnormal cells with uncontrolled proliferation. Cancer cells invade and populate tissues that are usually reserved for other cells. Cancer is classified according to the cell types and the origin of the tissues. For example, carcinomas are cancers from epithelial cells and sarcomas are cancer from muscle and connective tissue (Alberts *et al.*, 2007). There are many factors that could lead to the formation of cancer including environment causes, genetic constitution and life-styles. A person who smokes cigarettes has a higher potential of developing cancer compared to non-smokers. Carcinogens in a cigarette's tobacco have been identified as the main cause of lung cancer (~90%) and other cancers such as those of the esophagus, stomach, pancreas, larynx, lung, bladder, kidney and in leukaemia (Boyle, 1997).

1.1.1 Cancer incidence in Malaysia

Cancer is the second leading cause of death in developing countries. In 2008, cancer cases worldwide is estimated to be about 12.7 million with 7.6 million deaths of which 64 % of deaths occurring in developing countries (Garcia *et al.*, 2007). The increasing number of cancer cases in developing countries such as Malaysia are associated with unhealthy lifestyle including smoking and imbalanced diets (Jemal *et al.*, 2011).

National Cancer Registry reported, about 18,219 new cancer cases were diagnosed and registered in 2007. Cancers represent the third cause of death in Malaysia after heart disease and septicaemia. The ten leading cancers in Malaysian include those of the breast, colorectal, lung, nasopharynx, cervix, lymphoma, leukaemia, ovary, stomach and liver (Table 1). Breast cancer is the most common cancer among Malaysian female with 3,242 cases diagnosed and reported to the National Cancer Registry in 2007. This represented 18.1% of all cancer cases in Malaysia (Zainal Arifin and Nor Saleha, 2011).

Ageing, growth of the world populations and increase in cancer-causing behaviours (e.g smoking) result in increasing number of cancer cases. World Health Organization (WHO) predicted that the number of cancer cases will increase to 11.8 million in 2030. This global burden of cancer could be prevented through application of cancer knowledge including vaccination for liver and cervical cancer, anti-smoking campaign, early detection and treatment, as well as healthy lifestyle campaigns (Lim, 2002; Jemal *et al.*, 2011).

Table 1.1 Ten most frequent cancers in Malaysia.

No	Sites	Case	Percentage (%)
1	Breast	3292	18.1
2	Colorectal	2246	12.3
3	Trachea, bronchus, lung	1865	10.2
4	Nasopharynx	940	5.2
5	Cervix	847	4.6
6	Lymphoma	776	4.3
7	Leukaemia	741	4.1
8	Ovary	656	3.6
9	Stomach	630	3.5
10	Liver	605	3.3

(Extracted from Zainal Arifin and Nor Saleha, 2011)

1.1.2 Diagnosis of cancer

Cancer can be diagnosed through several ways such as the detection of biochemical markers (Voorzanger-Rousselot and Garnero, 2007). Biochemical markers are molecules that can be detected in the blood, urine or tissues. These markers are produced either by the metastasized tissues, surrounding normal tissues or by the tumor itself.

DNA, mRNA, antigens and hormones are biochemical markers that can be measured quantitatively or qualitatively using immunohistochemical (IHC) test, polymerase chain reaction (PCR), Western blotting and microarrays. Tumor marker assays confer several benefits in cancer diagnosis; not only to identify the disease, but to determine the severity of the disease. However, most of tumor markers are not specific to a particular cancer because such markers are also present in elevated levels in benign conditions (Voorzanger-Rousselot and Garnero, 2007).

There are many tumor markers that can be detected in patients. In the HUSM Chemical Pathology Laboratory, three markers are normally selected for cancer diagnosis. The markers are carcinoembryonic antigen (CEA), prostate specific antigen (PSA) and CA 125. These markers are detected in human sera using immunoassay method. CEA is a tumor antigen that can be found in elevated levels in tumors and in the developing fetus. CEA is a glycoprotein (molecular weight of 180 kDa) that is metabolized by the liver. Thus, abnormal level of CEA in serum can be sign of liver disease (Maestranzi *et al.*, 1998). The level of CEA is also high in colon,

breast, lung, pancreas, stomach and ovarian cancers. However, the presence of high levels of CEA can also be detected in benign condition including bronchitis, cirrhosis and hepatic diseases indicating that CEA test is not specific for cancer diagnosis. Therefore, other pathological tests are required to confirm the cancers (Maestranzi *et al.*, 1998; Hammarstrom, 1999). In contrast to CEA test, PSA and CA 125 tests are more specific. PSA and CA 125 are found in high levels in prostate and ovarian cancers compared to other cancer sites (Catalona *et al.*, 1994; Suh *et al.*, 2010).

Besides screening for tumor biomarkers, other methods are also used to diagnose cancers such as imaging and surgery. Imaging can provide information such as the stage of cancer. For example, computerised tomography (CT) not only diagnoses the presence of non-small cell lung cancer (NSCLC) but also provides information to guide its intervention. CT and magnetic resonance imaging (MRI) detect the abnormality of node sizes, status of metastases and anatomical information of lung cancer (Rankin, 2004).

Cancer diagnosis enables early detection of cancer, especially before any evident symptoms. By detecting tissue abnormalities at the early stage, the presence of malignant tissues could be eliminated and controlled.

1.2 Breast cancer

Breast cancer remains the most common cancer among females in the world comprising about 14 % of all cancer deaths (Jemal *et al.*, 2011). In 2007, approximately 32.1 % of breast cancer cases were diagnosed in Malaysian females and more than 40 % cases were presented at advanced stages (stage III and IV). Breast cancer rates were increased in Malaysian females within the age of 45 to 60 per 100,000 populations (Zainal Arifin and Nor Saleha, 2011). Females within this age group are recommended to perform breast cancer screening including breast self-examination and mammography as an early detection of breast cancer. Mammography screening could reduce breast cancer mortality rates by up to 30 % in women over 50. However, for young women (below 50 years old), mammography screening could result in false-positive diagnosis result which may lead to unnecessary biopsy (Kollias *et al.*, 1998).

Breast cancer risks are primarily due to environmental and hereditary factors. Environmental risk factors include reproductivity status, general lifestyle, age, weight, age at the birth of the first child and alcohol intake (Bland, 1987; Tavani *et al.*, 1999). Tavani *et al.* (1999) reported that breast cancer cases in women under the age of 40 were highly related to abnormal body mass index ($\geq 30 kg/m^2$), age at first menstrual cycle and age at birth of first child.

Family history is the most common risk factor for breast cancer in young women. This is because the inherited genes of breast cancer are mostly found in young

women (Kollias *et al.*, 1998). In a study, where 1371 asymptomatic women with family history of breast cancer were screened, 29 patients were detected with invasive and *in situ* breast cancer (Kollias *et al.*, 1998).

Studies on breast cancer led to the discovery of many susceptible genes such as BRCA1, BRCA2, ataxia telangiectasia (ATM and ATR) and p53 (Ford and Easton, 1995). BRCA1 and BRCA2 genes are responsible for familial breast cancer in young women (Rebbeck *et al.*, 1996). BRCA1 gene located at chromosome 17q, was identified in 1994 (Mak and Yeh, 2002). Mutations of BRCA1 gene are responsible for approximately 40-45 % of hereditary breast cancers. However, as the BRCA1 gene is rarely mutated, this gene accounts for only 2-3% of all breast cancers (Rosen *et al.*, 2003). BRCA1 gene has multiple functions including regulation of cell cycle via DNA damage-related cell cycle checkpoints, maintenance of genome stability, DNA repair, and apoptosis (Ford and Easton, 1995).

Another breast cancer susceptible gene is known as BRCA2 (Duncan *et al.*, 1998). This gene accounts for 35-40 % of hereditary breast cancer cases. BRCA2 gene is located at chromosome 13q. The function of BRCA2 gene is closely related to BRCA1 gene. In addition, the expression of both genes is co-regulated during cell cycle progression and in response to DNA damage. Some studies have suggested that BRCA1 and BRCA2 have overlapping functions in DNA repair and their functions in molecular pathways are quite similar (Rosen *et al.*, 2003). In contrast to BRCA1, the mutation of BRCA2 gene is commonly associated with breast cancer in men (Couch *et al.*, 1996). Ford and colleagues (1994) have demonstrated that mutations

occur in both BRCA1 and BRCA2 genes could increase the risk of hereditary prostate cancer but are rarely found in sporadic prostate cancer.

Besides BRCA1 and BRCA2 genes, other susceptibility genes have been identified to causing 20 % of breast cancer cases. For example, mutations in the p53 gene was lead to increased risk of Li-Fraumeni syndrome (LFS) which is characterised by early onset sarcomas, premenopausal breast cancers and other cancers (Li *et al.*, 1988). Approximately 50 % of LFS families were identified with germline mutations in p53 gene and it was estimated that at least 50 % of carriers would have breast cancer risk by the age of 50 (Li *et al.*, 1988).

The overexpression of epidermal growth factor receptor families such as EGFR/HER1 and HER2/*neu* also lead to the breast cancer cases. HER2/*neu*, a 185 kDa protein contributes for approximately 20-40 % of breast cancer. The activated protein of HER2/neu will interact with mitogen-activated protein kinase (MAPK) and other signal transduction pathways to increase the oncogenic potential (Hibshoosh and Mansuklani, 2001).

1.2.1 Anatomy and types of breast cancer

Breast is a special organ that can produce milk for lactation. The component of breast is shown in figure 1.1 which consists of ducts, lobules and other important parts. There are 15-20 ducts and 10-15 lobes/lobules of breasts that are formed

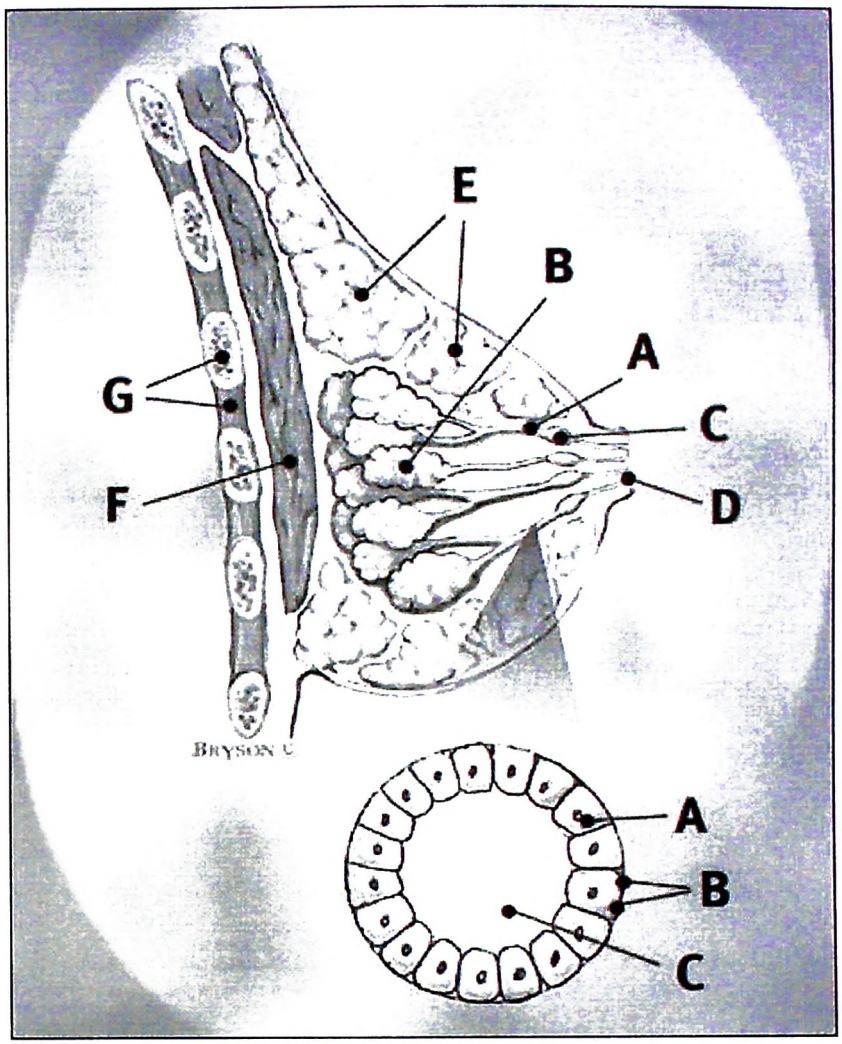

Figure 1.1 The anatomy of a breast.

The anatomy of a breast was illustrated above: A-ducts, B-lobules, C-dilated section of duct to hold milk, D-nipple, E-fat, F-pectoralis major muscles and G-chest wall. A duct section was enlarged and labelled as: A-normal duct cells, B-basement membrane and C-center of duct.

(Extracted from website: http://www.breastcancer.org/pictures/breast_anatomy/image_1)

during puberty. The milk is produced in the lobules which are connected to ducts for milk secretions. Most of the breast cancers arise from the cells of the lobules and ducts (Farrar *et al.*, 1995).

Breast malignancies can be divided into epithelial and non-epithelial lesions. Non-epithelial breast malignancies account for less than 1 % which includes sarcomas, primary lymphomas and haematological malignancies. Non-epithelial breast malignancies show similar demographic and clinical features to the epithelial breast cancer but the prognosis and therapeutic options are dissimilar (Chugh and Baker, 2004).

The epithelial breast malignancies can either be non-invasive or invasive. There are two histological types of non-invasive cancer which are ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). Both non-invasive types remain within the milk ducts or lobules of breasts. Both carcinomas arise from atypical proliferation of cells and do not invade normal tissues, thus they cannot cause serious morbidity unless they became invasive (Goldschmidt, 2000).

The most common breast cancers are the invasive type. The progression of cancer within the ducts from epithelial hyperplasia to atypical hyperplasia can cause non-invasive carcinoma which subsequently becomes invasive (Donegan, 1995). The invasive ductal carcinoma accounts for 85-90 % of invasive breast cancer cases (Goldschmidt, 2000).

1.2.2 Stages of the breast cancer

Breast cancer can be classified either as invasive or non-invasive which is dependent on the size of tumor (T), number of lymph nodes involved (N) and metastasis status (M) (Donegan, 1995). Currently the TNM classification is used worldwide for breast cancer classification.

The stages of breast cancer are expressed in the Roman numerals starting from stage I (the least advanced stage), stage II, stage III and to stage IV (the most advanced stage). Stage 0 is considered as non-invasive or in situ cancer stage. The stages of breast cancer are listed in Table 1.2.

Stage I to stage IV breast cancers are considered invasive as the tumor cells invade into the neighboring cells and are palpable. In addition, the existence of tumor cells in axillary lymph nodes including internal mammary lymph nodes is known as the advanced stages (stage II to stage IV). Stage II and III breast cancer patients are presented with various sizes of tumor and multiple lymph nodes are attacked, thus these stages are divided into several categories. Stage IV breast cancer is the most advanced stage characterized by distant metastasis of cancer cells. At this stage, the cancer cells spreads to other parts of the body including the bones, lungs, liver or brain (Donegan, 1995).

Table 1.2 Breast cancer stages

Stage	Tumor size (T)	Nodes involvement (N)	Distant metastasis (M)
0	DCIS; cancer cells grow in a duct and do not invade into the surrounding tissues	Not spread	None
I	Tumor size ≤ 2 cm	Not spread	None
II	- < 2cm	Spread to 1 to 3 axillary lymph nodes (with <2 mm across)	None
	- between 2 to 5 cm	Either localized or spread to 1 to 3 axillary lymph nodes (with <2 mm across)	None
	- > 5 cm	Not spread	None
III	- < 5cm or none	Spread to 4 to 9 axillary lymph nodes	None
	- > 5 cm	Spread to 1 to 9 axillary lymph nodes	None
	- any size (tumor has grown into the chest wall or skin	Either localized or spread to axillary lymph nodes	None
	- any size	Spread to 10 or more axillary lymph nodes and internal mammary lymph nodes including clavicular bone	None
IV	-any size	Either localized or spread to nearby lymph nodes	Distant metastasis present

The stages of breast cancer according to the TNM classification (Adapted from http://www.cancer.org/acs/groups/cid/documents/webcontent/003090-pdf.pdf and Donegan, 1995).

1.2.3 Breast cancer management

The possibilities of developing breast cancer among relatives of breast cancer patients require accurate and effective management. Based on the number of breast cancer patients in a family, the age at which breast cancer was first diagnosed and hormone levels, a clinician would predict the individual's risk of developing inherited breast cancer (Mackay and Ponder, 2001). Mammography screening programme is used to diagnose cancer for female at age 50 years and above. Interestingly, mammogram screening has reduced breast cancer mortality up to 30 %. Besides mammography, genetic testing is also used to identify the two important breast cancer susceptible genes, BRCA1 and BRCA2. Mutations of these genes may indicate the possibilities of developing breast cancer. However, the absence of mutation does not necessarily indicate a person is free from cancer (Mackay and Ponder, 2001).

Once breast cancer is diagnosed and localized, the cancer patient has to undergo treatment. The treatment of breast cancer depends on the grade and the site of tumor. There are several choices of breast cancer treatments including surgery, radiotherapy, chemotherapy and hormone therapy (Cristofanilli *et al.*, 2000).

1.2.3.1 Surgery

Surgery of the breast is the primary choice of breast cancer treatment and has been used for many years. The procedures include mastectomy (removal of the whole breast) and lumpectomy (removal of regional site). Lumpectomy is part of a breast conservation therapy (BCT) and has replaced mastectomy as a preferable treatment in early stage breast cancer (Spratt and Donegan, 1995).

1.2.3.2 Radiotherapy

Radiotherapy becomes an important choice of breast cancer treatment after surgery. Breast conservation surgery followed by whole breast irradiation (BCS+RT) is commonly used in women at early stage of breast cancer (stage 0, I and II). Radiation is generally performed with megavoltage photon energy of 4 to 10 MV to deliver 45 to 50.4 Gy to the whole breast at 180-200 cGy per fraction. Radiotherapy confers favourable cosmetic outcomes with minimal long-term complication. However, due to the lack of facilities and to avoid long term treatment, many patients prefer either mastectomy or lumpectomy alone compare to BCS+RT (Goyal *et al.*, 2010). Radiotherapy can be used to treat metastatic cancer but the physician has to consider the patient's treatment history. The patients who previously had bone marrow treatment are discouraged to undergo radiotherapy. This is because large dose of radiation may destroy the normal cells as well as abnormal cells in the bone marrow (McCormick, 1995).

1.2.3.3 Chemotherapy

Chemotherapy using cytotoxic anti-neoplastic drugs can be performed either alone or in combination with other drugs/agents. The combination treatment is more effective than single drug therapy especially for women with hormone receptor-positive breast cancer (Lopez *et al.*, 2008). Adjuvant therapies that are currently used in clinical practice are; cyclophosphamide, methotrexate and 5-fluorouracil (CMF). These adjuvants have resulted in successful outcome such as cancer-free survival rate (Murphy *et al.*, 2010). Besides CMF, other polychemotherapy regimens have been developed including antracycline- (doxorubin and epirubicin) and taxane- (paclitaxel and docetaxel) based. Antracyclines and taxanes based drugs have successfully reduced the annual risk of death compared to conventional CMF and have become the most active agents in breast cancer treatment (Murphy *et al.*, 2010).

Despite the benefit of chemotherapy, the presence of undesirable side effects is worrying. Chemotherapeutic drugs not only can kill cancer cells but also result in deaths of normal cells. Furthermore, it has been reported that chemotherapeutic drugs could increase the expression of xenobiotic efflux pumps that are associated in modulating breast cancer chemotherapy responses. Xenobiotic transporters, including P-glycoprotein (Pgp) and multidrug resistance-associated protein 1 (MRP1) are transmembrane efflux pumps that are responsible for removal of any potential damaging molecules from the intracellular environment. Unfortunately, the ability of these transporters to efflux the chemotherapeutic drugs results in drug therapy resistance in many cancers (Kim *et al.*, 2013). Thus, new agents with low

susceptibility to common drug resistance are required to improve the response rate and potentially extend survival.

1.2.3.4 Hormone therapy

Most patients with invasive breast cancer present with hormone receptor-positive cancer and they are normally offered adjuvant endocrine/hormonal therapy. The agent that is commonly used in endocrine therapy in both pre- and postmenopausal women is tamoxifen (TAM). TAM is a non-steroidal triphenylethylene derivative that is metabolized to 4-hydroxy-TAM (OHT). This metabolite has high affinity towards estrogen receptor (ER) but the mechanism of action is different from estrogen as it exhibits anti-estrogenic and anti-neoplastic activities (Cianfrocca, 2010).

Besides TAM, aromatase inhibitors (e.g aminoglutethimide) are used in endocrine therapy especially in postmenopausal women (Bonadonna *et al.*, 2001). Aromatase inhibitors prevent estrogen biosynthesis by inhibiting aromatase enzymes that catalyse the conversion of adrenal androgen to estrogen. Estrogen plays an important role in breast cancer progression. In postmenopausal women, the increased risk of breast cancer associates with high level of estrogen. Thus, the presence of aromatase inhibitors reduces estrogen production and significantly affects the progression of hormone-responsive breast cancers (Bonadonna *et al.*, 2001; Balunas *et al.*, 2008).

1.2.3.5 The use of TAM in hormone therapy

TAM is a selective ER modulator (SERM) that induces ERα-mediated anti-estrogenic activity in ER-responsive breast cancer which leads to cell growth arrest and cell death (Weng *et al.*, 2008). However, *in vitro* studies demonstrated that TAM also act as an antiproliferative agent in ERα-negative cell lines, possibly by ER-independent pathway (Budtz, 1999; Mandlekar and Kong, 2001; Salami and Karami-Tehrani, 2003; Weng *et al.*, 2008). The ER-independent antiproliferative effects of TAM in ER-negative breast cancer could possibly be exploited for the treatment of ER-unresponsive tumors (Weng *et al.*, 2008).

Despite the benefits of TAM in the treatment of both ER-responsive and unresponsive breast cancer, the presence of negative side effects cannot be denied. TAM induces aneuploidy and stimulates the formation of DNA adducts which results in carcinogenesis such as uterine and hepatic cancers (Mandlekar and Kong, 2001; Petinari *et al.*, 2004a). Long term treatment of TAM can also diminish both the antiproliferative and apoptotic effects in breast and ovarian cancers which increase the cancer resistance against TAM (Zhou *et al.*, 2005).

1.3 Hormone receptors in breast cancer therapy

Removal of ovaries (oophorectomy) from premenopausal women gives a better prognosis against metastatic breast cancer. This is because the circulating estrogen from the ovaries can be reduced and this can prevent breast cancer progression. This

finding shows that hormones play a critical role in breast carcinogenesis and provides a rationale for their used as anti-cancer therapies (Love and Uy, 2008). Since the prevalence of breast cancer in women is higher than men, researches focus on the dominant sex hormone in women which includes estrogen, progesterone and its receptors.

1.3.1 Estrogen receptors (ERs)

The identification of ERs has led to the development of anti-hormonal therapy and target for a development of new drugs for breast cancer treatment. ERs act as a transcription factor which induces the expression of associated-genes in cell division and breast cancer progression (Carroll and Brown, 2006).

In the hormone therapy, many strategies were applied to deplete the ER signalling pathways such as oophorectomy, the use of selective ER modulators (SERMs) and aromatase inhibitors. SERM such as TAM interferes with ligand-receptor interaction which causes reduction of cell cycle protein expressions including cyclin D1 and cyclin E. Furthermore, TAM inhibits the phosphorylation of the retinoblastoma (Rb) gene which is important in cell cycle progression and cellular proliferation (Keen and Davidson, 2003). The use of SERMs in women with high risk of breast carcinoma could reduce this disease. Previous study reported that TAM reduced the risk of developing breast tumors up to 50% in high-risk women following 5-years period of treatment (Chlebowski *et al.*, 1999).

There are two types of ERs known as ERα and ERβ. Both ERs are members of the nuclear hormone receptor family. ERs are inactive in the absence of ligand. Upon ligand binding (estrogen), ER dissociates from its binding to HSP90. Once activated, ERs undergo conformational change, dimerize and autophosphorylate through intrinsic tyrosine kinases. ER dimers will then bind to the estrogen response elements (ERE) within the promoter region of target genes to regulate gene transcription (Figure 1.2) (Keen and Davidson, 2003).

ERα was identified in 1960 (Jensen *et al.*, 2010). This receptor exists in almost 70 % of breast cancer cases. ERα-positive tumors are less aggressive and have better prognosis compared to ERα-negative tumors. ERα expression is strongly associated with breast cancer and overexpression of ERα marks a significant event of breast cancer progression (Hayashi *et al.*, 2003). ERα is an important indicator of potential response to hormone therapy in breast cancers. Unfortunately, the increased numbers of ERα-positive tumor resistant against hormone therapy limit the effectiveness of this treatment. A study showed that loss of ER responsiveness in breast cancer associated with extensive methylation of the ER gene at 5' exons which caused transcription repression of this gene (Lapidus *et al.*, 1996).

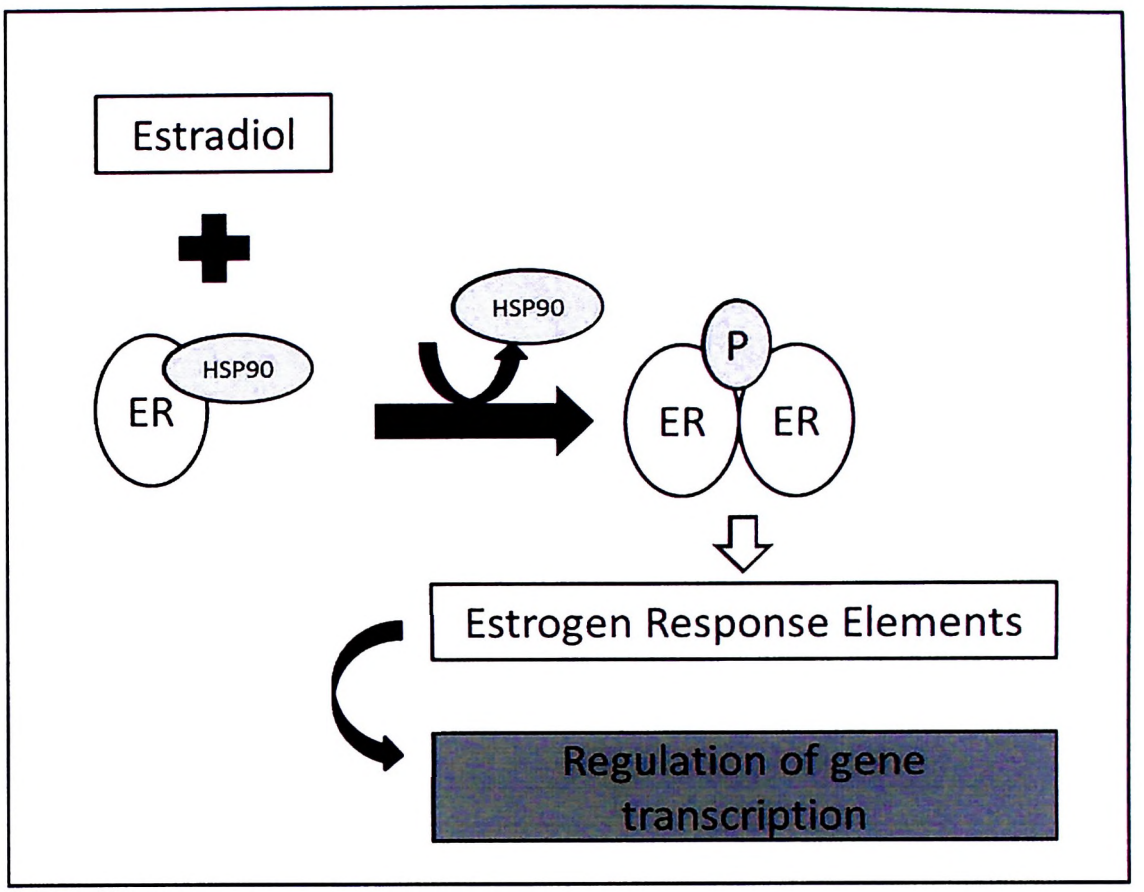

Figure 1.2 Activation of ER and the signalling pathway

Ligand binding (estrogen) leads to disassociation of ERs from its binding to HSP90. Once activated, ERs undergo conformational change, dimerize and autophosphorylate through intrinsic tyrosine kinases. ER dimers then bind to ERE to regulate the transcription of a variety of genes. (Adapted from Ross and Hortobagyi, 2005).

The ERβ gene was demonstrated in 1996 (Kuiper *et al.*, 1996). ERβ expression exists in various cancer tissues including breast, lung and stomach. ERβ is also expressed in ERα-positive breast cancers and in some cases ERβ could suppress the function of ERα. ERβ could be an important functional modulator of estrogen-signaling pathways in breast cancers (Hayashi *et al.*, 2003).

1.3.2 Progesterone receptors (PRs)

Like ERs, PRs are members of the nuclear receptor superfamily. There are two isoforms of PRs known as PR-A and PR-B. Both isoforms are encoded by the same gene but initiated by two distinct transcriptional start sites and produced proteins with different biological activities. Both PRs were highly expressed in normal tissues but PR-B levels are elevated in breast cancer (Ross and Hortobagyi, 2005).

PRs are useful as an indicator of hormone therapy responsiveness. PRs expressions are associated with response to hormone therapies in ER-positive breast cancer. This is because both ER and PR co-expression are involved in the estrogen response pathway. Studies have shown that poor prognosis of ER-positive breast cancer associated with loss of PR as well as ER expression (Lapidus *et al.*, 1996; Bardou *et al.*, 2003). These results suggest that high level of PRs expressions could be an indicator for ER-positive patients to benefit from hormonal therapy (Bardou *et al.*, 2003; Hefti *et al.*, 2013).

1.4 Cell death mechanisms

The main objective of cancer treatment is to stimulate signalling pathways associated with cancer cell death. In this sub-topic, several types of cell death mechanisms will be discussed such as apoptosis, necrosis and autophagy.

1.4.1 Apoptosis

Apoptosis is programmed cell death. It is characterized by distinct morphological characteristics such as cell shrinkage, alterations in nuclear morphology and formation of apoptotic bodies. Apoptosis play important roles in many pathological conditions which include proper development and function of the immune system and embryonic development (Alison and Sarraf, 1998).

Apoptosis occurs through the activation of specific sets of genes or apoptosis-related genes. There are six major domains or repeating structural elements that associate with mammalian apoptosis and over 100 genes have been identified in these six groups which are: caspase (cysteine-aspartic proteases), caspase recruitment domain, death domain, death effector domain, BIR (Baculovirus Inhibitor of apoptosis protein Repeat) domain and Bcl-2 homology domain (Kiechle and Zhang, 2002). These genes interact with each other in two distinct pathways of apoptosis known as extrinsic/death receptor and intrinsic/mitochondria-mediated pathways.

1.4.1.1 The extrinsic/death receptor pathway

The extrinsic/death receptor pathway is initiated when the ligand binds to the death receptor on the cell surface. Death ligand such as TNF binds to the death receptor known as TNF receptor (TNFR) which subsequently recruits two signal transducing molecules: TNFR 1-associated death domain protein (TRADD) and Fas-associated protein with death domain (FADD) to form death-inducing signalling complex (DISC). The complex binds to procaspase-8 and activates caspase-8 through self-cleavage. Caspase-8 then activates downstream executioner caspases known as caspase-3, -6 and -7, which cleave their target substrates for apoptosis (Kiechle and Zhang, 2002). Caspase-3 induced apoptosis is associated with biochemical alterations such as cleavages of α-fodrin and p21-activated kinase 2 (PAK2). The alteration of these molecules contributes to membrane blebbing and cleavage of the inhibitor of the caspase-activated DNase (ICAD) which causes DNA fragmentation. In addition, caspase-3 is also responsible for generating the 'eat me' signal which evokes the migration of phagocytes to the site of apoptosis (Lauber *et al.*, 2003). Death receptor signalling pathway can also activate mitochondria-mediated pathways through caspase-8. Activation of caspase-8 cleaves BID (Bcl2 Interacting Protein) to generate a C-terminal fragment which then translocates to the mitochondria and causes disruption of mitochondrial membrane potential. The mitochondrial membrane disruption triggers cytochrome c (cyto c) release. The release of cyto c results in binding of APAF-1 (Apoptotic Protease Activating Factor-1) to procaspase-9 and activates caspase-9. Caspase-9 then activates a series of executioner caspases (caspase-3,-6 and -9) for induction of cell death (Zou *et al.*, 1997). The summary of this pathway is illustrated in figure 1.3.

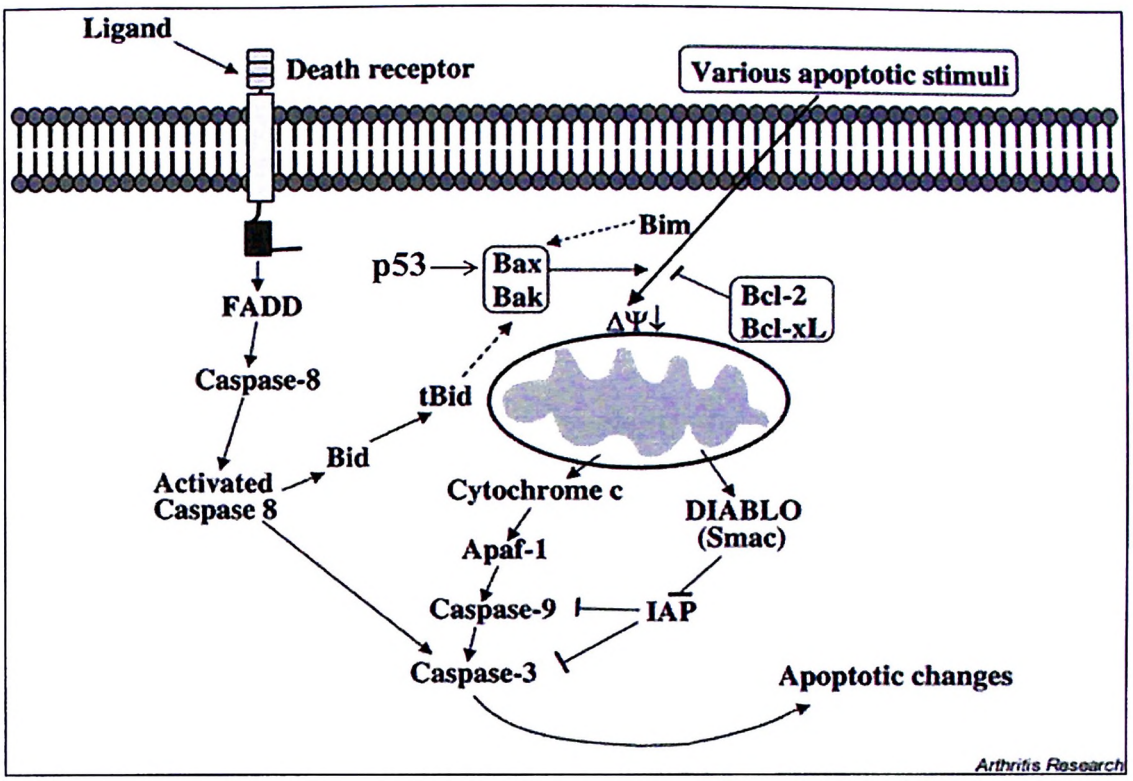

Figure 1.3 Extrinsic and intrinsic apoptotic pathways

The extrinsic/death receptor pathway is triggered by death receptor-ligand engagement, which subsequently activates caspase-8. Activated caspase-8 leads to caspase-3 activation, stimulates degradation of cellular proteins and other biochemical alterations of apoptosis. Activated caspase-8 also activates intrinsic/mitochondrial-mediated pathways by triggering BID cleavage, which then translocates into mitochondria and causes cyto c released. Besides BID, Bax also induces cyto c released from the mitochondria. Cyto c then binds to APAF-1 and caspase-9 which results in activation of caspase-3 and initiates apoptosis. (Adapted and extracted from Mak and Yeh, 2002)