IN VIVO EFFECTS OF SIROLIMUS AND SUNITINIB ON BREAST CANCER PROGNOSTIC MARKERS

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleic acid
ER	Estrogen receptor
PgR	Progesterone receptor
HER2/neu	Human epidermal growth factor receptor 2
DMBA	7,12-Dimethylbenz(a)anthracene
DEN	Diethylnitrosamine
NMU	N-Nitroso-N-methylurea
MNU	N-methyl-N-nitrosourea
AOM	Azoxymethane
mTOR	Mechanistic Target of Rapamycin
mTORC1	Mechanistic Target of Rapamycin Complex 1
mTORC2	Mechanistic Target of Rapamycin Complex 2
TKI	Tyrosine kinase inhibitor
ATP	Adenosine triphosphate
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
FLT1	Fms-related tyrosine kinase 1
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
FLT3	Fms-related tyrosine kinase 3
RET	Rearranged during Transfection
FDA	Food and Drug Administration
GIST	Gastrointestinal stromal tumour
RCC	Renal cell carcinoma

pNET	Pancreatic neuroendocrine tumour
HCC	Hepatocellular carcinoma
TDLU	Terminal duct lubular unit
IBC	Invasive breast carcinoma
NST	No special type
BRCA1	Breast cancer type 1
BRCA2	Breast cancer type 2
ATM	Ataxia-telangiectasia mutated
p53	Tumour protein p53
CHEK2	Checkpoint kinase 2
PTEN	Phosphatase and tensin homolog
CDH1	Cadherine-1
STK11	Serine/threonine kinase 11
LKB1	Liver kinase B1
PALB2	Partner and localizer of BRCA2
NBN	Nibrin
NBS1	Nijmegen breakage syndrome 1
NF1	Neurofibromatosis type 1
IHC	Immunohistochemistry
GEP	Gene expression profiling
DBD	DNA-binding domain
ERE	Estrogen response element
CDK	Cyclin-dependent kinase
S phase	Synthesis phase
G_1 phase	Gap1 phase
NFKB1	nuclear factor kappa-B 1
RANK	Receptor activator of nuclear factor kappa-B

RANKL	Receptor activator of nuclear factor kappa-B ligand
WNT	Wingless-type
WNT4	Wingless-type 4
mRNA	Messenger ribonucleic acid
HR	Hormone receptor
SR	Steroid receptor
EGFR	Epidermal growth factor receptor
ErbB1/2/3/4	Erythroblastic oncogene B 1/2/3/4
CSC	Cancer stem-like cell
TFAP2C	Transcription Factor AP-2 Gamma
MAPK	Mitogen-activated protein kinase
P13K	Phosphoinositide 3-kinase
AKT	Protein kinase B
Ras	Rat sarcoma
RAF	Rapidly Accelerated Fibrosarcoma
MDM2	Mouse double minute 2
GSK3	Glycogen Synthase Kinase 3
PIKK	Phosphoinositide 3-kinase-related kinases
HIF-1α	Hypoxia-inducible factor 1α
STAT3	Signal transducer and activator of transcription 3
PP2A	Protein phosphatase 2A
SGK	Serum glucose kinase
PKC	Protein kinase C
IRS	Insulin receptor substrate
RTK	Receptor Tyrosine Kinase
c-KIT	Stem cell factor receptor
CSF-1R	Colony stimulating factor 1 receptor

RET	Neurotrophic factor receptor
IRE1α	Inositol-requiring enzyme 1 α
GIST	Gastrointestinal stromal tumour
RCC	Renal cell carcinoma
mRCC	Metastatic renal cell carcinoma
MBC	Metastatic breast cancer
MMTV	Murine mammary tumour virus
DCIS	Ductal carcinoma in situ
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
DMSO	Dimethyl sulfoxide
PEG300	Polyethylene glycol 300
PEG(80)	Polyethylene glycol 80
NBF	Neutral Buffered Formalin
HCl	Hydrochloric acid
TBS	Tris-buffered saline
EDTA	Ethylenediaminetetraacetic acid
HRP	Horseradish peroxidase
DAB	3, 3′ diaminobenzidine tetrahydrochloride
ARASC	Animal Research and Service Centre
H&E	Hematoxylin and Eosin
FFPE	Formalin fixed paraffin embedded
cDNA	Complementary deoxyribonucleic acid
RT	Reverse transcriptase
CSC	Cancer stem cells
IP	Intraperitoneal
RICTOR	Rapamycin-insensitive companion of mechanistic target of rapamycin

RAPTOR	Regulatory-associated protein of mechanistic target of rapamycin
g	Gram
mg	Milligram
kg	Kilogram
ml	Milliliter
mM	Milimolar
M	Molar
mm	Millimeter
V	Volume

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KESAN *IN VIVO* SIROLIMUS DAN SUNITINIB PADA PENANDA PROGNOSTIK KANSER PAYUDARA

ABSTRAK

Kanser payu dara merupakan penyakit heterogen yang mempunyai kepelbagaian ciri-ciri klinikal, patologikal, dan molekul. Kanser payu dara merupakan kanser yang paling banyak didiagnos dalam kalangan wanita, dan merupakan punca utama kematian wanita di seluruh dunia. Reseptor hormon seperti *Estrogen Receptor* (ER), *Progesterone Receptor* (PgR) dan *Human Epidermal Growth Factor Receptor-2* (HER2/neu) adalah penanda rutin dalam prognosis kanser payu dara, dan membantu dalam menentukan jenis perawatan yang terbaik. Sirolimus, merupakan sejenis ubat semulajadi mikrolid daripada bakteria yang mampu menyekat imuniti dan menghalang percambahan sel kanser dengan cara menghalang pengaktifan mTOR. Sunitinib pula merupakan perencat *tyrosine kinase* yang bersifat menghalang proses angiogenesis. Oleh itu, ianya menarik untuk mengkaji kesan Sirolimus dan Sunitinib dalam menghalang perkembangan kanser payu dara daripada aruhan hormon. Dalam kajian ini, kanser payu dara diaruh dengan menggunakan N-Nitroso-N-Methylurea (NMU) dengan dos 70mg/ kg berat badan terhadap 32 ekor tikus betina strain Sparague Dawley. Pengekspresan gen dan protein untuk ketiga-tiga reseptor ini ditentukan dengan mengggunakan teknik imunohistokimia dan Real-Time PCR. Hasilnya, semua tumor payu dara merupakan 100% malignan, mempunyai ciri *invasive breast carcinoma (IBC)* yang kebanyakannya adalah jenis *cribriform, papillary* dan *no special type (NST)*. Perawatan dengan Sirolimus menunjukkan penyekatan perkembangan tumor dan mengurangkan pengekspresan protein ER dan PgR. Walaubagaimanapun, berlaku

peningkatan ekspresi pada tahap gen mungkin disebabkan Sirolimus menggalakkan regulasi pos-transkripsi berlaku. Manakala, perawatan dengan Sunitinib merencat perkembangan tumor selepas rawatan pertama, tetapi berlaku peningkatan diameter tumor selepas rawatan kedua. Perawatan dengan Sunitinib juga tidak menunjukkan pengurangan pengkspresan yang signifikan bagi ER dan PgR. Walaubagaimanapun, dari sudut histologi, perawatan dengan Sunitinib tidak menghasilkan sebarang jenis ductal NST yang agresif. Dalam kajian ini, semua kanser payu dara diaruh dengan NMU menunjukkan skor negative pengekspresan HER2/neu. Perawatan kombinasi menyebabkan tumor berjaya direncat, dan ianya dijangka disebabkan oleh Sirolimus lebih menunjukkan kesan antikanser berbanding Sunitinib. Oleh itu, kajian ini mencadangkan bahawa Sirolimus bukanlah penggalak atau sinergi dengan Sunitinib.

IN VIVO EFFECTS OF SIROLIMUS AND SUNITINIB ON BREAST CANCER PROGNOSTIC MARKERS

ABSTRACT

Breast cancer is a heterogeneous disease with a wide variety of clinical, pathological, and molecular characteristics, the most commonly diagnosed cancer among females and the leading cause of women cancer death. Hormone receptor studies such as estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor-2 (HER2/neu) are routinely done in prognosis of breast carcinoma and helps in deciding the best treatment. Sirolimus is a natural macrocyclic lactone drug from bacteria with immunosuppressive and anti-proliferative properties by inhibiting mechanistic target of rapamycin (mTOR). Sunitinib is a tyrosine kinase inhibitor (TKI) with antiangiogenic properties. Therefore, it will be interesting to analyse the effect of Sirolimus and Sunitinib in blocking the growth of breast cancer from responding to hormone stimulation. In this study, invasive mammary carcinoma was induced by using 70mg/kg body weight N-Nitroso-N-Methylurea (NMU) in 32 young female Sprague Dawley rats. The gene and protein expressions of ER, PgR and HER2/neu markers were evaluated by using semi-quantitative immunohistochemistry analysis and quantitative real-time PCR assay. Findings from the untreated-control group demonstrated that all mammary lesions are 100% malignant, histopathological characterized with invasive breast carcinoma (IBC) of three major patterns; cribriform, papillary and no special type (NST). Sirolimus treatment showed significant inhibition of mammary tumour progression and downregulate the protein expressions of ER and PgR. However, high expressions of ER and PgR genes expressed on mRNA level might due to Sirolimus

cause post-transcriptional regulation in gene. Meanwhile, tumour treated with Sunitinib reduced in diameter after first treatment, but the diameter increased after second treatment, and consequently showed no significant downregulation of ER and PgR. Histologically, Sunitinib treated tumour did not show any aggressive ductal NST histological subtypes. All NMU-induced tumours were HER2/neu-negative scoring. Tumour regression in combination treatment shown was predicted due to Sirolimus predominantly showed anticancer effect rather than Sunitinib. Thus, present findings suggested that Sirolimus is neither synergistic nor additive with Sunitinib.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Breast cancer, commonly diagnosed cancer encountered in females which lead to mortality with various characteristics in clinical, pathological, and molecular (Bray *et al.*, 2018). In Malaysia as reported in Malaysia National Cancer Registry Report (2019), breast cancer is the leading cause of female cancer death with 21,634 cases of breast cancer reported on 2012-2016, accounted for 34.1% of all female cancer cases (Azizah *et al.*, 2019). Hence, it is compulsory to conduct research to understand the pathogenesis of breast cancer and discover the targeted therapy for the detection and therapy of breast cancer.

Estrogen hormone is important in normal mammary cell to regulate growth, differentiation and maintain homeostasis. Estrogen can cause cancer cells to develop by stimulating mammary tissue to mitosis; acts as a mitogen, and damaging DNA by acting as carcinogens (Cavalieri and Rogan, 2011). However, the effects of estrogen hormone alone do not fully lead for breast cancer development. Breast cancer tumours are dependent on estrogen and progesterone hormones binding to their own receptor. Human epidermal growth factor receptor-2 (HER2/neu) is a member of four homologous receptors family which actively involved in the tyrosine kinase mediated regulation, responsible for normal mammary tissue growth and development (Iqbal, 2014). The overexpression of HER2/neu in breast cancer associated with more tumour aggressiveness and poor prognosis. These three prognostic markers are routinely done in breast carcinoma screening. It not only helps in the prognosis of the tumour but also helps in deciding the best treatment.

In order to understand the biology of cancer and develop cancer prevention strategies, chemically induced carcinogenesis models in rat are widely used. There are several types of carcinogen used to induce cancer in animal model such as 9,10-Dimethyl-1,2-benzanthrazen (DMBA), Diethylnitrosoamine (DEN), Azoxymethane (AOM), and N-Nitroso-N-Methylurea (NMU). NMU is a common inducer to establish rat mammary carcinoma models in human breast cancer study. NMU is administrated intraperitoneally (IP) to animals to induce the oncogenesis of the mammary ducts with high incidence of ER and PgR expressed in mammary tumours (Alvarado *et al.*, 2017). NMU-induced mammary carcinoma is age dependent; and the model is widely used to screen and evaluate the potency of cancer-suppressing and promoting agents.

Sirolimus, also known as Rapamycin is isolated from bacterium *Streptomyces hygroscopicus* which initially developed as an antifungal agent until recently discovered with effective immunosuppressive and anti-proliferative characteristics by inhibiting mechanistic target of rapamycin (mTOR) (Li *et al.*, 2014). Sirolimus is a mechanistic target of rapamycin inhibitor that has been shown to inhibit rather than promote cancers in experimental models. Sirolimus target mechanistic target of rapamycin complex 1 (mTORC1). Inhibition of mTORC1 will inhibit cell growth and proliferation by limiting nutrients, energy and oxygen status. However, long-term exposure to Sirolimus will inhibits mechanistic target of rapamycin complex 2 (mTORC2) by isolating newly synthesized mTOR molecules (Guduru and Arya, 2017).

Sunitinib (Sutent) is a tyrosine kinase inhibitor (TKI) indicated for first-generation multi-targeted ATP-competitive TKIs including the vascular endothelial

growth factor receptors (VEGFRs) types 1 and 2 (FLT1 and FLK1/KDR), the platelet-derived growth factor receptors (PDGFR-α and PDGFR-β), the Fms Related Receptor Tyrosine Kinase (FLT3), Rearranged during Transfection (RET) kinases, and the stem cell factor receptor c-Kit (Kaji and Yoshiji, 2017). The vascular endothelial growth factor (VEGF) family are frequently overexpressed in various solid tumours including mammary tumour and bind to vascular endothelium to induce angiogenesis. Inhibiting these tyrosine kinase receptors will block downstream signal transduction, thus inhibiting tumour growth and angiogenesis. Sunitinib antiangiogenic properties is use against treatment of gastrointestinal stromal tumor (GIST), renal cell carcinoma (RCC) (Adams and Leggas, 2007; Rizzo and Porta, 2017), adjuvant treatment of adult patients at high risk of recurrent RCC following nephrectomy (Fadil Hassan, 2018), and pancreatic neuroendocrine tumours (pNET) in patients with not resectable locally advanced or metastatic disease (Delbaldo et al., 2012), and approved by Food and Drug Administration (FDA) (Lopes and Bacchi, 2010).

1.2 Problem Statement

For decades, researchers all around the world have identified the important role of mTOR and tyrosine kinases in the breast cancer development and progression. In this study, the role of Sirolimus as anti-mTOR and Sunitinib as multi-targeted tyrosine kinase inhibitor agents were used and analyzed towards retarding breast tumour growth. Sirolimus and Sunitinib were thought to downregulate the expressions of breast cancer prognostic markers such as ER, PgR, and HER2/neu. This can be a novel targeted therapy strategy to treat the specific molecular subtypes of breast cancer.

1.3 Objectives of the Study

The general objective of the study is to investigate the expression of breast cancer prognostic markers (ER, PgR and HER2/neu) of NMU induced breast cancer under the influences of Sirolimus and/or Sunitinib in *in vivo* model.

1.3.1 Specific Objectives

The specific objectives of the study are:

1. To investigate the morphological changes of NMU-induced breast cancer under the influence of Sirolimus and/ or Sunitinib.

2. To analyze the effect of Sirolimus and/ or Sunitinib on molecular biomarkers of ER, PgR and HER2/neu of treated tumours using immunohistochemistry and quantitative Real-Time PCR

CHAPTER 2

LITERATURE REVIEW

2.1 Overview on Breast Cancer

2.1.1 Breast

Breast is an organ from modified skin gland lies on the chest wall, sits atop the pectoralis muscle. Breast develops well in females as a vital accessory organ of the female reproductive system and rudimentarily develops in the males. The epithelial tissue of the breast contains lobules where milk is produce, and connects to ducts that lead out to the breast nipple. The major purpose of breast is to secrete milk for breastfeeding of the infants in a process called lactation, and also plays an essential role in female sexuality (OpenStax, 2013). However, breast generally non-functional form in males. Breast is divided into three parts; skin, parenchyma, and stroma (Pandya and Moore, 2011).

The skin covering the breast is alike with the skin in another place on the body except at nipple and areola parts (Cimino-Mathews *et al.*, 2020). The nipple contains circular and longitudinal smooth muscle fibres help in erecting the nipple upon stimulation, and is rich in the nerve supply. Areola is the dark pinkish-brown pigmented area around the nipple, rich in modified sebaceous glands that secrete oily secretion to prevent cracking of the nipple, and to provide lubrication for the nipple during nursing.

Parenchyma is the glandular tissue of the breast made up of branching ducts and terminal secretory lobules. There are 15 to 20 lobes, and a lactiferous duct drains each of them. Each lobe is subdivided into many smaller lobules, separated by broad fibrous Cooper’s ligaments, which connect the skin with the fascia, or sheet of

connective tissue, that covers the pectoral muscles beneath the breast. Each lobe is drained by a separate excretory duct. These arborizing networks lobe is like a tree whose trunk, branches, and with hollow leaves to conduct mammary milk from the lobules to the nipple. The lobule consists of multiple blunt-ending ducts in a cluster like the fingers of a glove. These fingers form the glandular acini of the lobule. They are surrounded by specialized connective tissue called fascia. The acini and fascia together form the lobule. A terminal duct and its lobule are collectively called the terminal duct lobular unit (TDLU) (Figure. 2.1)(Pathology, 2020).

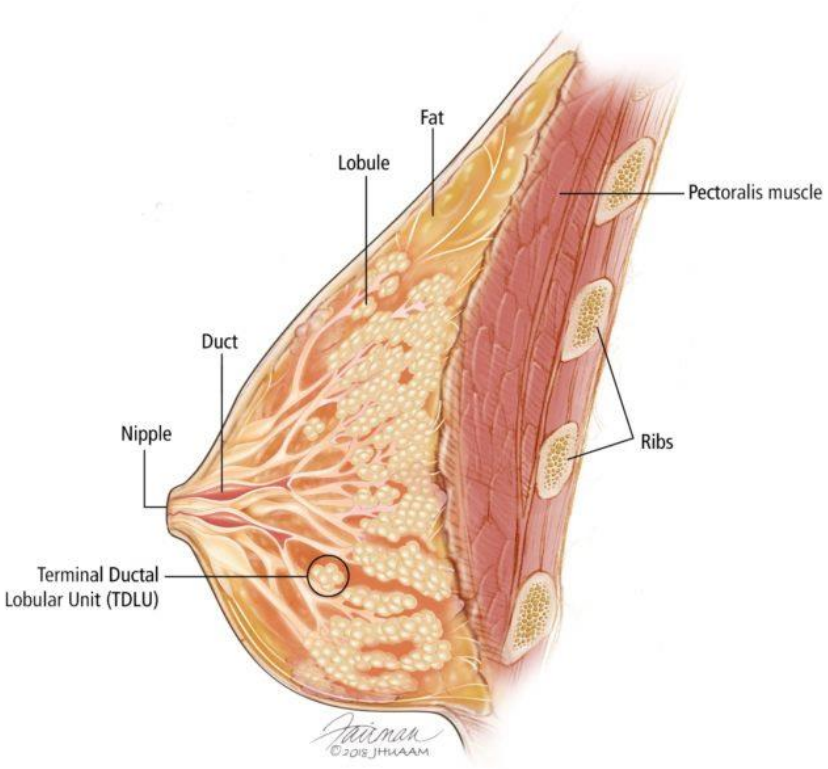

Figure 2.1 Anatomy of the breast

The female breast starts to develop and enlarge when reach puberty. Estrogen and progesterone stimulation involved in the development of the mammary glands and also associated in proliferation of epithelial and connective tissue (Pandya and Moore, 2011). The structure of male breast is almost identical with the

female breast, except lacking of the specialized milk producing lobules, since male does not breastfeeding the baby.

2.1.2 Breast Cancer Pathogenesis

Cells within tissue normally communicate with each other using networks of locally produced chemicals such as hormones, growth factors and cytokines. These signals are crucial in numerous cellular homeostasis. Balance of proto-oncogenes and tumour suppressor genes are required for normal cell functions. However, mutations of these genes through insertions, deletions, or substitutions will resulting in gain or loss of functions, and will activate the signalling pathways which lead to tumorigenesis (Tuna and Amos, 2012).

According Sever and Brugge (2015), cancer is determined by genetic and epigenetic alterations that allow cells to escape the normal cell cycle including cell proliferation and division, cell survival, cell death and apoptosis, cell differentiation and fate, cell motility and migration signalling pathway. The activating mutations of proto-oncogenes cause hyper activation of these signalling pathways, whereas inactivation of tumour suppressors reduces critical negative regulators of signalling (Sever and Brugge, 2015).

For rationalizing the complexities of neoplastic disease, Fouad and Aanei (2017) have attempted to re-postulate previous seven hallmarks of cancer which are cell proliferation, altering stress response favouring overall survival including apoptosis and autophagy, inducing angiogenesis and vascularization, invading and metastasis, rewiring metabolic, abetting microenvironment, and modulating immune system (Fouad and Aanei, 2017).

Tumour are divided into two types; benign (not harmful to health) and malignant (very virulent or infectious) (Pietrangelo, 2019). The benign tumours or also called benign neoplasms are non-cancerous and only grow in one place. They are unable to spread or invade to other parts of the body (Kennecke *et al.*, 2010; Liu *et al.*, 2012). Differing from benign, malignant tumours are cancerous and can invade to other parts of the body (Yanhua *et al.*, 2012). Benign tumour have potential in becoming malignant tumour in woman who have family history which had altered genetic mutation (Zeinomar *et al.*, 2019b).

Breast cancer is a malignant tumour that has developed from cells in the breast. Breast cancer may develop in the cells of the lobules (lobular cancer), or the ducts (ductal cancer), or stromal tissues of the breast (Sharma *et al.*, 2010). Breast tumour prognostic is based on degree of tubular formation, mitotic count, and nuclear pleomorphism (Rakha *et al.*, 2010).

Invasive breast carcinoma (IBC) of no special type (NST) pattern is the most commonly diagnosed breast cancer accounted for 75% of breast cancers (Sinn and Kreipe, 2013). IBC metastasize via lymphatics system from terminal duct lobular unit through the basement membrane of a breast duct with no specific histologic characteristics (Peter Abdelmessieh, 2018).

2.1.3 Aetiology of Breast Carcinoma

2.1.3(a) Gene mutation

Gene and chromosome mutations are currently considered to be important end-points linked to heritable defects and to cancer stimulation. Generally, 5 to 10% emergence of this correspond cancer is due to inheritance of commonly mutated gene such as Breast Cancer Type 1 gene (BRCA1) or Breast Cancer Type 2 (BRCA2)

gene (Colditz *et al.*, 2012). Statistically, a woman at 80 years old had 70% chance in developing breast cancer with the mutation of these two genes. Women with a BRCA1 mutation have a 55–65% lifetime risk of developing breast cancer statistically, while for women with a BRCA2 mutation, the lifetime risk is 45%. Women with one of these two mutations are also more likely to be diagnosed with breast cancer at a younger age, as well as to have cancer in both breasts. The impact of the BRCA1 and BRCA 2 mutation also associated with an increase of ovarian cancer risk as well (Petrucelli *et al.*, 2010).

Compared to BRCA mutations, there are less common and less drastic inherited mutations in other genes that also lead to increase of breast cancer risk. Some of the mutated genes involved in breast cancer development include Ataxia–telangiectasia gene (ATM) (Jerzak *et al.*, 2018), p53 gene (Kaur *et al.*, 2018), Checkpoint kinase 2 (CHEK2) (Apostolou and Papasotiriou, 2017), phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Zhang *et al.*, 2013), cadherine-1 (CDH1) (Corso *et al.*, 2018), PALB2 (Li *et al.*, 2017), nibrin (NBN) gene (Uzunoglu *et al.*, 2016), and Neurofibromatosis type 1 (NF1) genes (Salemis *et al.*, 2010). Women with the high risk factor is advisable for screening with precise genetic testing on these genes mutations (Lynch *et al.*, 2015).

2.1.3(b) Non-genetic aetiological factors

Several aetiological factors that involved in the breast cancer pathogenesis comprises of late age, gender, family pedigree, food intake, alcohol consumption, overweight, sedentary lifestyle, and presence of hormone factors (Abdulkareem, 2013).

Increasing age may increase aetiological risk of breast cancer. Breast cancer also associated in menopause women around 50 years (Kamińska *et al.*, 2015). Additionally, according to epidemiological data, 50% of breast cancers occur in women aged from 50 to 69 years. Breast cancer is very uncommon before the age of 20 years, but the incidence gradually increases with age, and by the age of 90 years, one-fifth of women are affected (Akram *et al.*, 2017).

Woman is highly risk of getting breast cancer due to sex hormones produced by the ovaries and the adrenal glands involved in the pathogenesis of breast cancer. Breast cancer is the most common cancer affecting women and accounts for approximately one quarter of all female cancers (Siegel *et al.*, 2016), and only less than 1% of patients with breast cancer are males. The differences are thought to be due to sex hormonal factor. Increased percentage of positive Estrogen Receptor (ER) tumours diagnosed in women after menopause showed an interesting correlations between the age when this neoplastic disease is diagnosed (Ban and Godellas, 2014).

Low in phytoestrogen diet, high intake of alcohol, obesity, and sedentary lifestyle increased the aetiology of breast cancer. Phytoestrogens diets have the ability to inhibit local estrogen synthesis, induce epigenetic changes, inhibit the transcriptional growth-promoting activity of ERα, and thus exert tumour growth inhibitory effects. Food with 35-40% of fat increased incidence of obesity which leading to breast cancer due to rich in cholesterol, source of steroid hormones production (Sieri *et al.*, 2014). In addition, breast cancer risk increases with moderate alcohol intake, particularly for women with ER-positive breast cancer (Zeinomar *et al.*, 2019a).

2.1.4 Hormonal and growth receptors role in carcinogenesis of breast cancer

These three aforementioned receptors are IHC markers that routinely performed in pathology laboratories, with well-established staining and evaluation protocols. These prognostic markers are responsible to mediate cell growth signalling and classically used for breast tumour subtyping (Park *et al.*, 2012).

2.1.4(a) Estrogen and Estrogen Receptor (ER)

Estrogen hormone generally is a pace maker for female reproductive system and multi organ such as breast, bone, brain, and cardiovascular system. In breast, estrogen is vital in the normal breast epithelium development by promoting epithelial cell proliferation. Estrogen also act as pivotal mediators of ductal morphogenesis which occurs mostly postnatally under endocrine control (Brisken and O'Malley, 2010). This ligand is a membrane-soluble ligand which activates gene expression through intracellular receptors. In premenopausal women, estrogen is synthesized primarily in the ovary (especially membrane granulose and luteinized granulosa cells), and in postmenopausal women, estrogen primarily synthesized in peripheral tissues. However, the proliferation and genetic instability induced by estrogen have been considered to increase transformation of normal cells into malignant cells through their expression of Estrogen Receptor (ER).

Estrogen effects are mainly mediated through heptahelical receptor and binding to two nuclear ligand-activated transcription factors; ERα and ERβ. Estrogen-responsive elements bind to ERα and ERβ in the DNA to regulate the transcription of targeted genes. Estrogen receptor is the key in breast carcinogenesis and metastasis (Saha Roy and Vadlamudi, 2012b). Recent gene expression profiling (GEP) studies reported that ER status is the main predictor in breast cancer. ER positive tumours are mostly well-differentiated, attrite aggressive, and associated

with better recovery rate after surgery compared to ER-negative tumour. Powell *et al.* (2012) suggested that targeting both ER receptors offer better therapeutic management of breast cancer (Powell *et al.*, 2012).

These two transcriptional factors works by either initiate or suppress the expression level of related targeted genes such as ERα (NR3A1) and ERβ (NR3A2), encoded by two different genes called Esr1 and Esr2. Both Esr1 and Esr2 have common structural features to uphold receptor-specific signal transduction through estrogen response elements (EREs) (Kulkoyluoglu and Madak-Erdogan, 2016).

In the normal breast, ERα is found in luminal epithelial cells, whereas ERβ has been shown to be expressed in luminal, myoepithelial cells, and stromal cells (Brisken and Ataca, 2015). The major mediator of estrogen action is ER-α because it has a higher affinity to the physiological form of estrogen. ER-α is the main molecule associated with breast cancer development and progression. Thus, the ER-α expression status is widely used with other prognostic markers receptors in order to classify the breast cancer subtypes.

Breast cancer cells have relatively high ERα expression and low ERβ expression (Huang *et al.*, 2014). Upon formation of homo- or heterodimers, these complexes are translocating into the cell nucleus and regulate gene transcription. ER dimers bind to the estrogen response elements (EREs) region of targeted genes and convert co-regulators to achieve the regulation of transcriptional activity (Renoir *et al.*, 2013). The activity was simplified as shown in Figure 2.2 (Feng et al., 2018a).

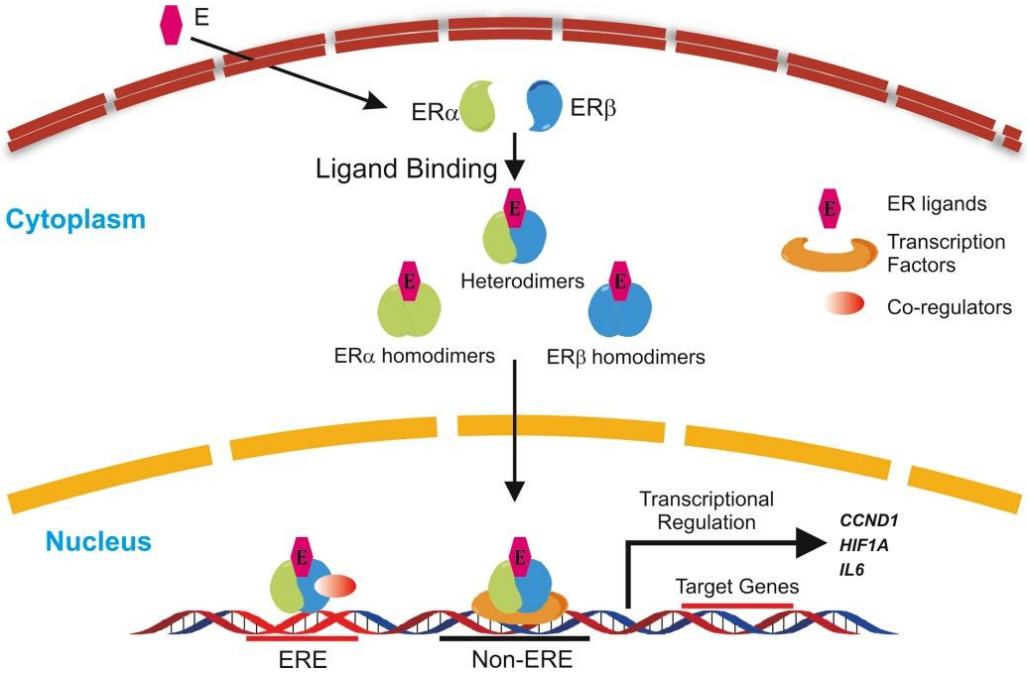

Figure 2.2 ER signalling pathway

ERα in breast cancer tumorigenesis involved many factors and various occurrences of cross-talk (Saha Roy and Vadlamudi, 2012a). ERα promotes the breast tumour cell growth mainly characterized by mechanisms through interaction with cyclin D1. In cancer cells, cyclin D1 control the progression of cell cycle from G1 to S phase by activating cyclin-dependent kinases (CDKs) 4 and 6. Mechanism of anti-estrogen therapy resistance also been explained from the synergism within the ERα and cyclin D1 feedback loop, and suggesting the rationale for the combined use of selective CDK4 and 6 inhibitors with hormonal therapy in ER positive breast cancer (Finn *et al.*, 2016; O'Leary *et al.*, 2016).

2.1.4(b) Progesterone and Progesterone Receptors (PgR)

Progesterone is an ovarian hormone that soluble in membrane. Binding of progesterone to the intracellular receptors generate epithelial growth in the mammary gland (Macias and Hinck, 2012). Progesterone involved in alveologenesis and required for preparation for lactation-competent gland formation during pregnancy.

The progesterone signal is transmitted by the Progesterone Receptors (PgR), which encompasses of two isoforms; PgR-A and PgR-B that are only differentiated by 164 additional N-terminal residues in PgR-B (Abdel-Hafiz and Horwitz, 2014). Imbalanced of PgR-A and PgR-B expression occurs early in carcinogenesis with predominance of one protein, usually PgR-A. However, the ratio of PgR-A:PgR-B imbalance in breast cancers is not associated with lifetime endogenous endocrine (Mote *et al.*, 2015).

There are diverse mechanisms that have different biological functions, but have been associated in the biological response to progesterone that may promote tumorigenesis such as RANKL, WNT4, and CyclinD1. Apart from that, progesterone also involved in RANK/RANKL signalling pathway. Upon binding with NFKB1 ligand mediate the cell proliferation. Both RANKL and progesterone genes are co-expressed in luminal epithelial cells during the morphogenesis of mammary lactation (Tanos *et al.*, 2013).

In luminal cells that expressed progesterone receptors (PgR), progesterone leads to the upregulation of RANKL expression. Recent studies demonstrating central role of RANKL in generating the pro-growth response to progesterone to allow cell proliferation in progestin-dependent breast cancers. In this regard,

progesterone has dual prominence works (Figure. 2.3) either by autocrine and paracrine.

WNT signalling pathway is another downstream pathway that has been identified as oncogenic and may promote tumorigenesis in the mammary gland as reported by Tanos *et al*. (2013) using freshly isolated human breast tissue microstructures that found expression of both RANKL and WNT4 mRNA is induced by PgR signalling (Tanos *et al.*, 2013).

In short, progesterone binds its receptor in a subset of hormone receptor (HR) luminal cells or the sensor cells which is surrounded by myoepithelial or basal cells, which are in contact with the basal lamina. In certain PgR cells, it induces cell proliferation by a Cyclin D1-dependent mechanism (cell intrinsic signalling). It induces RANKL, which elicits cell proliferation in neighbouring HR cells (paracrine homotypic) and WNT4, which acts on myoepithelial cells (paracrine heterotypic) and increases stem cell activity (Figure 2.3) (Brisken *et al.*, 2015) .

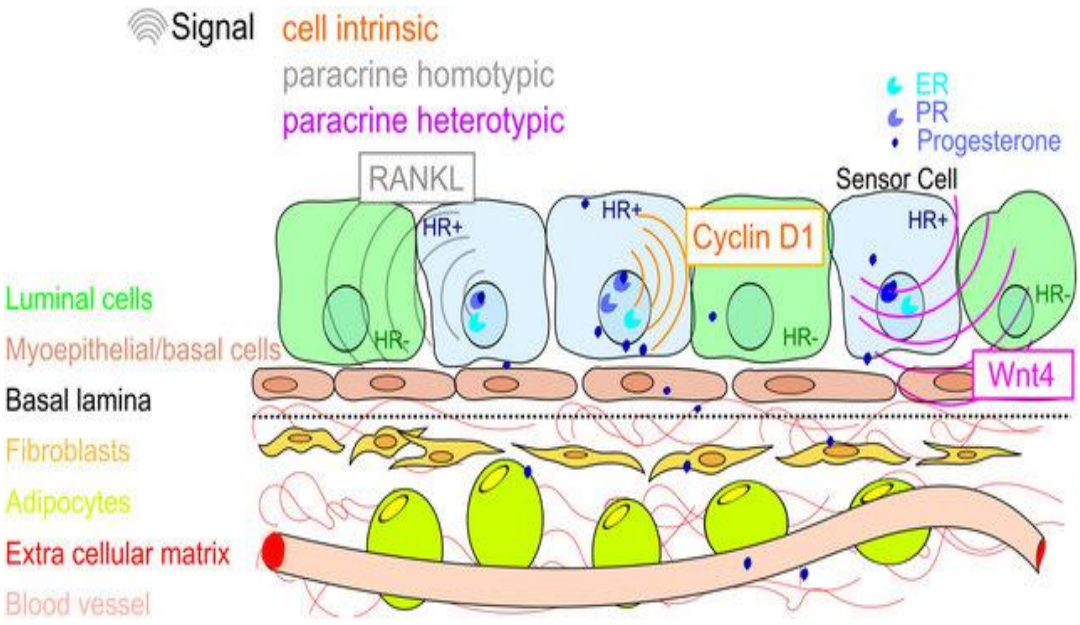

Figure 2.3 Signalling downstream of progesterone.

The major downstream effector on estrogen action and act as the main ER target gene is PgR. Remarkably, there are broad cross-talk occurred between PgR with ER since both are required for mutual signal transduction pathways in mammary gland development and are most often elevated in breast cancer. For instance, the cross-talk between PgR-B and the tyrosine kinase growth factor receptors (Egfr) pathway. Synergistic effect between progesterone and EGF on numerous endogenous genes increase incidence of breast cancer carcinogenesis (Migliaccio *et al.*, 2010). The functional significance of EGF-induced and PgR-B hyper activation along with ERα mediate proliferation of massive alveolar during mammary gland growth (Wu *et al.*, 2015).

2.1.4(c) HER2 signalling and HER2-Positive breast cancer

Human epidermal growth factor receptor-2 (HER2/neu) or erythroblastic oncogene B 2 (c-ERBB2) one of the Epidermal Growth Factor (EGF) Receptor (EGFR) family among ErbB1/HER1, ErbB3/HER3, and ErbB4/HER4. HER2/neu may express in both normal and pathological tissues (Pines *et al.*, 2010; Roskoski Jr, 2014). HER2/neu is a proto-oncogene product from transmembrane tyrosine kinase growth receptor, thus involved in cancerous signalling pathway including proliferation, survival, cell motility, and invasion (Appert-Collin *et al.*, 2015).

HER2/neu positive breast cancers are more likely to metastasize, associated with inflammation and also expansion of cancer stem-like cells (CSCs) (Liu *et al.*, 2018b). A newly identified enhancer located at the 3′ gene body of HER2/neu was reported to be the target locus of known HER2 regulator, TFAP2C (Liu *et al.*, 2018a).

HER2/neu comprise of three multi-domains which are presence as extracellular, transmembrane, and intracellular domain (Arteaga and Engelman, 2014). In the intracellular domain of HER2/neu, phosphorylation of tyrosine residues stimulated by binding of ligand and subsequent dimerization, affecting many cellular functions, which lead to the intracellular activation (Figure 2.4) (Feng *et al.*, 2018). The downstream targeted pathways such as mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) pathways which are heavily associated with breast tumorigenesis (Mayer and Arteaga, 2016). HER2/neu as well as the others member of the EGFR family is located on the cell membrane and responds to a wide variety of ligands. Phosphorylation of the tyrosine kinase domain in the cytoplasm initiates downstream oncogenic signalling pathways such as PI3K/AKT pathway and Ras/MAPK pathway.

Mammary tumour progression and proliferation is related with HER2/neu gene expression results in HER2/neu protein overexpression. A novel targeted treatment targeting to inhibit the signalling pathways that are important for cancer development and progression such as HER2/neu monoclonal antibodies are developed, and improved the prognoses of patients with positive HER2/neu breast cancer (Swain *et al.*, 2015).

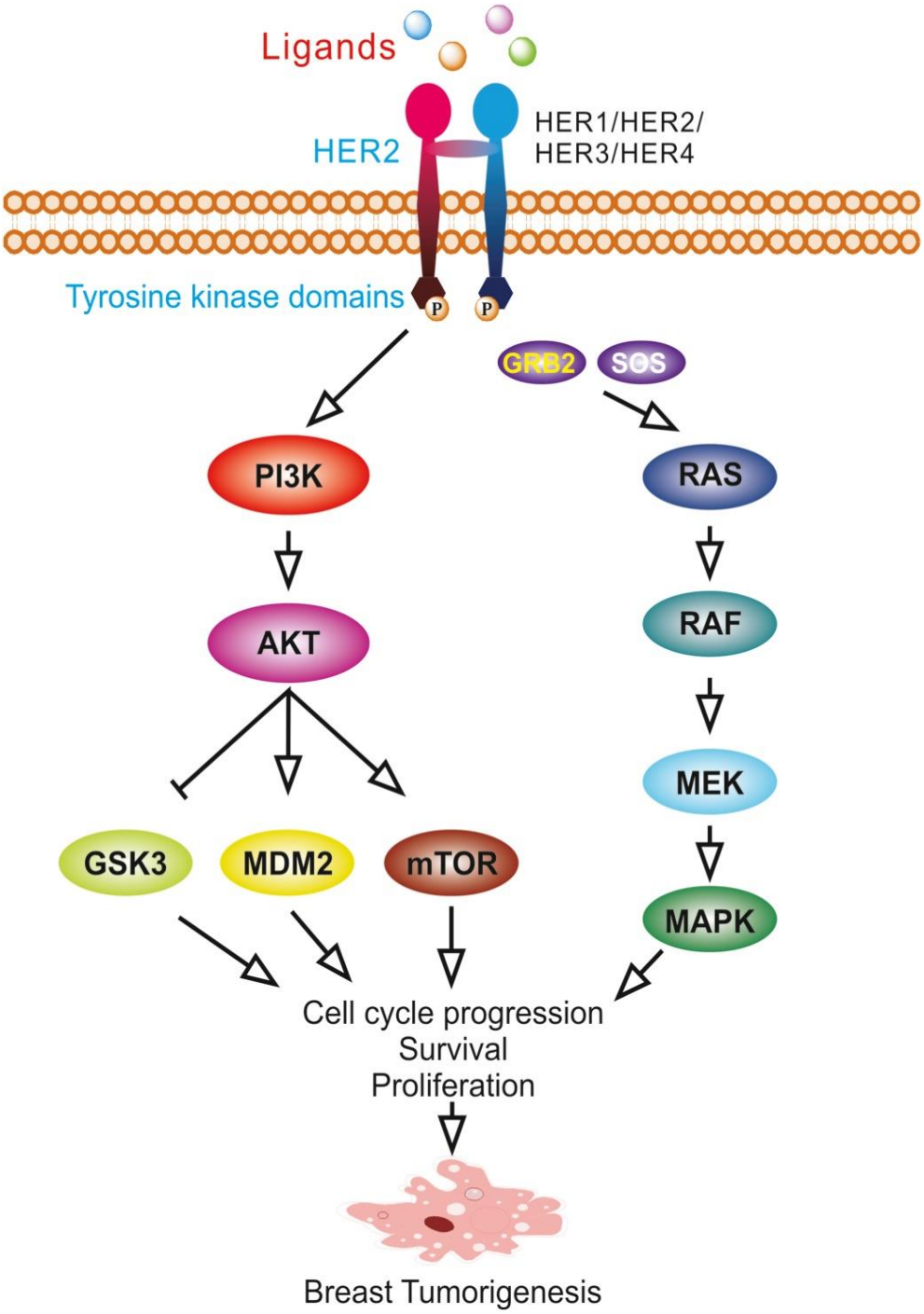

Figure 2. 4 HER2/neu signalling pathway

2.1.5 Breast Cancer Classification

Breast cancer demonstrated variety of biological and clinical behaviours. For several years, pathologists have recognized the biological and clinical heterogeneity of breast cancer. Understanding the morphology, molecular variation, histological structures and molecular pathological markers of breast cancer are used by pathologist in predicting clinical outcome and deciding appropriate treatment.

IHC detection of estrogen receptor (ER), progesterone receptor (PgR), and HER2/neu are routinely been done for histopathological sub-classification of breast cancer, with or without additional cell proliferation markers such as Ki-67 (Ki-67). Positive hormone receptor of ER and PgR shows the tumour types targetable by hormone targeted therapy such as tamoxifen and aromatase inhibitors. Similarly, positive overexpression of HER2/neu can be treated with trastuzumab. Triple negative breast cancers (TNBC) referred to lack of ER, PgR and HER2/neu which are not suggested for hormonal targeted therapies. TNBC are frequently associated with poor prognosis, exhibited a more aggressive behaviour, earlier and more frequent recurrence, and worse survival compared with positive prognostic breast cancer markers (Gonçalves *et al.*, 2018).

In order to classify the breast cancer subtypes, the ER, PgR and HER2/neu expression statuses have been considered as the most important features, where has been used in the dichotomized semi-quantitative immunohistochemistry evaluation. Breast cancer is classified into 5 molecular subtypes as summarized in Table 2.1 (Guiu *et al.*, 2012).

Table 2.1 Molecular subtypes of breast cancers

Subtype	Markers features	Characteristics	Treatment options
Luminal A	ER+, PR±, HER2/neu -, Ki67 <14%	Most common Best prognosis	Hormonal therapy Targeted therapy
Luminal B	ER+, PR+, HER2/neu ±, Ki67 ≥14%	10-20% Lower survival than Luminal A	Hormonal therapy Targeted therapy
HER2/neu overexpression	ER-, PR-, HER2/neu +	5-15%	Targeted therapy
Basal like	ER-, PR-, HER2/neu -	15-20%, worst prognosis, diagnosed at younger age	Limited targeted therapy
Normal like	ER+, PR±, HER2/neu -, Ki67 low	Rare, low proliferation and low gene expression	Hormonal therapy Targeted therapy

2.1.6 mTOR signalling pathway and cancer

The atypical phosphoinositide 3-kinase related kinase (PIKK) family mechanistic target of rapamycin (mTOR) is a member of the serine and threonine protein. mTOR is intracellular protein which is found downstream PI3K and protein AKT. mTOR signalling is critically important in regulating cell homeostasis and normal mammary development such as metabolism, protein and lipid production, cell survival, and organization of cell skeletal (Watanabe *et al.*, 2011).

Due to mutations of mTOR, commonly mTOR is over active in multiple cancer types including breast cancer. However, besides mTOR mutation, increases in activity of HER family receptors or alterations and mutations of PI3K signalling also related to breast cancer incidence (Hare and Harvey, 2017). mTOR interacts with different proteins and comprises of two functionally different complexes, each defined by the specific co-factors in complex with mTOR kinase and by their relative sensitivity to rapamycin: mTORC1 and mTORC2 (Laplante and Sabatini, 2012).

Both receptor-ligand complexes are involved in tumorigenesis through different mechanisms. mTORC1 is responsive to control several cellular processes, including protein and lipid synthesis, autophagy and lysosome biogenesis, nutrients, hormones, amino acids, hypoxia and growth factor signalling (Saxton and Sabatini, 2017). Phosphoinositide 3-kinase/ Protein kinase B (PI3K/Akt) and Rat sarcoma - Mitogen activated protein kinase (Ras-MAPK) regulate mTORC1 signalling, and lead to activation of Signal transducer and activator of transcription (STAT3), Hypoxia-inducible factor 1α (HIF-1α), and Protein phosphatase 2A (PP2A) in tumorigenic (Figure 2.5)(Meng *et al.*, 2018). mTORC1 requires the co-factor regulatory-associated protein of mTOR (Raptor), whereas mTORC2 requires the co-factor rapamycin-insensitive companion of mTOR (Rictor) (Luo *et al.*, 2015).

mTORC2 plays role in cytoskeletal remodelling, responsible in ion transportation and cell cycle by regulating Serum glucose kinase (SGK) and Protein kinase C (PKC) (Ebner *et al.*, 2017). However, IRS (insulin receptor substrate) indirectly regulates mTORC2 by mTORC1 via different feedback loops. mTORC1 negatively regulates mTORC2 by two mechanisms. First, decrease the insulin signalling through phosphorylating insulin receptor substrate (IRS), and second inactivate of Akt through Akt phosphorylation and through the phosphorylation of

Rictor (Dalle Pezze *et al.*, 2012). Akt is the main modulator for varies cellular processes begin with mTORC2 through phosphorylating at S473 directly by mTORC2.

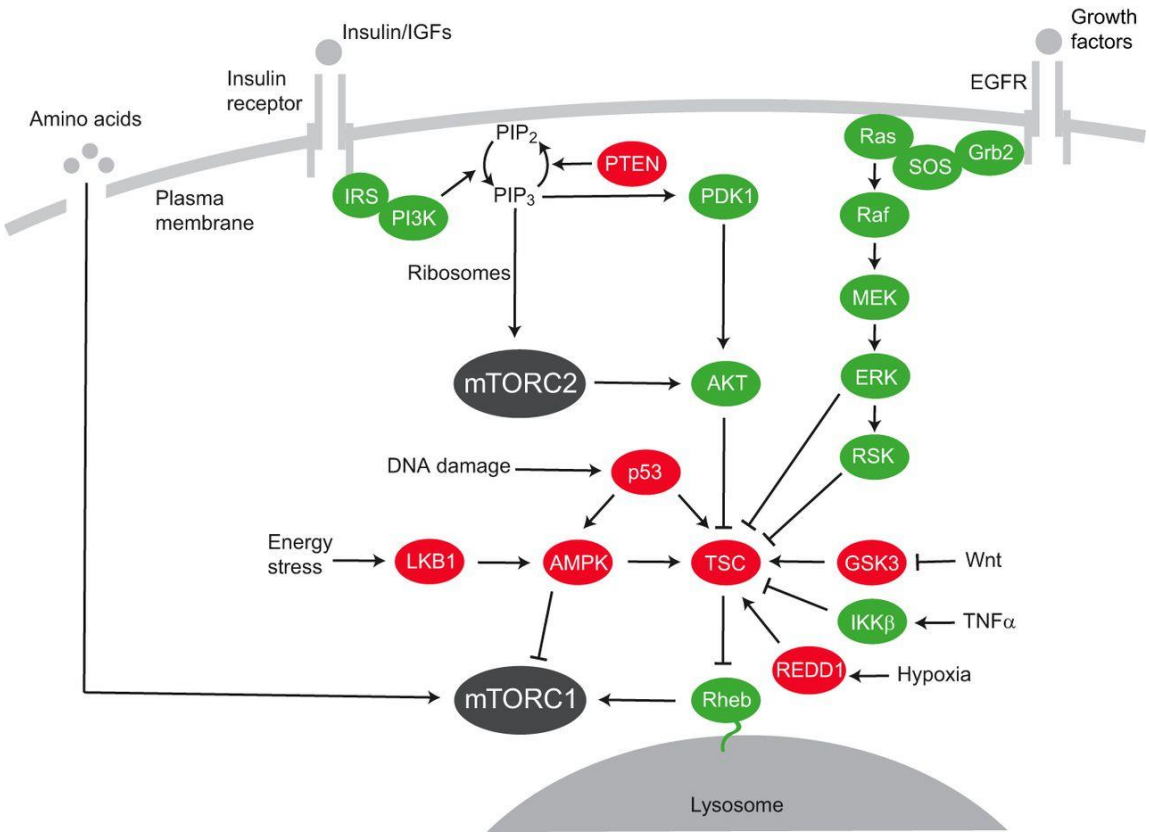

Figure 2. 5 mTOR signalling pathway

2.1.7 Angiogenesis in Breast Cancer

Angiogenesis is referred to formation of new blood vessel which also involved in breast cancer initiation, progression, and malignancy (Paduch, 2016). Angiogenesis also involved in both local tumour growth and distant metastasis in breast cancer. A major pathway involved in angiogenesis is from hypoxic tumour cells release vascular endothelial growth factor (VEGF), and it is binding to the VEGF receptor (VEGFR), located on endothelial cells. Angiogenesis is cause by

transcription of pro-angiogenic genes within the nucleus of the endothelial cell, which was induced by activation of signalling cascade promoted by VEGFR (Ziyad and Iruela-Arispe, 2011).

A ubiquitous feature of solid cancers is hypoxia. Hypoxia is a situation of incompatible between cellular oxygen supply and cellular oxygen consumption. Hypoxia able to stimulate the formation of neo-genesis (angiogenesis) and lymphatic vessels (lymphangiogenesis) to allow the cancer cells to escape the unfavourable tumour microenvironment and metastasis into secondary sites. Thereby, hypoxia is highly associated with metastatic disease and mortality (Schito, 2019). Lack of oxygen stimulates hypoxia-induced factor 1 alpha (HIF-1α), which then activates transcription of various proangiogenic cytokines such as VEGF (Schito and Rey, 2017). In targeted genes including VEGF, the HIF-1 complex binds to hypoxia-responsive elements in the promoter region which lead to over expression and contribute to angiogenesis.

In breast cancer, the level of angiogenesis is associated with survival of tumour. VEGF is a major transcriptional target for HIF-1, thus is considered as vital factor playing a role in angiogenesis. The high levels of VEGF and other angiogenic factors indicate the high-risk disease with poor prognosis. In addition, VEGF also promotes vascular permeability, vasodilation, recruit endothelial progenitor cells from the bone marrow and inhibit apoptosis (Hoffmann *et al.*, 2013).

Recognition of the importance of angiogenesis for tumour growth and metastasis led researcher to lead advance research for therapeutic purpose by inhibiting this pathway (Wang *et al.*, 2015). Since then, tyrosine kinase inhibitors targeting angiogenic factors such as VEGFR, platelet-derived growth factor receptor,

and others, were developed such as bevacizumab (anti VEGF-A), ramucirumab (anti-VEGFR2) and Sunitinib (multi-targeted receptor tyrosine kinase).

2.1.8 Prevalence of Breast Cancer

Breast cancer is highly associated with female at advance age and lead to death (Desreux, 2018). Figure 2.6 shows the most common type of cancer incidence in 2018 worldwide. Breast cancer (presented in pink colour) showed the most incidence number and mortality rate among female globally. GLOBOCAN 2018 reported that breast cancer (2,088,849 numbers of new cases) is the second common cancer diagnosed after lung cancer (2,093,876 numbers of new cases) on 2018 with a significant mortality at 626,679 number of death after lung cancer 1,761,007 (Bray *et al.*, 2018).

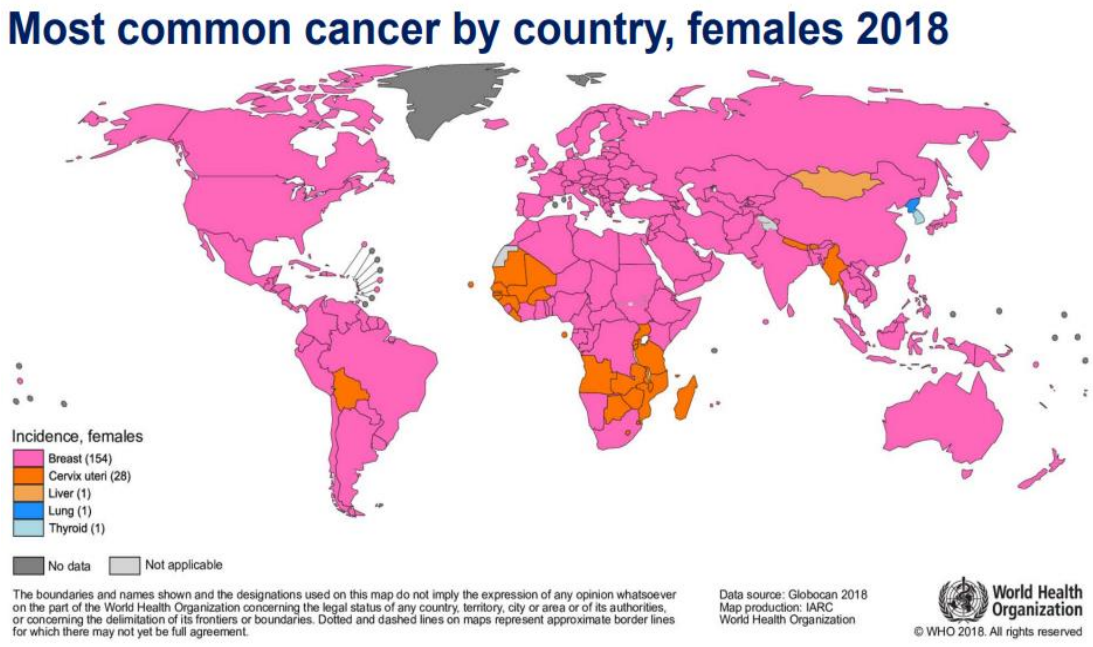

Figure 2. 6 Global Maps Presenting the Most Common Type of Cancer Incidence in 2018 in Each Country Among Women.

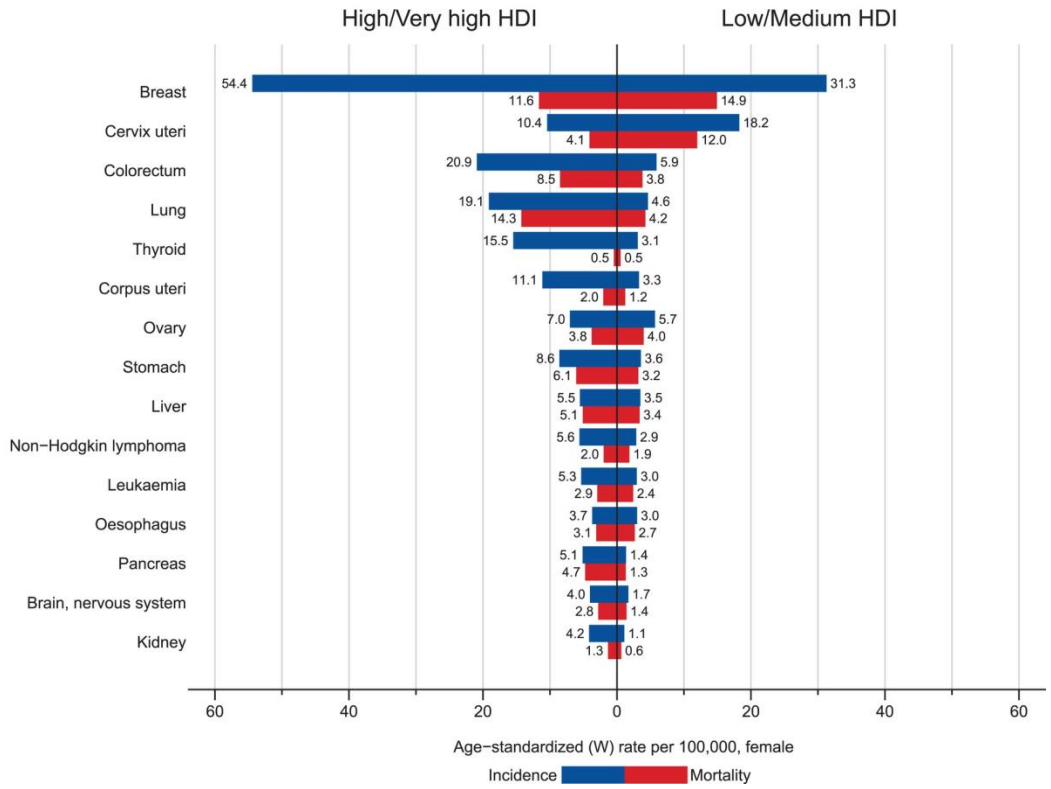

Figure 2.7 Bar Charts of Incidence and Mortality Age-Standardized Rates in High/Very-High Human Development Index (HDI) Regions Versus Low/Medium HDI Regions Among Women in 2018.

In women (Figure 2.7), incidence rates for breast cancer far exceed those for other cancers in both transitioned and transitioning countries, followed by colorectal cancer in transitioned countries, and cervical cancer in transitioning countries.

As in Malaysia, according to the Malaysia National Cancer Registry Report (2019), breast cancer accounted for 34.1% of all female cancer cases. Majority of the cases were Chinese (43.2%) followed by Malays (40.7%), Bumiputra (8.6%), Indians (6.6%) and Other Ethnic groups (0.8%). Most of the cases were females 43621 (59.8%) and 29263 (40.2%) were males. Among them, 98% of the total cases from 21,634 cases were adult (45- 64 years old) (Azizah *et al.*, 2019).

For male in Malaysia, the cancer incidence from 2012 to 2016 reported by National Cancer Registry Report 2012–2016 (MNCRR) was 86 and in female was 102 per 100,000 populations (Azizah *et al.*, 2019). Cancer is the fourth leading cause of death in Malaysia which contributes to 12.6% of all deaths in government hospitals and 26.7% in private hospitals in 2016 (National Cancer Registry, 2018). However, there has been an increasing trend especially in private hospital on 2018 which contributes to 11.82% mortality rate in government hospital and 30.11% in private hospitals in 2018 (*Health Facts 2019 (Reference Data for 2018)*, 2019).

Table 2.2 Number and percentage of cancers in Malaysia by age groups in adults

Cancer types	15-44 years		45-54 years		55-64 years		65-74 years		75+ years		Total
	NO.	(%)	NO.	(%)	NO.	(%)	NO.	(%)	NO.	(%)	
Female Breast	4435	(26.1)	5936	(34.9)	4152	(24.4)	1829	(10.8)	657	(3.9)	**17009**
Cervix Uteri	971	(24.2)	1244	(31.0)	969	(24.1)	602	(15.0)	229	(5.7)	**4015**
Ovary	934	(30.3)	989	(32.1)	692	(22.4)	354	(11.5)	115	(3.7)	**3084**
Corpus Uteri	363	(17.8)	626	(30.7)	673	(33.0)	279	(13.7)	97	(4.8)	**2038**
Stomach	304	(10.8)	480	(17.0)	689	(24.4)	800	(28.4)	545	(19.3)	**2818**
Colon	731	(11.7)	1131	(18.0)	1772	(28.2)	1618	(25.8)	1021	(16.3)	**6273**
Rectum	592	(10.2)	1069	(18.4)	1682	(28.9)	1636	(28.1)	841	(14.5)	**5820**
Liver	349	(12.6)	610	(22.1)	888	(32.1)	638	(23.1)	281	(10.2)	**2766**
Pancreas	140	(9.7)	284	(19.7)	455	(31.6)	382	(26.6)	177	(12.3)	**1438**
Lung, T & B	657	(8.2)	1473	(18.4)	2308	(28.8)	2452	(30.6)	1131	(14.1)	**8021**
Prostate	24	(0.8)	114	(3.9)	661	(22.7)	1328	(45.6)	786	(27.0)	**2913**
Nasopharynx	1459	(31.1)	1433	(30.5)	1101	(23.4)	568	(12.1)	136	(2.9)	**4697**
Thyroid	868	(42.2)	472	(22.9)	370	(18.0)	273	(13.3)	75	(3.6)	**2058**
Brain & NS	556	(41.1)	304	(22.5)	266	(19.7)	176	(13.0)	50	(3.7)	**1352**
Lymphoma	1468	(32.9)	885	(19.8)	1019	(22.8)	767	(17.2)	326	(7.3)	**4465**
Leukaemia	1235	(45.8)	510	(18.9)	495	(18.4)	317	(11.8)	137	(5.1)	**2694**
All cancers	**15086**	**(21.1)**	**17560**	**(24.6)**	**18192**	**(25.5)**	**14019**	**(19.6)**	**6604**	**(9.2)**	**71461**

Source: Malaysian Study on Cancer Survival Ministry of Health (2018)

Early detection determines the cancer survival rate. However, early detection is highly dependent on cancer awareness and uptake of screening (NCD, 2017). Survival analysis in Malaysia was done for all cancer types. Analysis was done from total of 69,011 cases. Out of these, 17,009 were breast cancer cases in female. Study

show that most of detected breast cancer in Malaysia was in late stage (56%) (National Cancer Registry, 2018). Less eligible Malaysian women performed regular mammography screening which shows poor awareness of breast cancer in Malaysian women. Thus, it is crucial to improve awareness on benefits of early breast cancer screening and proper treatment.

2.2 Sirolimus

In 1970s, Sirolimus (Figure 2.8) also known as rapamycin was first discovered from the bacterium *Streptomyces hygroscopicus* that presence in plants and soil sample in Rapa Nui Island (Sehgal *et al.*, 1975). Initially, Sirolimus was used as antifungal agent, but later its anti-tumour property was discovered (Martel *et al.*, 1977; Vezina *et al.*, 1975). Sirolimus complex also able to inhibit cell proliferation (Chung *et al.*, 1992). In 1993, researchers performed genetic screening in *Saccharomyces cerevisiae* and discovered protein target of rapamycin (TOR) that were resistant to growth inhibition (Kunz *et al.*, 1993). Further studies showed Sirolimus acts on mTOR (Sabatini *et al.*, 1994; Sabers *et al.*, 1995). Nowadays, Sirolimus and the analogues are recently prescribed clinically as cancer drug as well as immunosuppressant in organ transplantation (Blagosklonny, 2013).

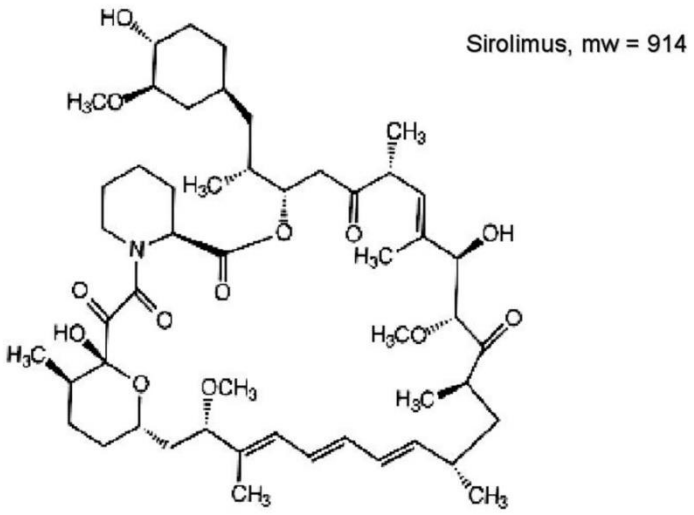

Figure 2.8 Structures of Sirolimus

Source: National Center for Biotechnology Information. PubChem Database. Sirolimus, CID=5284616, https://pubchem.ncbi.nlm.nih.gov/compound/Sirolimus (accessed on Apr. 10, 2020)

mTOR, as the name implies, is targeted by rapamycin (Sirolimus). Varies studies was conducted trying to understand the mode of action of Sirolimus. The binding of Sirolimus causes conformational changes in mTOR that can disturb functional mTOR complex. Sirolimus only works on mTORC1 and show insensitiveness towards mTORC2 (Mukhopadhyay *et al.*, 2016). Due to its mTOR inhibitory effect, and thus affecting cellular growth, Sirolimus was discovered as an anti-cancer agent. It was shown to possess cell cycle inhibitors capacity in several cancer including colon cancer (He *et al.*, 2016), pancreatic cancer (Xu *et al.*, 2015), and breast cancer (LoRusso and LoRusso, 2013). However, Sirolimus has not been taken forward for cancer monotherapy because of low solubility with poor pharmacokinetic properties. To tackle these problems, Sirolimus rapalogues (derivatives) such as everolimus, temSirolimus, ridaforolimus and zotarolimus have been developed to open up new ways for treatment.

2.3 Sunitinib

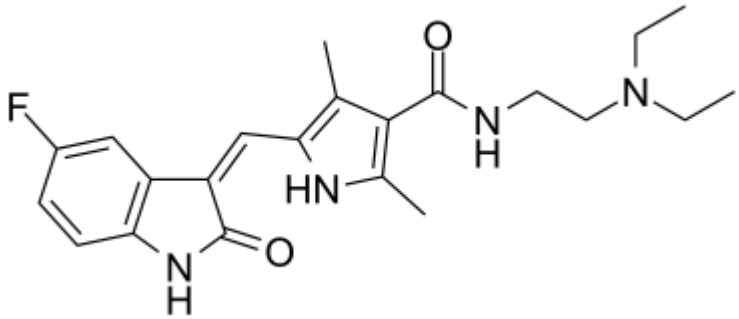

Figure 2.9 Sunitinib Chemical Structure
Source: https://www.medchemexpress.com/Sunitinib.html

Sunitinib is a potent and clinically approved as multi-targeted tyrosine kinase inhibitor that able to block different signalling pathways acted on different Receptor Tyrosine Kinases (RTKs). Sunitinib effectively inhibits variant of VEGFR and PDGFR and some other type of receptor tyrosine kinases, including stem cell factor receptor (c-KIT), FMS-like tyrosine kinase-3 receptor (FLT3), the receptor for macrophage colony-stimulating factor (CSF-1R), and glial cell-line-derived neurotrophic factor receptor (RET) (Kim *et al.*, 2014). Sunitinib also act as ATP-competitive inhibitors which effectively inhibits phosphorylation of Ire1α, thus consequent to RNase activation (Ali *et al.*, 2011). All these tyrosine kinases signalling pathway are associated in the pathogenesis of breast cancer (Butti *et al.*, 2018).

Sunitinib can suppress tumour growth by inhibiting tumour angiogenesis. The efficacy of Sunitinib has been demonstrated in patients with gastrointestinal stromal tumours (GIST) and renal cell carcinoma (Mulet-Margalef and Garcia-Del-Muro, 2016; Rizzo and Porta, 2017). Sunitinib also has been shown to extend progression

free survival and overall survival in patients with metastatic renal cell carcinoma (mRCC) and is now used as first line treatment for this disease (Rini *et al.*, 2018).

In short of mechanism of action of Sunitinib (Figure 2.10) (Delbaldo *et al.*, 2012), Sunitinib penetrate into the cytoplasm and enters into competition with ATP for the VEGFR ATP-binding pocket. The activated VEGFR can no longer activate its intracellular kinase domain, thus preventing further downstream cell signalling (B). However, in comparison absence of Sunitinib, the binding of vascular endothelial growth factors (VEGFs) to VEGFR leads to the dimerization of VEGFR and the activation of the intracellular kinase domain of VEGFR. The activation of VEGFR involves the presence of adenosine triphosphate (ATP), thus activate signal transduction of cell (A).

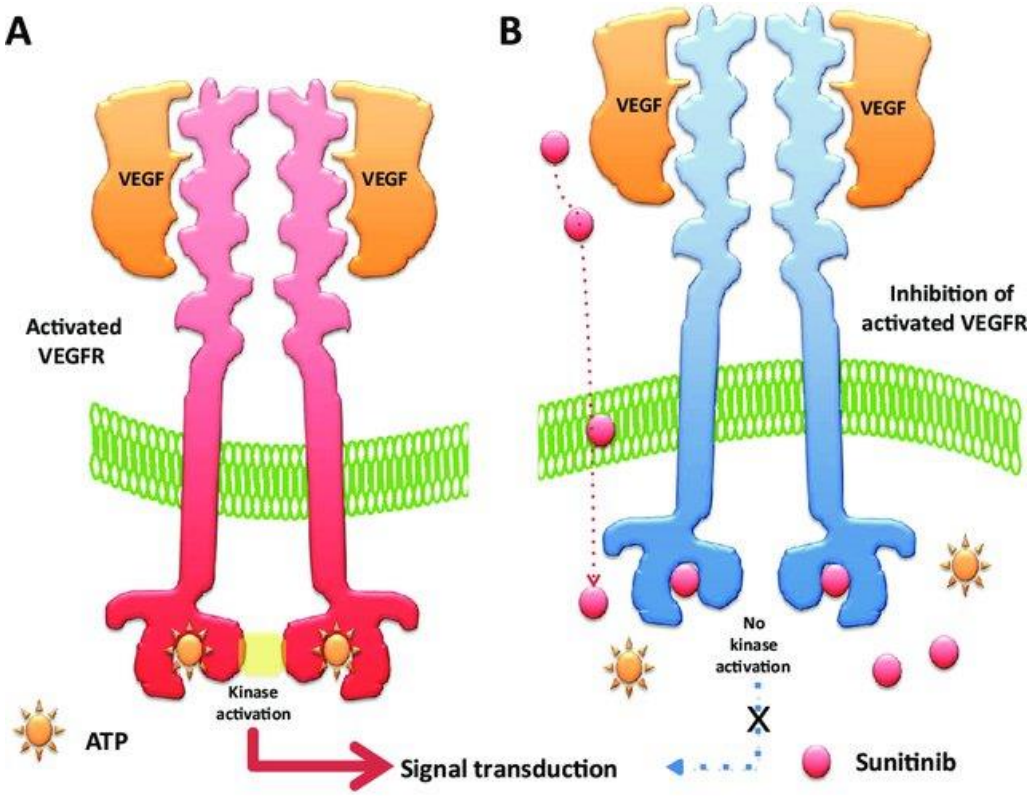

Figure 2.10 Mechanism of action of Sunitinib in endothelial cells expressing the vascular endothelial growth factor receptors (VEGFRs)

For breast cancer, a xenograft study has proved that Sunitinib inhibit angiogenesis in breast cancer. In the first and second phase of preclinical studies of Sunitinib has demonstrated modest monotherapy effect (Burstein *et al.*, 2008b; Kozloff *et al.*, 2007). In consequent third phase of clinical trials, Sunitinib also failed to increase survival of metastatic breast cancer (Crown *et al.*, 2013), thus further as targeted combination treatment in breast cancer.

2.4 NMU induced mammary carcinoma

The N-methyl-N-nitrosourea (NMU) also known as 1-methyl-1-nitrosourea (MNU) is an N-nitroso compound ("Nomenclature of Organic Chemistry," 2014). NMU is potent mutagens and carcinogens which can alter the DNA structure that are left damaged. The accumulation of damaged DNA can cause DNA mutations and finally develop cancer risk (Faustino-Rocha *et al.*, 2015). NMU has never been produced in commercial quantities; therefore, no human case reports or epidemiological studies are available (Tsubura *et al.*, 2011). In addition, when the DNA damage is very severe, NMU acts as a cell-disrupting agent that can causes cell death in subjected organs and tissues. NMU induced mammary cancer model is relevant to human disease and can be used for therapeutic trials purposes (Faustino-Rocha *et al.*, 2015).

The NMU-induced mammary carcinoma model is frequently used to screen and assess the potency of cancer suppressor or inducer for the breast cancer treatment research (Liu *et al.*, 2015). NMU is a highly specific carcinogen with no metabolic activation required for the breast cancer carcinogenesis to occur. NMU-induced breast cancer development by increasing the expression level of estrogen and progesterone receptor.

In addition, the NMU-induced rat breast cancer seems similar to human breast cancer. The NMU induced is originally developed tumour from terminal end buds of the terminal ductal lobular unit. NMU induction resulted in similar morphology of the breast tumour and the pre-invasive stage (hyperplasia, ductal carcinoma in situ) as in human (Saminathan *et al.*, 2014). Thus, this model is suitable for this *in vivo* study.

2.5 Mammary carcinoma in rats model

Rat is the major murine species used in the fundamental study as well as in prevention and treatment of breast cancer research. Rats are free from murine mammary tumour virus (MMTV) with highly susceptible to various carcinogen agents (Russo, 2015). Rats have a high frequency of hormone-dependent tumours that are ductal in origin (Rajmani *et al.*, 2011). According to Tsubura *et al*. (2011), NMU-induced mammary carcinoma is age dependent; rats that are between 3 and 7 weeks of age are most susceptible to NMU (Tsubura *et al.*, 2011). Mammary tumours can be easily induced by NMU with no need for irradiation. It is easy to prepare an injectable NMU solution because it is water soluble. The intraperitoneal (i.p.) route is the simplest way to administer NMU to animals (Saminathan *et al.*, 2014).

Thompson and his colleagues experienced mammary tumorigenesis was NMU dose-dependent. At low dosage as 25 mg/kg body weight NMU administration were grown both benign and malignant tumours. Induction of NMU intraperitoneally at the dose at 50 mg/kg body weight and above resulted 100% malignant tumours with latency period as short as 28 days (Thompson *et al.*, 1992). However, most animal model for breast cancer applied NMU system work at dosage of 50 mg/kg

body weight (Liska *et al.*, 2000; Shilkaitis *et al.*, 2000; Thompson and Adlakha, 1991; Thompson *et al.*, 1998). Histology of mammary malignancy was identified both adenocarcinomas and papillary carcinomas, whilst benign as fibroadenomas, fibromas, and adenomas (Thompson and Adlakha, 1991). Other variants of carcinomas that are seen in humans have not been observed in the rat tubular carcinoma such as colloid or mucinous carcinoma, adenoid cystic carcinoma etc. instead of invasive adenocarcinoma seen such as cribriform, comedo, and papillary (Thompson *et al.*, 2000).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Design

The idea of this *in vivo* study was started by inducing carcinogenic chemical (NMU) to develop breast tumorigenesis in rodent, followed by classifying histological subtypes of tumour tissue and observing the efficacy of targeted therapy treatment on breast tumour receptors. The study design was summarized as shown in Figure 3.1.

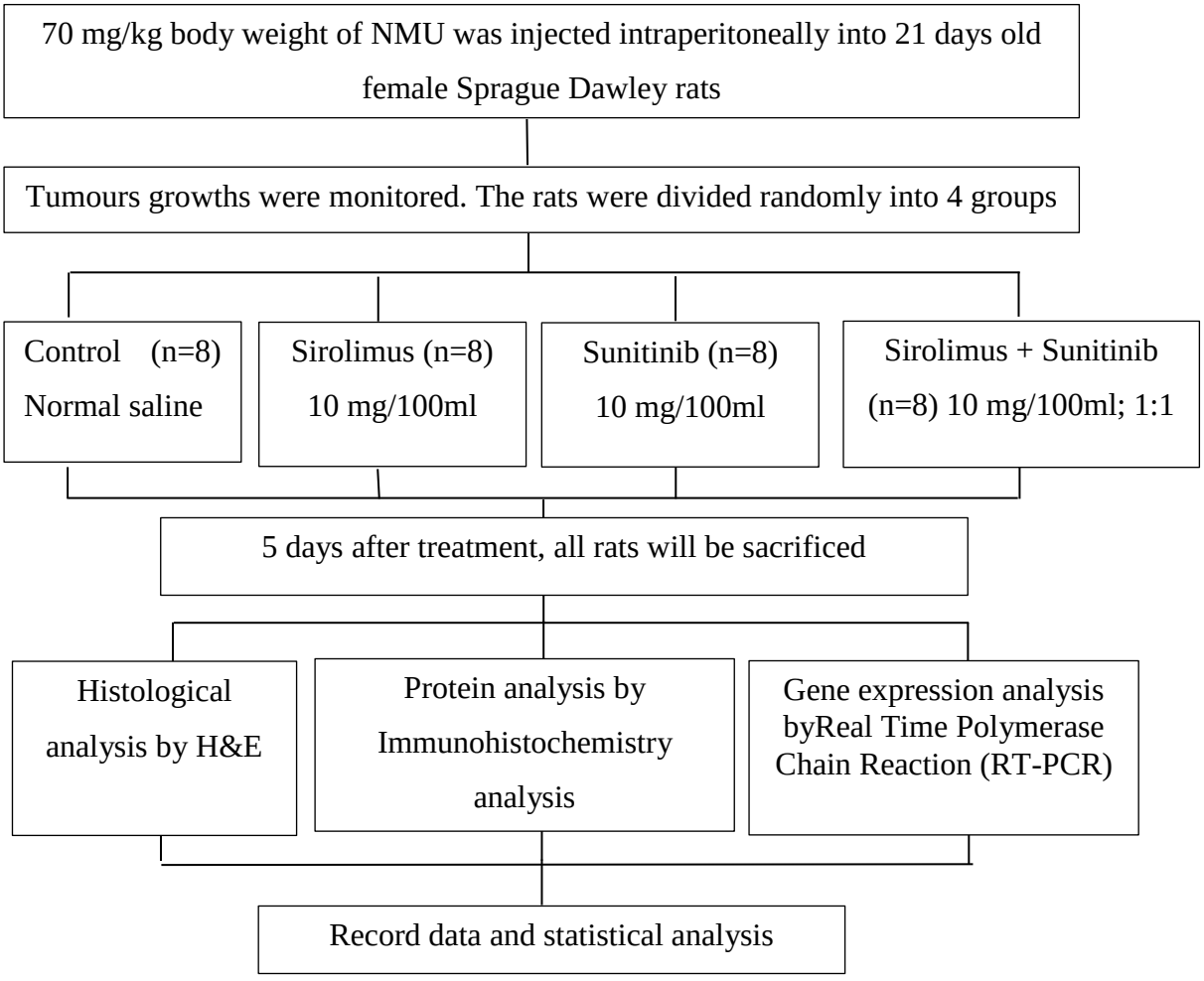

Figure 3.1 Flowchart of Study Design

3.2 Reagents and materials

3.2.1 Reagents and materials for mammary tumour induction and interventions

The materials needed for inducing mammary tumour and treatment were vernier calliper, BD Luer-Lok 1 ml syringe, sterilized surgical tools, electrical shaver, gauze, 1.5 mL tube, aluminium foil, ice, NMU solution, Sirolimus, Sunitinib, 0.9% sodium chloride (NaCl), DMSO, ethanol, PEG300 solution and PEG (80) solution.

3.2.1(a) Preparation of NMU solution

NMU (Cat. No. M325815, Toronto Research Chemicals, Canada) was freshly prepared before injection based on individual body weight of the rats. Seventy milligram per kilogram body weight of NMU was homogenously dissolved in 0.9% normal saline followed by mild heating in water bath and vigorous shaking using vortex (Jaafar *et al.*, 2009). The 1.5 mL tube containing NMU solution was wrapped with aluminium foil due to NMU was highly light sensitive.

3.2.1(b) Preparation of Sirolimus solution

Sirolimus (Cat. No. HY-10219, MedChemExpress, USA) was prepared to final dosage of 20 µg/0.2 ml dosage per intralesional injection (Al-Astani Tengku Din *et al.*, 2014). 0.1 mg of Sirolimus powder was dissolved by adding one by one solvent of 10% DMSO, 40% PEG300, 5% PEG (80) and 0.9% normal saline to make up 1 ml solution. Since the PEG (80) solution is a light-sensitive chemical, the Sirolimus working solution was covered with aluminium foil and kept on ice until treatment process.

3.2.1(c) Preparation of Sunitinib solution

Sunitinib Malate (SU 11248 Malate) was purchased from MedChemExpress, USA (Cat. No. HY-10255). The 100 µg/ml Sunitinib solution was freshly prepared by dissolving 0.1 mg of yellowish Sunitinib powder in 1000 µl solvent of 10% DMSO, 40% PEG300, 5% PEG (80) and 0.9% normal saline. The solvent was added one by one and vortex for fully dissolved. Solution preparation was done on ice and the tube was covered with aluminium foil to avoid light exposure.

3.2.1(d) Preparation of 10% (V/V) DMSO

One ml of DMSO was mixed with double distilled water to the final volume of 10 ml for preparation of 10% DMSO solution.

3.2.1(e) Preparation of 40% (V/V) PEG300

Forty percent of PEG300 solution was prepared by dissolving 4 ml of PEG300 in 6 ml of distilled water.

3.2.1(f) Preparation of 5% (V/V) PEG (80)

Five millilitre of PEG (80) was dissolved in 95 ml double distilled water to make up 100 ml of final volume.

3.2.2 Reagents and materials for Histology analysis

Materials for histopathological analysis (Hematoxylin & Eosin staining) were cassettes, forceps, slides, cover slips, staining jars, slide staining rack, mounting medium, 10% normal buffered formalin, paraffin wax, xylene, 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, distilled water, Harris Hematoxylin solution, Eosin Y solution, 1 % acid alcohol, and 0.2% ammonia water.

3.2.2(a) 10% Neutral Buffered Formalin (NBF) solution

The pre-mix 10% Neutral Buffered Formalin (NBF) solution (Cat. No. 5701, Richard-Allan Scientific, USA) was aliquoted evenly into graduated container for tissue fixation.

3.2.2(b) Preparation of Harris Hematoxylin working solution

Harris Hematoxylin commercially prepared solution (Cat. No. 3136, Sigma-Aldrich, Germany) was filtered by using filter paper before used.

3.2.2(c) Preparation of Eosin working solution

Commercially prepared Eosin working solution (Sigma, USA) was filtered by using filter paper prior to use.

3.2.2(d) Preparation of different percentage of ethanol

The 95% (V/V) ethanol was prepared by diluting 950 ml absolute ethanol in 50 ml distilled water. The 80% (V/V) ethanol was prepared by diluting 800 ml absolute ethanol in 200 ml distilled water. The 70% (V/V) ethanol was prepared by diluting 700 ml absolute ethanol in 300 ml distilled water. The 50% (V/V) ethanol was prepared by diluting 500 ml absolute ethanol in 500 ml distilled water.

3.2.2(e) Preparation of 1% (V/V) acid alcohol

Ten millilitre of concentrated hydrochloric acid (HCl) was mixed with 700 ml absolute ethanol. The solution was then diluted with distilled water to the final volume of 1000ml.

3.2.2(f) Preparation of 0.3% (V/V) ammonia water

Three millilitre of concentrated ammonia (NH_4) was diluted with one litre of distilled water to produce 0.3% ammonia water.

3.2.3 Reagents and materials for protein expression analysis

Reagents and materials for immunohistochemistry analysis were poly-L-lysine coated slides, PAP pen (Cat No. ab2601, Abcam), cover slips, mounting medium, absolute ethanol, 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, xylene, Washing buffer of 1X TBS Tween-20 solution, 3% perhydrol solution, 1X citrate buffer, 1X Tris EDTA buffer, primary antibodies, antibody diluent of Large Volume UltraAb Diluent Plus kit, secondary antibody of Ultra Vision ONE Large Volume Detection system HRP Polymer kit and DAB Plus substrate system.

3.2.3(a) Preparation of washing buffer

1X TBS/0.1% (V/V) Tween-20 (1X TBST) washing buffer was prepared by dissolving 100 millilitres of 10 X Tris Buffered Saline solutions (Cat No. T5912, Sigma Aldrich) in 900 ml distilled water to yield 1X Tris Buffered Saline (20 mM Tris, pH 8.0, and 0.9% NaCl) at 4 °C. 1 ml of Dako Tween-20 (Cat No. S196630-2, Agilent) was then added to 1X TBS and mixed well.

3.2.3(b) Preparation of different concentration of ethanol

95%, 80%, 70%, and 50% (V/V) ethanol were prepared using the same method as in H&E before.

3.2.3(c) Preparation of 3% (V/V) perhydrol

3% (V/V) perhydrol was prepared by adding 10 ml 30% perhydrol solution, H_2O_2 (Cat No 107209, Merck) to 90 ml distilled water.

3.2.3(d) Preparation of 1X Citrate Buffer (10mM Citric Acid, 0.05% (V/V) Tween 20, pH 6.0)

1.92 gram of Citric acid (anhydrous) powder (Cat No 100241, Merck) was dissolved in one litre distilled water and mixed well. The pH was adjusted to 6.0 by

adding 1N Sodium Hydroxide (NaOH) drop by drop, followed by adding 0.5 ml of Tween 20 and was then mixed well. The buffer was stored at 4ºC for longer storage.

3.2.3 (e) Preparation of Tris-EDTA Buffer

1.21 gram of Tris Base powder (Cat No 648311, Merck) and 0.37 gram of disodium salt EDTA (Cat No 324503, Merck) was dissolved in one litre distilled water and mixed well. The pH was adjusted to 9.0 by 1N hydrochloric acid (HCl). 500 µl of Tween 20 was then added and mixed to form the final working solution of Tris-EDTA Buffer contains 10mM Tris Base, 1mM EDTA Solution, and 0.05% Tween 20. Store this buffer at 4º C.

3.2.3(f) Preparation of primary antibodies

The primary antibodies of Rabbit polyclonal to Estrogen Receptor alpha (Cat. No. ab75365), Rabbit polyclonal to Progesterone Receptor (Cat. No ab191138), and Rabbit polyclonal to ErbB 2 or HER2/neu (Cat. No ab47262) (Abcam, UK) were diluted by using antibody diluent (Cat No 00-3218, Invitrogen, USA) followed the dilution factor of 1: 100, 1: 200 and 1:100 respectively.

3.2.3(g) Preparation of Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse

EnVision Systems are based on dextran polymer technology which permits binding of a large number of enzyme horseradish peroxidase to a secondary antibody via the dextran backbone. Dako kit of REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse were consists of 3 bottles.

Bottle A was ready-to-use 100 mL Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV). This buffer was against rabbit and mouse immunoglobulin, consisted of dextran coupled with peroxidase molecules and goat secondary antibody

molecules. Bottle B was 250 mL Dako REAL™ Substrate Buffer. This solution contained hydrogen peroxide (H_2O_2) and preservative. Bottle C was 5 mL, 50x concentrated Dako REAL™ DAB+ Chromogen

Before used, DAB+ Chromogen was diluted in Substrate Buffer in a dropper bottle. The DAB-containing Substrate Working Solution was freshly prepared by mixing thoroughly 20 µL Dako REAL™ DAB+ Chromogen (Bottle C) and 1 mL Dako REAL™ Substrate Buffer (Bottle B). The Substrate Working Solution must be used within 5 days and stored away from light at 2–8 °C. The substrate system produced a crisp brown end product at the site of the targeted antigen.

3.3 Methodology

3.3.1 *In vivo* study

3.3.1(a) Animal preparation

32 female of Sprague Dawley rats were acquired from the Animal Research and Service Centre (ARASC), USM. The rats were then caged in environmentally controlled conditions (temperature 23 ± 2 °C, relative humidity 70 ± 5%, and alternate 14 h day 10 h night cycle) one to three rats per cage in polycarbonate cages with wood chip bedding (Figure 3.2). They were fed with food pellets and tap water *ad libitum*. The care and use of animals for research was conducted with the proper code of practice for research in compliance with applicable national and USM laws and regulations governing the use of animals, with supervision and husbandry facilities provided by ARASC (USM/IACUC/2017/(108)(876).

Figure 3.2 Rats in polycarbonate cages

3.3.1(b) Tumour Induction and Detection

The NMU at a dose of 70 mg/kg body weight was injected intraperitoneally two times (Figure 3.3). The first NMU injection was administrated when the rat's age were 21 days old, followed by second injection at the alternate days. The rats were administered with NMU at 21 days old due to at the younger age, the TDLU of rats were susceptible to NMU for promoting mutation and induce carcinogenesis. The rats were weighed daily and palpated once a week for the detection of breast tumours. The mammary lesions growths were observed and their diameter size was measured by using Vernier calliper, and recorded (Figure 3.4). The symptoms of illness or side effects which may cause by NMU toxicity were also observed.

Figure 3.3 Intraperitoneal injection of NMU

Figure 3.4 Measure tumour size by using vernier calliper

3.3.1(c) Experimental Design

All rats were randomly grouped into four groups. Group Control (n=8) served as an untreated control group and were sacrificed after 5 days injection with physiological normal saline (used as a placebo) at size of 14.5 ± 0.5 mm. For the treated groups, the rats were anesthetized by inhaled anaesthetics Isoflurane (Figure 3.5). Then, the rats in Group Sirolimus (n=8) were treated with Sirolimus, Group Sunitinib (n=8) with Sunitinib, and Group Sirolimus + Sunitinib (n=8) with Sirolimus and Sunitinib via an intratumoral injection (Figure 3.6) when the tumour lesions reached diameter size of 14.5 ± 0.5 mm. 14.5 ± 0.5 mm size was choose due to NMU induced breast cancer show peak aggressiveness on this size with clear vascularization and histologically start developed the papillary and NST histological patterns. The tumours were treated twice for alternate days. Intratumoral administration of treatment was chosen to deliver the drug directly into an established mammary carcinoma and spare the host from systemic adverse effects. Intratumoral injection of treatment into breast tumours was choose due to it was safe, feasible, and provide the opportunity to evaluate the direct effects of therapy onto solid breast tumour (Tchou *et al.,* 2017). The diameter of tumours were measured using Vernier calliper after first treatment injection and second treatment injection, and the readings were recorded. The treatment solutions were freshly prepared prior to injection and kept on ice until intervention process. The rats in Group Sirolimus-treated, Sunitinib-treated and Sirolimus + Sunitinib treated groups were euthanized when the lesions regressing post 5 days of second treatment injection.

Figure 3. 5 Anesthetize the rat by inhaled anaesthetics Isoflurane

Figure 3.6 Intratumoral treatment injections.

3.3.1(d) Tumour samples collection

After reaching endpoints, rats were euthanized through exposure to 100% carbon dioxide gaseous in a closed plastic bag (Figure 3.7). The final diameters of the tumours were measured and recorded (Figure 3.8). All grossly visible breast tumours and normal breast pad were removed. A portion about 5 mm of each tumour sample was fixed in RNA later solution while the remaining was fixed at room temperature in 10% normal buffered formalin (NBF). Tumour tissues were fixed in 10% NBF for at least 24 hours at room temperature to allow the NBF to penetrate into every part of the tissue and to allow the chemical reactions of fixation to reach equilibrium. Sufficient fixation was important to preserve the tissue structure, prevent tissue degradation, stop cellular processes, and kill pathogens within tumour lesions to get the ideal histology result. The tissues were automated processed in tissue processor machine provided in Pathology Laboratory, and embedded in paraffin for further histological analysis. Then, all tissues were sectioned and coloured with Hematoxylin and Eosin staining.

Figure 3. 7 Euthanize process through exposure to carbon dioxide gaseous in a closed plastic bag

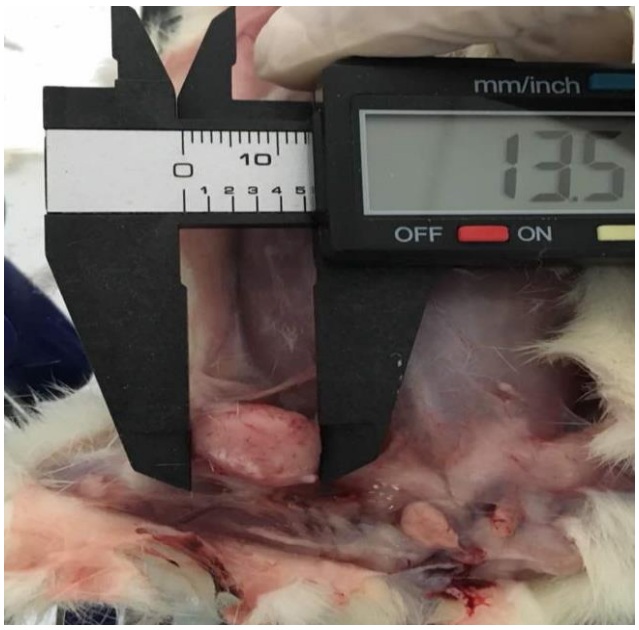

Figure 3. 8 Measuring of the final diameters of the tumours

3.3.2 Histological study

According to Anderson (2011), histological study required a sequence of processes starting with the preparation of tissue sample for histological staining. The process takes five key stages which involved; fixation, processing, embedding, sectioning and staining (Anderson J., 2011). After tissues getting adequate fixation in 10% normal buffered formalin, the tissues were processed and embedded in paraffin, being sectioned and were stained with Hematoxylin and Eosin staining. Slide readings and histological analysis were conducted and supervised by two pathologists.

3.3.2 (a) Fixation, tissue grossing, and tissue processing

The tumour tissues were fixed in 10% NBF, then, were grossed to appropriate size and areas. The specimens were then placed in suitable labelled cassettes and subjected to tissue processing procedures by using an automated fully enclosed system of tissue processor (Leica ASP300S, USA). The automated tissue processing procedure started with fixation (10% formalin), followed by dehydration in a series of graded ethanol (80%, 95%, and absolute ethanol), clearing in xylene and finally completed with cleaning ethanol and distilled water. The summary of tissue processing six hour schedule is listed in Appendix A in appendix section.

3.3.2(b) Tissue embedding and sectioning

The excised tissue were then processed and embedded in paraffin wax. The embedding process was done conventionally using tissue embedding machine (Tissue-Tek TEC 6 Embedding Console System, Sakura Finetek USA) provided in Pathology Laboratory. The mould was prefilled with paraffin wax, and the tissue was introduced into the mould by using warm forceps. Gentle amount of pressure was channelled to ensure evenly distribute surface followed with chilling step on the cold

plate. A cassette base was inserted onto the mould, and remaining spaces were filled with additional paraffin wax. The block was then been cooled on the -15 ºC cold plate until the block could be removed from the mould.

The formalin fixed paraffin embedded (FFPE) tissue blocks were trimmed (10 µm) and sectioned using a microtome to obtain 3 µm thick tissue sections. The ribbons of sectioned tissue were floated on the water in water bath at the temperature between 41 to 42^{o}C subsequently loaded onto two types of microscopic glass slide which are standard microscopy frosted end glass slide for H&E staining and Poly-L-Lysine slides for the immunohistochemistry staining.

3.3.2 (c) Harris Haematoxylin and eosin staining

Before staining, the tissues were de-paraffinized first. This step was important to remove paraffin wax from the tissues and to attach the sections completely on the slides. Prior to de-paraffinization, the 3 µm thick tissue sections on slides were heated on a hot plate at 60^{o}C for 30 minutes to melt the wax. This was followed by de-paraffinizing step by immersing them into two changes of xylene each for 5 minutes to solubilize and remove the paraffin. Next, the xylene is removed by graded washes with xylene and ethanol. Finally, the sample is rehydrated through graded concentrations of ethanol in water, ending in a final rinse in water.

The rehydration process was commenced by immersing the tissues in descending concentrations of ethanol, 2 minutes for each step including two changes in absolute ethanol for 1 minute, one time for 95% ethanol (1 minute) and 80% ethanol (1 minute). The section was now hydrated so that aqueous reagents will readily penetrate the cells and tissues elements.

The sections subsequently immersed in deionised water for 1 minute before stained in Harris Hematoxylin solution for 5 minutes. Harris Hematoxylin which consists of a dye (oxidized hematoxylin) and a binding agent (aluminium salt) in solution was specifically used for nuclear staining with reddish-purple colour. The tissue sections were then washed in running tap water for 5 minutes.

Next, the sections were differentiated by immersing the sections in 1% acid alcohol for 5 seconds followed by rinsing under running tap water. Differentiation step is required to take out excess Harris Hematoxylin from the tissues components in order to remove non-specific background staining and to improve contrast. After rinsing under running tap water for 5 minutes, the sections were blued for 10 seconds in 0.3% ammonia water. The sections were rinsed again under running tap water for 5 minutes and counterstained in Eosin solution for 2 minutes. Eosin counterstain stained many non-nuclear elements in different shades of pink colour.

The sections were then dehydrated for 1 minute immersion in ascending concentration of ethanol, one time for 80% and 95% ethanol and two changes of absolute ethanol. Next, the sections were cleared in two changes of xylene. Finally, they were dried and mounted with mounting media. Finally, coverslips were applied to cover the tissue for better viewing under microscope, to decrease the rate of evaporation from the sample, and to protect the sections from contamination by airborne particles.

3.3.2 (d) Tumour classification

Bloom-Richardson grading scheme were used for classification and grading of the breast carcinomas (Bloom and Richardson, 1957). The slide readings and histological evaluation were conducted under supervision of pathologists.

3.3.3 Immunohistochemical staining

The study of protein expression was carried out by using immunohistochemistry (IHC) technique due to IHC will show the expression and localization of the protein in a specific tissue. In this study, the mammary tumour samples which had been fixed, processed, paraffin embedded, and sectioned for histological assessment were subjected to IHC staining.

3.3.3(a) Tissue preparation for immnohistochemical staining

The FFPE tumour tissues preparation for IHC was briefly shown in the section 3.3.2.1 and 3.3.2.2.

3.3.3(b) Immunohistochemistry procedure

First, the slides were de-paraffinized for 30 minutes on a 60°C hotplate followed by clearing twice in xylene for 5 minutes. The tissues were then rehydrated in ethanol of decreasing concentration of ethanol, began with two changes of absolute ethanol, followed by 95%, 80%, 70% and 50% and distilled water.

Heat-induced epitope retrieval was then performed by boiling the tissue in the buffer of 1X Citrate pH 6.0 or 1X Tris-EDTA pH 9.0 using pressure cooker according to the preference of respective antibodies. Subsequently, endogenous peroxidase activity was quenched using 3% H_2O_2 in methanol for 15 minutes at room temperature. The tissues were then washed and rinsed by using TBS-tween 20 washing buffer.

After endogenous peroxidase blocking, the antigens were retrieved for 5 minutes incubation of Ultra V Block at room temperature to block the non-specific binding. The tissues were then incubated with representative primary antibodies as listed in Table 3.1 and then washed three times with 1X TBS-Tween 20.

Table 3. 1 Staining protocol of IHC

Primary antibody	Dilution	Antigen retrieval	Incubation time
Estrogen receptor (ER)	1:100	Tris-EDTA (pH 9)	2 hour
Progesterone receptor (PgR)	1:200	Citrate buffer (pH6)	2 hour
HER2/neu	1:100	Tris-EDTA (pH 9)	1 hour

Subsequently, immunoreactivity of the antibodies was determined by incubating the tissue sections with commercially available detection kit, UltraVision ONE Large Volume Detection System HRP Polymer (Ready-To-Use), followed by three times rinsing with 1X TBS-Tween 20. The expression was visualized using DAB Plus Substrate System as chromogen and counterstained with Harris Hematoxylin solution. Finally, the tissue sections were dehydrated in ascending concentrations of ethanol, cleared in xylene and mounted.

Positive tissue control is a specimen previously shown to stain specifically for the target antigen after exposure to primary antibody. It will be advantageous to monitor the presence of the antigen and determine any possible loss of sensitivity due to varies staining intensity with the degree of tumour differentiation. Therefore, positive control sample consisting tissues known to express ER, PgR and HER2/neu was included with each immunohistochemical staining batch. The tissues used for positive control in the study were breast cancer for all antibodies. For the negative control, the primary antibody was omitted and included also in every staining batch.

3.3.3(c) Immunohistochemistry scoring

The positivity of staining for all antibodies was evaluated by using a light microscope (Nikon, Japan), according to the brown DAB chromogen reaction uptake under 40X magnification. Scoring was performed in a double-blind manner by three independent investigators supervised by pathologist. Any disagreement was resolved by discussion to obtain a final score.

The expression of nucleus staining of ER and PgR were assessed using a semi-quantitative scoring system (Allred et al., 1998). Through this system, the final score ranged between 0-8 was obtained by the sum of proportion score and intensity score for 100 cells in 5 hot spots (Table 3.2). Briefly, the proportion score is an estimation of the proportion of positive cells from 100 cells (scored on a scale of 0-50), divided into the following categories: 0= no cells stained; 1= less than 1%; 2= 1% to 10%; 3= 11% to 33%; 4= 34% to 67%; and 5= more than 67%. Meanwhile, the intensity score is the average staining intensity of positive tumour cells (scored on a scale of 0-3): 0 = negative; 1= weak; 2= moderate; and 3= intense.

Table 3.2 Guidelines of scoring ER and PgR by Allred scoring system

Proportion score (PS)	**Positive cells**	**Intensity**	**Intensity score (IS)**
0	0	None	0
1	<1%	Weak	1
2	1% to 10%	Moderate	2
3	11% to 33%	Intense	3
4	34% to 67%	Allred score = PS + IS	
5	≥67%		

Membrane and/or cytoplasmic staining for HER2/neu antigen were scored using American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guideline (Wolff *et al.*, 2018). HER2/neu test result was positive for HER2/neu 3+ based on circumferential membrane staining that is complete, intense. HER2/neu 2+ was equivocal based on circumferential membrane staining that is incomplete and/or weak/moderate and within >10% of the invasive tumour cells; or complete and circumferential membrane staining that is intense and within ≤10% of the invasive tumour cells. HER2/neu test result is negative if; IHC 1+ as defined by incomplete membrane staining that is faint/barely perceptible and within >10% of the invasive tumour cells and; IHC 0 as defined by no staining observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤10% of the invasive tumour cells.

3.3.4 Gene Expression Study

The gene expression of ER (Esr1), PgR and HER2/neu (Egfr) were determined using quantitative Real-time polymerase chain reaction (q-PCR). After extraction, the tumour RNA was converted to complimentary DNA (cDNA) and served as a template for qRT-PCR reaction.

3.3.4(a) RNA Extraction

RNA extraction was performed using innuPREP RNA Mini Kit 2.0 (Analytik Jena, Germany) following the extraction kit protocol. The general procedure of RNA extraction by using the extraction kit is homogenization, selective removing of genomic DNA, selective binding of RNA onto filter, washing of RNA, and finally elution of RNA.

For homogenization of tumour tissue, initially, 20 mg of tumour tissues were weighed and grinded using pestle and mortar to a fine tissue powder under liquid nitrogen. The powder was then transferred into 1.5 ml reaction tube, 450 µl Lysis Solution RL was added. The sample was incubated for 5-30 minutes to allow a further lysis by permitting complete dissociation of the nucleoprotein complex under continuous shaking.

The material was then centrifuged at maximum speed for 1 minute to spin down the unlysed material. After placed the Spin Filter D (provided by kit) into a Receiver Tube, the supernatant of the lysed sample was transferred onto the Spin Filter D. The sample was centrifuged at 11,000 rpm for 2 minutes to remove genomic DNA. The Spin Filter D was then discarded and placed a Spin Filter R. Spin Filter R was used for selectively binding the RNA.

For RNA isolation procedure, RNA was washed by adding 400 µl of 70 % ethanol to the filtrate. The sample was mixed by pipetting up and down several times. The sample was then transferred onto the Spin Filter R and centrifuged at 11,000 rpm for 2 minutes. 500 µl of Washing Solution HS was added onto the Spin Filter R to wash the RNA, and centrifuged at 11,000 rpm for 1 minute. The Receiver Tube with the filtrate was discarded, and the Spin Filter R was placed into a new Receiver Tube. 700 µl Washing Solution LS was added into the Spin Filter R and centrifuged at 11,000 rpm for 1 minute. The Receiver Tube with the filtrate was discarded, and the Spin Filter R was place into a new Receiver Tube.

The Spin Filter R was centrifuged again at 11,000 rpm for 2 minutes to remove all traces of ethanol. The Spin Filter R was placed into an Elution Tube and 30–80 µl RNase-free Water was added to elute the RNA. The eluted RNA was then

incubated at room temperature for 1 minute and centrifuged at 11,000 rpm for 1 minute. The eluted RNA was stored at -80°C in the Elution Tube until use.

To assess the purity of RNA, the ratio of absorbance at 260 nm and 280 nm was observed by using NanoDrop ND-8000 spectrophotometer. A ratio of ~1.8 to ~2.0 is generally accepted as "pure" for RNA and will be used for further process.

3.3.4(b) cDNA synthesis

cDNA synthesis was done using SuperScript™ IV First-Strand Synthesis System (Invitrogen, USA). The SuperScript™ IV First-Strand Synthesis System for RT-PCR is optimized for synthesis of first-strand cDNA from total RNA. For complete cDNA reaction components, the RNA was mixed with the RNA-primer mix and Master Mix in RNAse free tube on ice by mixing the components according to manufacturer's guidelines:

The first step is annealing primer to template RNA. The following components were combined in PCR reaction tube, mixed and briefly centrifuged before heat the RNA-primer mix at 65°C for 5 minutes, and then incubated on ice for 1 minute. The RNA-primer mix components are:

- 50 µM Oligo d(T)20 primer = 1 µl
- 10 mM dNTP mix = 1 µl
- Template RNA (500 µg) = 2 µl
- DEPC-treated water = 9 µl

Then, the RT reaction mix was prepared followed the manufacturer protocol. The following components were combined, mixed, and briefly centrifuged in reaction tube. The RT reaction mix components are:

- 5× SSIV Buffer = 4 µl
- 100 mM DTT = 1 µl
- Ribonuclease Inhibitor = 1 µl
- SuperScript™ IV Reverse Transcriptase (200 U/µL) = 1 µl

The annealed RNA and RT reaction mix was combined to make the final volume for the mixture was 20µl per cDNA synthesis reaction. The mixture was mixed gently and incubated at 50^{o}C for 10 minutes followed by inactivating the reaction by incubating it at 80°C for 10 minutes. The reaction was terminated at 85^{o}C for 5 minutes and chilled on ice for 10 minutes. Then, 1 µL E. coli RNase H was added to the mixture and incubated at 37^{o}C for 20 minutes to remove the RNA template from the cDNA:RNA hybrid molecule after first-strand synthesis, thus increase the sensitivity in Real-time PCR. The cDNA was stored at -20^{o}C until further use.

3.3.4(c) Primer design and quantitative Real-Time Polymerase Chain Reaction (q-PCR)

The primers and probe used in this study was designed using Primer3web version 4.1.0 (Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M and Rozen SG. Primer3--new capabilities and interfaces. Nucleic Acids Res. 2012 Aug 1;40(15):e115). The primers and probe are listed in Table 3.3.

The primers and probe concentrations used in Real-Time PCR were optimized according to the manufacturer's protocol provided by Applied Biosystems. In addition, ß-actin, a constitutively expressed housekeeping gene of high abundance was used for normalization of target genes.

For a complete one quantitative RT-PCR assay component, the cDNA was mixed with SensiFAST™ Probe Hi-ROX master mix (Bioline, UK), 0.5µM of each primer, and 0.25µM of the probe in RNAse free tube on ice.

- SensiFAST™ Probe Hi-ROX master mix = 10 µl
- Forward primer = 0.8 µl
- Reverse primer = 0.8 µl
- Probe = 0.2 µl
- cDNA template = 1.0 µl
- Nuclease free water = 7.2 µl

The final volume for the mixture was 20µl per assay. The RT- PCR was undergone by using the Applied Biosystems™ StepOne™ Plus (PE Applied Biosystems, Foster City, CA). Negative control reactions were routinely run without cDNA template by replacing with 1 µl nuclease free water.

The thermal cycling started with 1 cycle of polymerase activation for 3 minute at 95°C, followed by 40 cycles of 10 seconds at 95°C (denaturation) and 60 seconds at 60°C for annealing. The expression of Esr1, PgR and Egfr was determined via comparative C_T method in the PCR system, where the amount of ER, PgR and HER2/neu was normalized to the reference gene Actb and a relative calibrator, $2^{-\Delta\Delta C}{}_T$ (Livak and Schmittgen, 2001). The summary of the relative expression level of transcripts in experimental groups compared to untreated control group after normalization with ß-actin was computed by mathematical model of REST-MCS.

Table 3. 1 List of primers and probes sequences used in Real-Time PCR

Targeted gene	GenBank Accession No.	Primers Sequences		Probe Sequence (5' to 3')
		Forward (5' to 3')	Reverse (3' to 5')	
ER	NM_012689.1	TCGCTACTGTGCTGTGTGTA	GCCTTTCATCATGCCCACTT	AGATGACTTGGAAGGCCGAA
PgR	NM_022847.1	GGATTTCATTCACGTGCCCA	GCTGGAATTCGCCGTAAACT	CCAGAAGGCGACCCTAAAGA
HER2/neu	NM_031507.1	CTGATAGCCGCCCAAAGTTC	TCAAGAGTGGAAGTCCGTGAC	GCACAAGTAACAGGCTCACC
Reference gene				
Actb	NM_031144	TCTTCCAGCCTTCCTTCCTG	CACACAGAGTACTTGCGCTC	GGCTCCTAGCACCATGAAGA

3.4 Calculation of sample size

The sample size calculation was carried out by using G*Power Version 3.1.9.7 software (Faul *et al.*, 2009). The alpha value was set as standard (0.05), power (0.80), and effect size of 2. The sample size of each group was 8 rats per group. Considering 20% dropout, 2 additional rats had been added into each group in the process of animal ethical approval. From total of 40 rats approved, the number of rats was allocated as follows:

(a) Untreated control group (n= 8)

(b) Sirolimus-treated group (n= 8)

(c) Sunitinib-treated group (n= 8)

(d) Sirolimus + Sunitinib treated group (n= 8)

3.5 Statistical analysis

Significant differences for histopathological characteristic, protein, and gene expression between the treatments groups were determined by the non-parametric Kruskal–Wallis test followed by Mann-Whitney test with Bonferroni correction for multiple testing. Statistical analyses were performed using SPSS for Windows, version 26.0 (SPSS Inc, Chicago, IL., USA). Statistical significance was set at $P < 0.05$.

CHAPTER 4

RESULTS

4.1 NMU induced rat mammary carcinoma *in vivo* study

4.1.1 Tumour incidence and latency of NMU induced mammary carcinoma

Throughout the study, injection of NMU in thirty two (32) female Sprague Dawley rats at age 21-day old caused no sign of toxicity such as hair loss or any obvious changes in rat's behaviour. No significant decreased in their body weight although some of them had shown slight decreased from 3 to 5 grams in body weight for several days after the NMU injections. Otherwise, their body weight increased up until the end of experiment.

The sums of mammary tumour which were successfully excised from the rats were 35 tumours. At least one mammary tumour arose nearest to the breast pads which are located at abdominal inguinal and cervical thoracic regions of the rats' body. Among them, 19 tumours (54.3%) were observed to be located in the abdominal inguinal region while 16 tumours (45.7%) located in cervical thoracic region of mammary gland chain.

4.1.2 Intervention of Sirolimus and Sunitinib

In Sirolimus treated group, the tumour diameter decreased from 14.5 ± 0.5 to 11.3 ± 4.0 mm after first treatment, and significantly regressed to 6.3 ± 3.0 mm after second treatment. The tumour diameter in Sunitinib treated group regressed after first injection from 14.5 ± 0.5 mm to 12.5 ± 2.2 mm. However, five days after second treatment intervention, the tumour diameter increased to 14.2 ± 2.5 mm. In Sirolimus

+ Sunitinib treated group, the tumour diameter also regressed to 12.6 ± 3.2 and 11.2 ± 2.4 mm after first and second treatment (Table 4.1).

Table 4.1 Tumour diameter (Mean ± S.D) of Intervention Groups after First Treatment and Five Days Post Second Treatment

Groups	Tumor diameter (Mean ± S.D), mm	
Control	14.5 ± 0.5	
	After first treatment	Five days after second treatment
Sirolimus	11.3 ± 4.0	6.3 ± 3.0
Sunitinib	12.5 ± 2.2	14.2 ± 2.5
Sirolimus + Sunitinib	12.6 ± 3.2	11.2 ± 2.4

4.2 Histopathological analysis

4.2.1 Characterization of NMU induced mammary tumour in untreated group

The histology of rat NMU-induced mammary tumours are characterized by a combinations of several morphologic patterns. All tumours were 100% malignant histologically with invasive carcinoma, without in-situ component or benign tumours. Invasive breast carcinoma (IBC) of no special type (NST) is the most common histological type encountered that making up about 80%. This type is recognized by the presence of unequivocal growth of malignant epithelial cells infiltrating into the adjacent stroma without any specific patterns that followed by desmoplastic stromal reaction.

There are three types of IBC patterns shown in our study; IBC of cribriform pattern, IBC of papillary and IBC of No Special Type (NST). In control group, a

total of 8% of NMU-induced mammary tumours were observed to show less aggressive IBC of cribriform pattern. The remainder 92% was aggressive subtypes (papillary and NST). However, no benign mammary tumour was observed. Figure 4.1 and 4.2 showed comparison of histological features of normal mammary gland with the malignant invasive carcinoma. The histology of IBC of cribriform, papillary and NST patterns were shown in 4.3, 4.4, and 4.5.

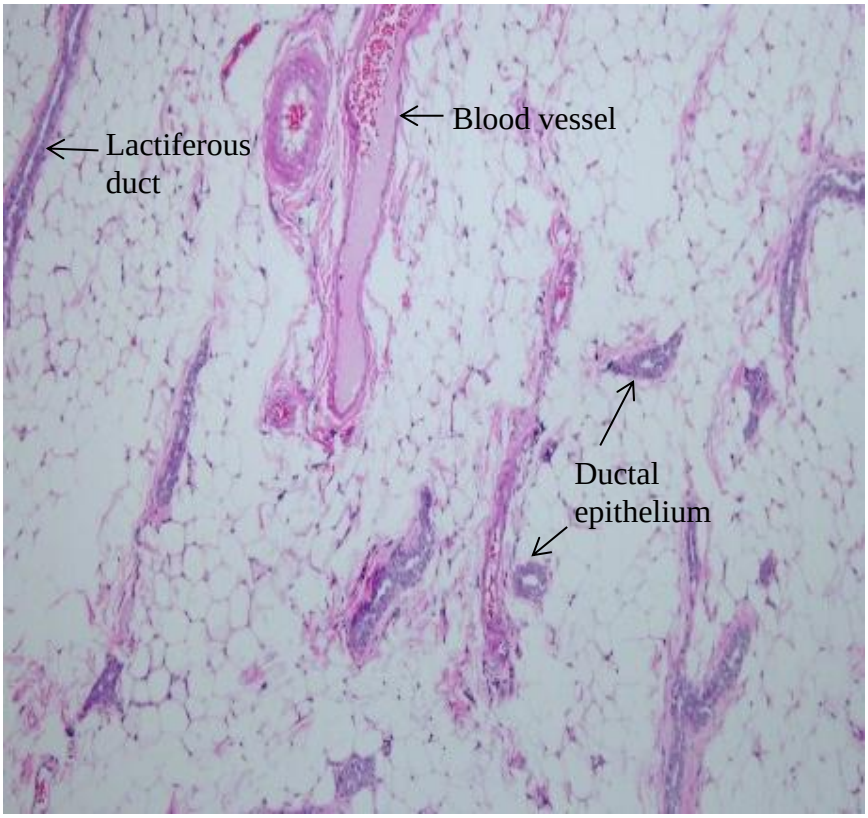

Figure 4.1 Histology of normal mammary gland of female Sprague-Dawley rat. Mammary ducts are surrounded by adipose and fibrous tissue with varied distribution. H&E staining magnification x100

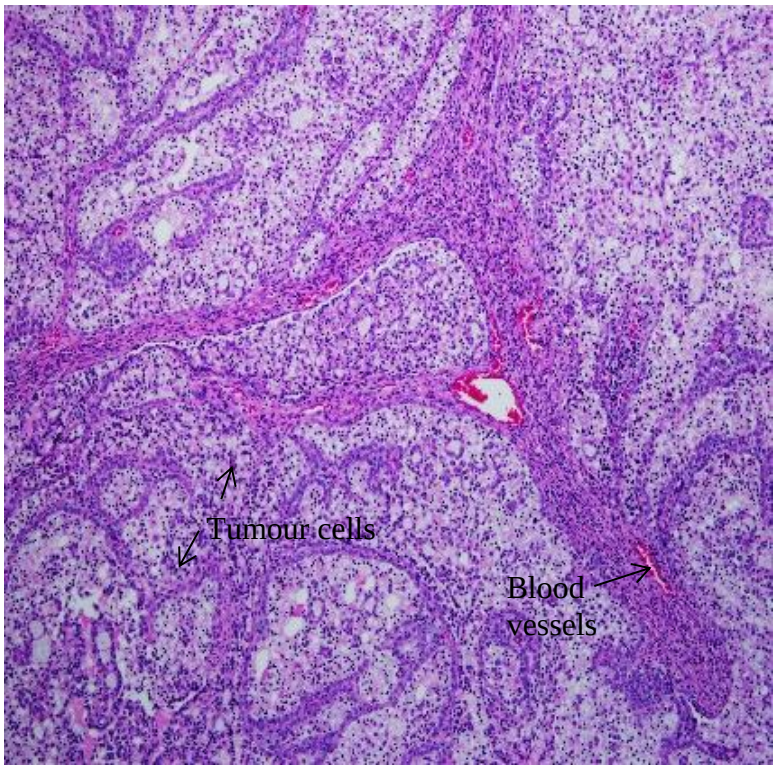

Figure 4.2 Histology of NMU-induced Invasive Breast Carcinoma. H&E staining magnification 100X. The carcinoma displayed diffuses infiltration of neoplastic cells, with less tubule formation, highly nuclear pleomorphism and high mitotic rate.

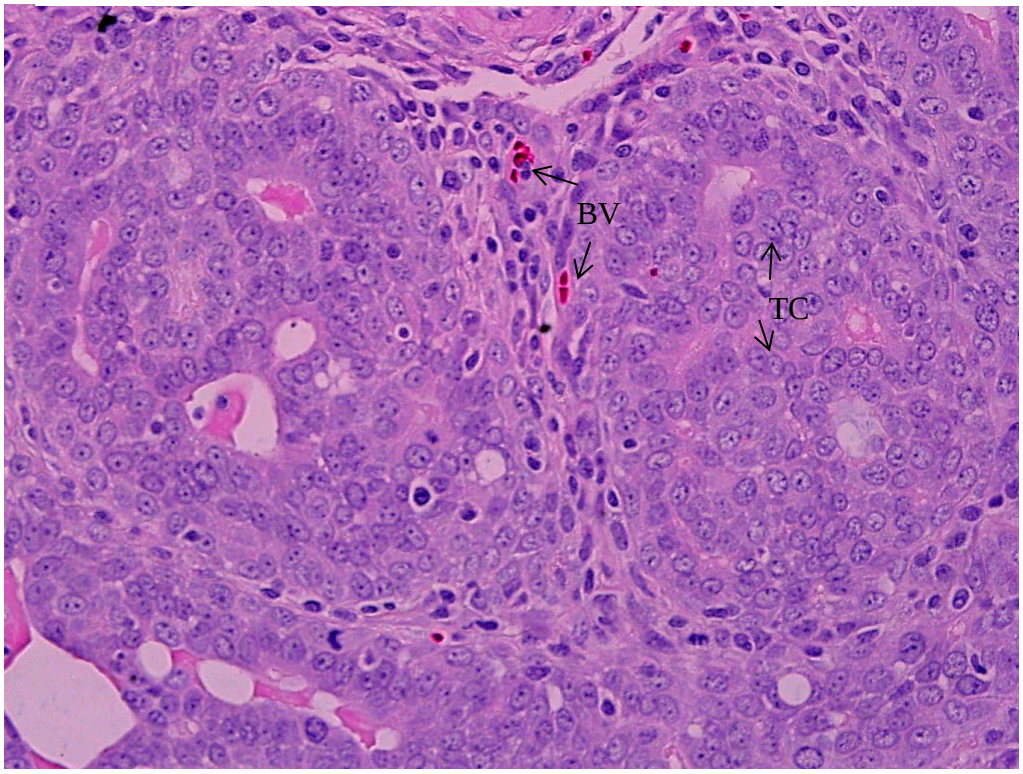

Figure 4.3 NMU-induced Cribriform Invasive Carcinoma. H&E staining magnification X 400. Tumour cell (TC). Blood vessel (BV)

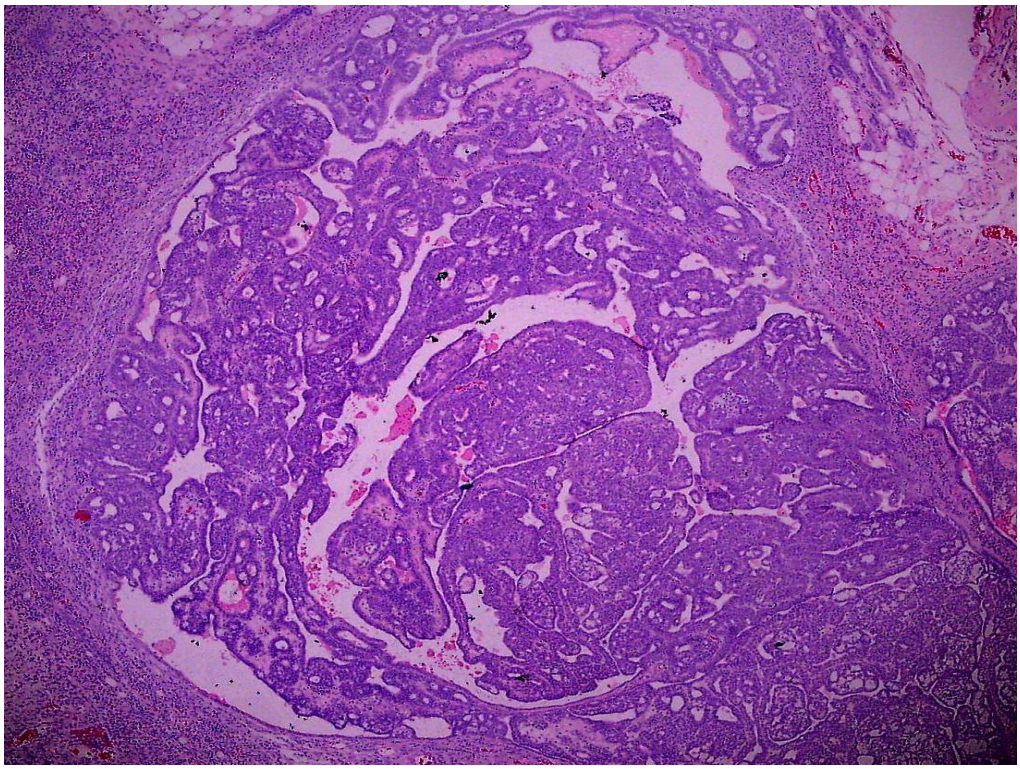

Figure 4.4 NMU-induced Papillary Invasive Carcinoma. H&E staining magnification X 400.

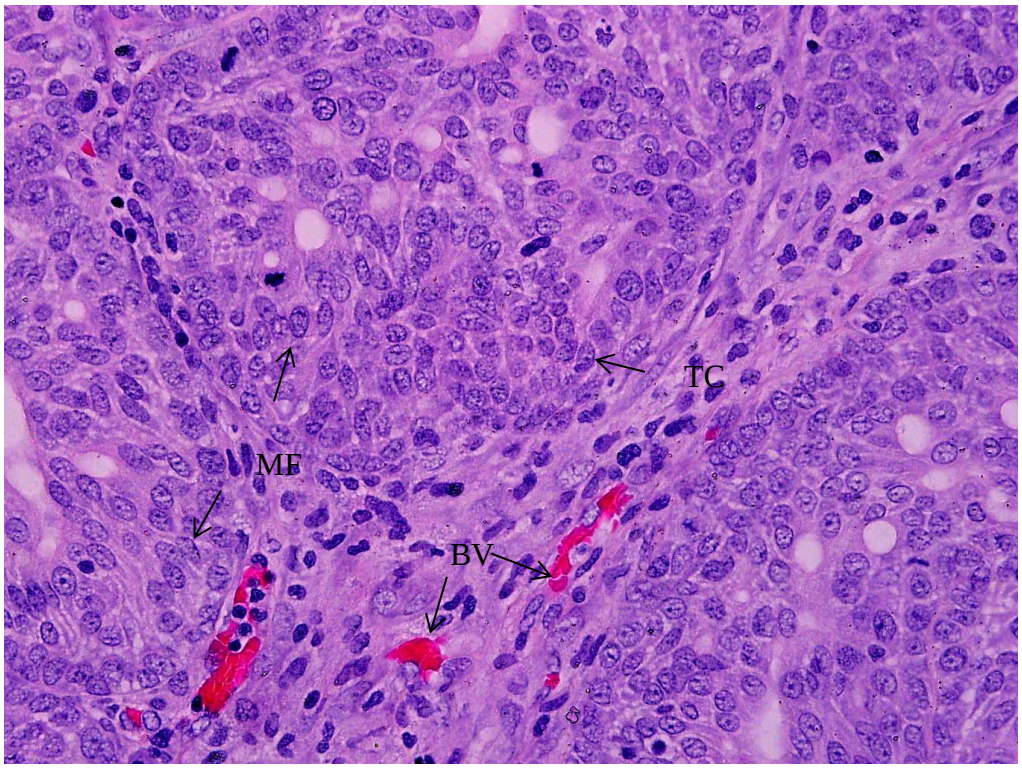

Figure 4.5 NMU-induced No Special Type Carcinoma. H&E staining magnification X 400. Tumour cell (TC), Blood vessel (BV), Mitotic Figures (MF)

4.2.2 Characterization of NMU induced mammary tumour in treated group

As shown in Table 4.2, the trend of invasive breast carcinoma pattern in tumour tissues for control group showed the highest papillary pattern (56.25%). However, the invasive carcinoma of papillary pattern decreased in Sirolimus-treated group (18.75%). Sirolimus-treated group showed the highest less aggressive IBC of cribriform pattern (56.25%). In contrast, the tumour tissues treated with Sunitinib and Sirolimus + Sunitinib treated groups showed the highest pattern of papillary (56% and 56.25% respectively) as compared to Sirolimus-treated group.

Table 4.2 The Tumour Types in the Intervention Groups.

Groups	Invasive Carcinoma		
	Cribriform (%)	Papillary (%)	No Special Type-NST (%)
Control	1/16 (6.25%)	9/16 (56.25%)	6/16 (37.5%)
Sirolimus	9/16 (56.25%)	3/16 (18.75%)	4/16 (25%)
Sunitinib	7/16 (44%)	9/16 (56%)	0/16 (0%)
Sirolimus + Sunitinib	5/16 (31.25%)	9/16 (56.25%)	2/16 (12.5%)

4.3 Protein expression analysis

Immunohistochemistry (IHC) technique was applied to determine ER, PgR and HER2/neu proteins localization. The ER and PgR were observed localize in nucleus while HER2/neu protein localize was in cytoplasm. The statistical analysis

of each marker was done by using multiple group comparison Kruskal-Wallis Test with Bonferroni correction followed by Pairwise test for multiple comparisons.

4.3.1 ER, PgR and HER2/neu expressions in control and treatment groups

Figure 4.6, 4.7, and 4.8 show representative for IHC staining of ER, PgR and HER2/neu. IHC statistical analysis of ER, PgR, and HER2/neu was significantly different for all groups comparison with ER (p= 0.001), PgR (p=0.001), and HER2/neu (p=0.043).

Table 4.3 ER localization in control and treatments groups

ER localization	N	Median (IQR)	X^2 Stat (df)	p value
Control	12	6.5 (1)		
Sirolimus	12	4.0 (1)		
			29.353 (3)	0.001 *
Sunitinib	12	5.0 (1)		
Sirolimus + Sunitinib	12	5.0 (1)		

Statistical analysis of ER expression at protein level by multiple group comparison Kruskal-Wallis Test

*significant value: $p < 0.05$

Table 4.4 PgR localization in control and treatments groups

PgR localization	N	Median (IqR)	X^2 Stat (df)	*p* value
Control	12	8.0 (1)		
Sirolimus	12	5.0 (2)		
			27.426 (3)	0.001*
Sunitinib	12	7.0 (1)		
Sirolimus + Sunitinib	12	6.0 (2)		

Statistical analysis of PgR expression at protein level by multiple group comparison Kruskal-Walis Test

*significant value: $p < 0.05$

Table 4.5 HER2/neu localization in control and treatments groups

HER2/neu localization	N	Median (IqR)	X^2 Stat[a] (df)	*p* value
Control	12	1.0 (1)		
Sirolimus	12	1.0 (1)		
			8.142 (3)	0.043*
Sunitinib	12	1.0 (1)		
Sirolimus + Sunitinib	12	1.0 (1)		

Statistical analysis of HER2/neu expression at protein level by multiple group comparison Kruskal-Walis Test

*Significant value: $p < 0.05$

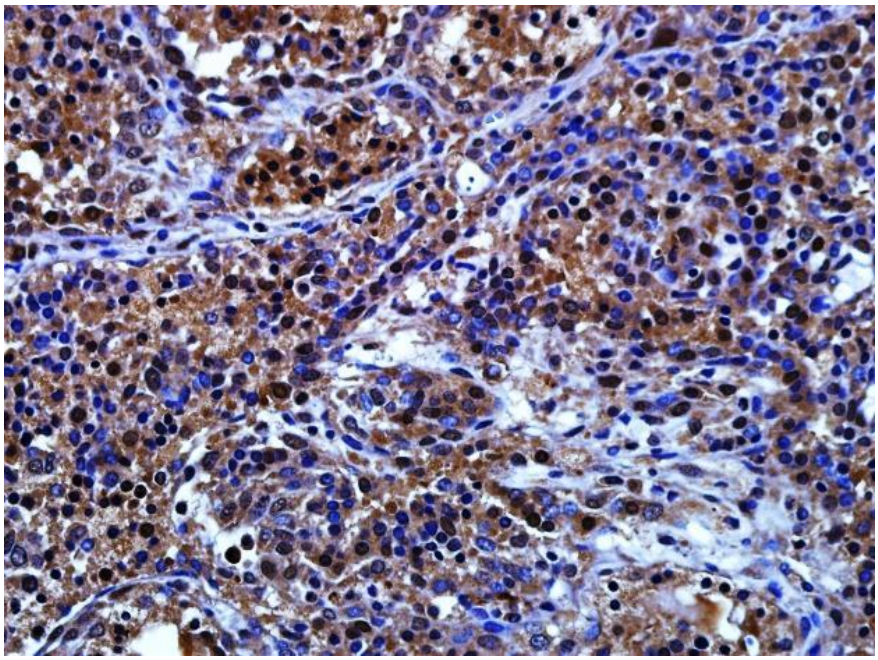

Figure 4.6 Representative of immunohistochemical nuclear expressions of ER on tumour specimens. 400X magnification

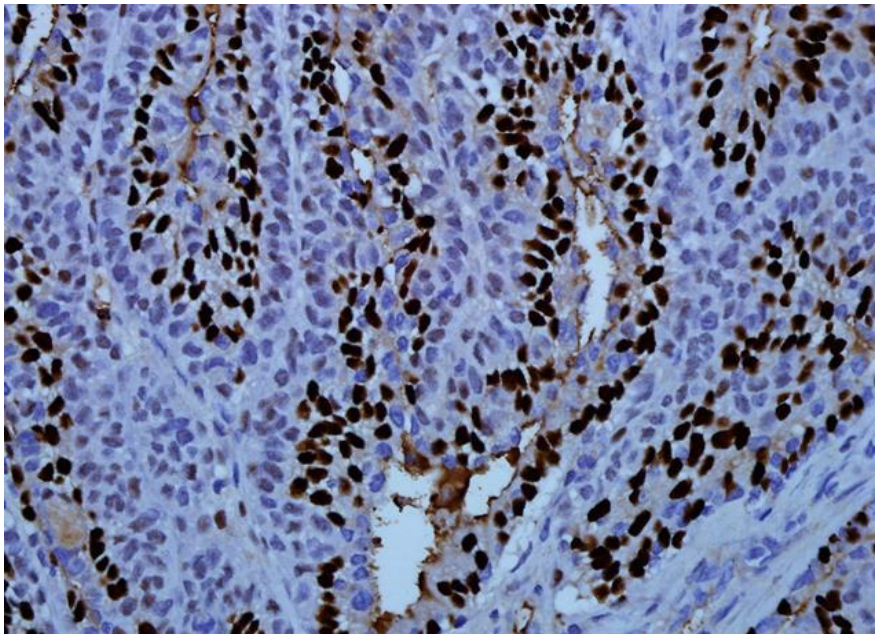

Figure 4.7 Representative of immunohistochemical nuclear expressions of PgR on tumour specimens. 400X magnification

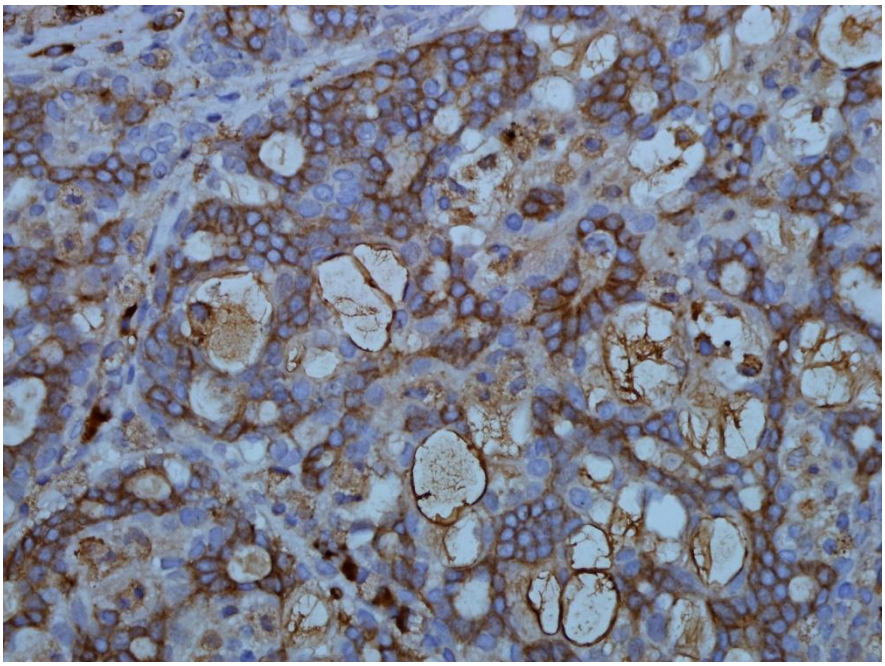

Figure 4.8 Representative of immunohistochemical low expressions (scored 1) of HER2/neu on tumour specimens. 400X magnification

4.3.2 Association of prognostic markers expression within intervention group

The association of all markers between intervention and control group were determined by Pairwise Comparisons Test and showed in Table 4.6, and the association of ER, PgR, and HER2/neu expressions amongst the intervention groups were summarized in Table 4.7.

Table 4.6 The Expressions of ER, PgR, and HER2/neu between Intervention and Control Groups

Group	*p* value		
	ER	PgR	HER2/neu
Control vs Sirolimus	0.001*	0.001*	0.446
Control vs Sunitinib	0.087	0.625	1.000
Control vs Sirolimus + Sunitinib	0.011*	0.038*	0.815

*Pairwise Test
*Significant value: $p < 0.05$

Table 4.7 The Expressions of ER, PgR, and HER2/neu amongst the Intervention Groups

Group	*p* value		
	ER	PgR	HER2/neu
Sirolimus vs Sunitinib	0.020*	0.003*	0.093
Sirolimus vs Sirolimus + Sunitinib	0.142	0.109	1.000
Sunitinib vs Sirolimus + Sunitinib	1.000	1.000	0.200

*Pairwise Test
*Significant value: $p < 0.05$

For Estrogen Receptor (ER), Kruskal-Wallis test provided very strong evidence of a difference (p= 0.001) between the median ranks of at least one pair of groups. Pairwise tests were carried out for the four pairs of groups. There were very strong evidence of ER total score readings between Sirolimus treated and Sunitinib treated group (p= 0.020, adjusted using the Bonferroni correction), Sirolimus treated and control group of a difference, (p= 0.001, adjusted using the Bonferroni correction), and ER total score of control and combinational-treated group (p= 0.011, adjusted using the Bonferroni correction). The median ER total score for the control group was 6.5 compared to 4.0 in the Sirolimus-treated group. There was no evidence of a difference between the other pairs (refer Table 4.3).

Progesterone Receptor (PgR) Kruskal-Wallis test showed significant difference (p= 0.001) between the median ranks of at least one pair of groups. Pairwise tests were carried out for the four pairs of groups. There were significant differences of PgR total score readings between Sirolimus treated and Sunitinib treated group (p= 0.003, adjusted using the Bonferroni correction), Sirolimus treated and control group of a difference, (p= 0.001, adjusted using the Bonferroni correction), and PgR total score of control and Sirolimus + Sunitinib treated group (p= 0.0038). The median PgR total score for the control group was 8.0 compared to 5.0 in the Sirolimus-treated group. There was no evidence of a difference between the other pairs.

Kruskal-Wallis test for HER2/neu expression also provided a difference (p= 0.043) between the median ranks of at least one pair of groups. However, the pairwise tests carried out for the four pairs of groups showed no strong evidence of HER2/neu total score readings between all four groups when the p-value for all comparisons are >0.05. The median for HER2/neu total score for all groups were 1. Hence, there was no evidence of difference between all pairs.

4.4 Gene expression analysis

4.4.1 Relative changes in gene expression of ER, PgR and HER2/neu mRNAs in Sirolimus and/or Sunitinib- treated groups

The primer specificity was confirmed by the absent of primer dimers or non-specific binding generated during the applied 40 amplification cycles applied in real-time RT-PCR reactions. Appendix B in appendix section shows amplification plots for ER, PgR, HER2/neu and β actin assays. The mRNA expression of each transcript was determined by using quantitative real time PCR method. The analysis of gene

expression was perform by using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). The C_T values provided from real-time PCR instrumentation were imported into Microsoft Excel program. The change in expression of the ER, PgR, and HER2/neu target gene normalized to β-actin was monitored. The mean CT values for both the target and internal control genes were determined and were analysed using $2^{-\Delta\Delta C}{}_T$ and were used in statistical analysis.

In this study, treatment of Sirolimus shows no significant changes in expression of ER, PgR, and HER2/neu compared to control group ($p>0.05$). Sunitinib treated also does not show significant downregulation of ER compared to control ($p= 0.500$), PgR ($p= 0.513$) and HER2/neu ($p= 0.500$). However, administration of combination of Sirolimus and Sunitinib significantly downregulated expression of HER2/neu compared to untreated control group ($p<0.05$), but there were no significant changes in ER and PgR expressions in combination treated group compared to untreated control group. The relative changes of mRNA expressions level of ER, PgR, and HER2/neu in all experimental groups compared to untreated control group are presented in Table 4.8.

Table 4.8 Relative changes of mRNA expression level ER, PgR, and HER2/neu mRNA expression level in treated groups relative to control group by using $2^{\wedge -\Delta\Delta C_T}$ method

Groups	ER (*Esr1*)		*PgR*		*HER2/neu*	
	E= 2.17		E=2.18		E=1.98	
	RC (S.D)	*p* value	RC (S.D)	*p* value	RC (S.D)	*p* value
Sirolimus	0.199 (0.21)	0.513	-0.098 (0.076)	0.513	1.247 (1.55)	0.827
Sunitinib	-1.802 (0.13)	0.500	-9.240 (0.00)	0.513	-6.490 (0.00)	0.500
Sirolimus + Sunitinib	1.556 (1.48)	0.827	1.887 (1.45)	0.275	-4.924 (0.00)	0.050**

* *p* value from T-test analysis to compare between mRNA expression level of intervention groups relative to control group
* The relative mRNA expression ratio ± S.D of $2^{\wedge -\Delta\Delta C_T}$ value of experimental groups for *ER*, *PgR* and *HER2/neu* expressions relative to untreated control after normalized to ß-actin. SD=standard deviation.
* Significant value: $p < 0.05$

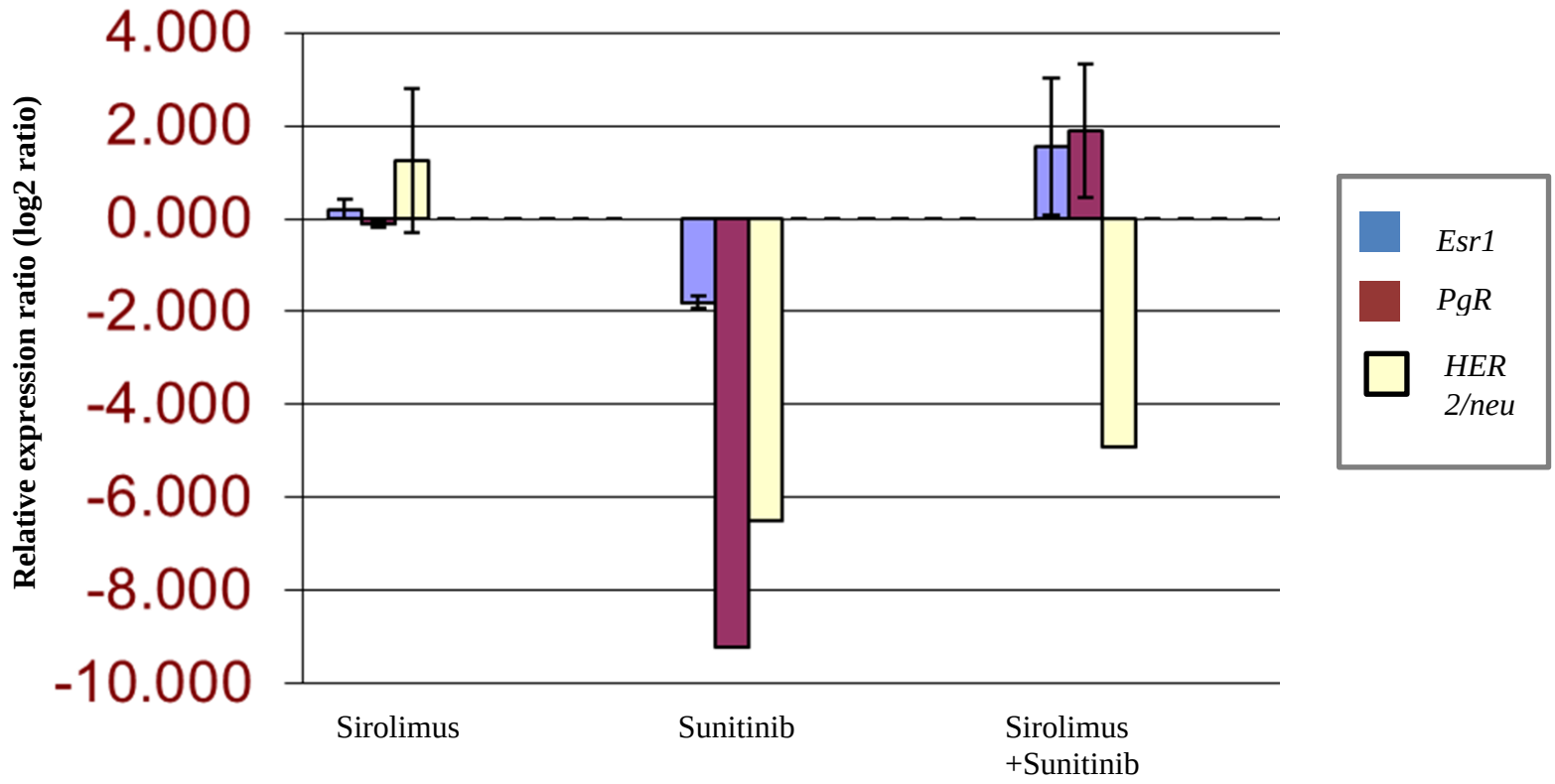

Figure 4. 9 Summary of the relative expression level of transcripts in experimental groups compared to untreated control group after normalization with ß-actin. The data are the log2 R ± SD (relative expression ratio ± standard deviation).

CHAPTER 5

DISCUSSION

5.1 Effects of Sirolimus and Sunitinib on Histological Features of NMU-induced Breast Carcinoma

From H&E histological staining, it was observed that intraperitoneal induction of NMU with dosage of 70 mg/kg body weight of Sprague Dawley rats were developed 100% malignant tumour and represented histological features of invasive breast carcinoma (IBC). Our result supported that the latency and the incidence of tumours vary according to the dosage of NMU and the route of carcinogen administration, as well as the age of rats. 50 mg/kg body weight was reported to develop 88.64% malignant with 66.67% invasive carcinoma (Saminathan *et al.*, 2014). On the other hand, findings by Jaafar et. al (2009) has proved that the induction of breast tumour by 70 mg/kg body weight intraperitoneally resulted in increasing trend of malignancy as the tumour size increased to 12.0 ± 0.5 mm, hence consistent with this recent findings.

Our NMU breast cancer model was fully developed invasive breast carcinoma (IBC) with three predominant patterns; IBC cribriform, IBC papillary and IBC-NST. IBC of cribriform is usually low grade cancer cells, nestlike formations between the ducts and lobules, and cells behave somewhat like normal breast cells. IBC papillary usually moderate grade, has a well-defined border, and is made up of small, finger-like projections. IBC-NST subtype of breast carcinomas exhibited high proliferation ratio and are associated with poor host cellular immune reaction. IBC-NST attributes translate to poor prognosis (Makki, 2015).

Histological analysis (refer Table 4.2) showed a reduction in aggressive pattern of IBC papillary and IBC-NST in Sirolimus treated group compared to untreated control group. Previous study conducted by Al-Astani et al (2014) also reported that Sirolimus lowered percentage aggressive high grade IBC-NST pattern and increased the percentage of low grade cribriform pattern (Al-Astani Tengku Din *et al.*, 2014). This was suggested that Sirolimus inhibited tumour growth by inhibiting the mTOR pathway, thus reduced tumorigenesis. Generally, in order to meet the high demands of cell proliferation, breast cancer cells altered the mechanism in nutrient uptake and energy metabolism, and these processes are directly controlled by the mTORC1 pathway. Activation of mTORC1 promotes glycolysis via upregulation of hypoxia-inducible factor alpha (HIF1a) and c-Myc (Gordan *et al.*, 2007). Thus, Sirolimus that selectively target mTORC1, are expected to impair cancer metabolism, reduce cell progression, decrease the severity and aggressiveness of malignancy, and are considered promising anticancer therapies.

Interestingly, Sunitinib treated group did not show any aggressive IBC-NST histological pattern, showed that presence of Sunitinib reduced aggressiveness of breast carcinoma. This result might relate with Sunitinib targeting not only endothelial cells and the endothelial proangiogenic factors, but also the tumour cells, that lead to inhibition of angiogenesis and regression of tumours aggressiveness (Kamli *et al.*, 2018). However, Sirolimus + Sunitinib treated group showed the highest papillary histological pattern compared to Sirolimus-treated group. There was no significant difference of IBC-papillary in Sunitinib and Sirolimus + Sunitinib treated group compared to control group. The IBC-papillary was assessed by the presence of invasive elements showing predominantly papillary architecture. The papillae formed by malignant cells are having mild-to-moderate nuclear atypia with

delicate fibrovascular core lacking of myoepithelial cells. High percentage of IBC-papillary subtype showed that the presence of Sunitinib reduce of aggressiveness of breast carcinoma, but not significantly lower the histological grade.

5.2 Effects of Sirolimus and Sunitinib on NMU-induced Breast Carcinoma Growth

After being administered with 70 mg/kg body weight dose of NMU at 21 days old rat, rats did not show any gross tumour development until subsequently the first palpable tumour mostly appeared on 60^{th} day post carcinogen injection. The gross tumour initially was solid and small less than 5 mm. Then, the gross tumour was seen growing fast and increased in size. In this study, 8 mammary carcinoma lesions after reached sizes within ranged 14.5 ± 0.5 mm were assigned in control group.

Sirolimus was proven to successfully inhibit NMU-induced mammary carcinoma progression and growth. In this study, mammary lesions diameter sizes regressed from 14.5 ± 0.5 mm to minimally 6.3 ± 3.0 mm five days post Sirolimus second administration. The results disclosed that treatment with Sirolimus alone significantly inhibited mammary tumour progression (Table 4.1). Other in vivo study conducted by Zeng et al (2010) also showed Sirolimus alone was significantly suppressed the orthotopic mammary MDA-MB-231 tumour growth of treated nu/nu mice compared with the control group through proliferation inhibition and apoptosis induction (Zeng *et al.*, 2010). This might relate with the mechanism of action of Sirolimus.

Sirolimus targeted directly mTORC1 by binding to FK506 Binding Protein 12 (FKBP12) which resulting in the unbinding RAPTOR from mTORC1 (Tian *et al.*, 2019). mTORC1 inhibition caused inactivation of S6K1 and 4E-BP1 by inhibiting phosphorylation, which leads to G1 phase cell cycle arrest, and reduce in protein synthesis (Fingar *et al.*, 2002). In addition, Sirolimus also can target indirectly mTORC2 by binding to FKBP12, leading to dissociation of RICTOR from mTOR, thus decreasing the levels of mTORC2 (Schreiber *et al.*, 2015). Low mTORC2 activity leads to reduce in cytoskeleton reorganization activity and cell movements involved in tumorigenesis.

Receptor tyrosine kinases (RTKs) are the major targets for targeted cancer therapies approaches, because of their critical roles in cell survival and proliferation, and activated in a wide range of cancers. The efficacy of Sunitinib has been demonstrated in patients with gastrointestinal stromal tumours (GIST) and renal cell carcinoma (RCC) (Mulet-Margalef and Garcia-Del-Muro, 2016; Rizzo and Porta, 2017). Studies confirmed that VEGF and PDGF signalling pathway implicated in the angiogenesis of breast cancer, and Sunitinib was inhibited angiogenesis in triple-negative breast cancer xenografts model (Chinchar *et al.*, 2014). Hence, we applied Sunitinib in our NMU model in order to investigate anticancer effect through angiogenesis inhibition. The findings (refer to Table 4.1) showed that Sunitinib treated tumours reduce in diameter post first Sunitinib administered (12.5 ± 2.2 mm), but the diameter increased to 14.2 ± 2.5 mm post second treatment.

Even though Sunitinib has been used as anticancer treatments in several types of tumour including breast cancer, however previous clinical observations of Sunitinib treatment showed that this therapy have limited efficacy. Researchers

figured out that when Sunitinib as anti-angiogenic agents administered on an intermittent schedule; 4 weeks on and 2 weeks off, tumour regrowth was seen during drug-free periods (Burstein *et al.*, 2008a) or upon discontinuation of the treatment (Mulet-Margalef and Garcia-Del-Muro, 2016). This result was explained due to Sunitinib inhibits primary tumour growth, but the inhibition is exceptional lasting responses and only show moderate increases in progression-free survival and little benefit in overall survival. This might relate with our studies when Sunitinib treated tumours reduce in diameter after first treatment, but the diameter increases after second treatment. Sunitinib might generate intratumoral hypoxia modulating the metastatic process (Lu and Kang, 2010) and stimulating cancer stem cells (CSC) (Seidel *et al.*, 2010), thus resulting in tumour regrowth after first treatment.

The combinational treatment was administered when the tumour size reach 14.5+ 0.5 mm; whereby Sunitinib was injected initially on the first day followed by Sirolimus on the next day. In this present study demonstrated that combination of Sirolimus and Sunitinib was regressed the tumour size to 11.2 ± 2.4 mm post 5 days of second Sirolimus + Sunitinib treatment. From these findings, it was assumed that combining two anti-cancers Sirolimus and Sunitinib might yield synergistic effects for anti-cancer activity thus suggesting a more effective cancer cell inhibition in breast cancer cells.

Previous study conducted by Yin et al (2014) reported that the combinational strategy of Sirolimus + Sunitinib was effective to retard tumour progression in murine breast tumour models. The researchers also reported that Sirolimus alone significantly reduced the tumor growth, and there was limited tumour growth in mice

treated with Sunitinib alone. In addition, the combinational strategy reduced splenomegaly in 4T1 breast cancer models (Yin *et al.*, 2014).

We hypothesized through targeting anti-mTOR of Sirolimus and inhibits tyrosine kinase of Sunitinib will significantly resulted more effective inhibition of tumour growth. However, since Sunitinib monotherapy negatively inhibited tumour growth in this study (refer to Table 4.1), we assumed that the tumour size reduction and growth inhibition in combination treatment of Sirolimus + Sunitinib mainly influenced by Sirolimus action.

5.3 Effects of Sirolimus and Sunitinib on Protein Expressions of ER, PgR, and HER2/neu of NMU-induced Breast Carcinoma

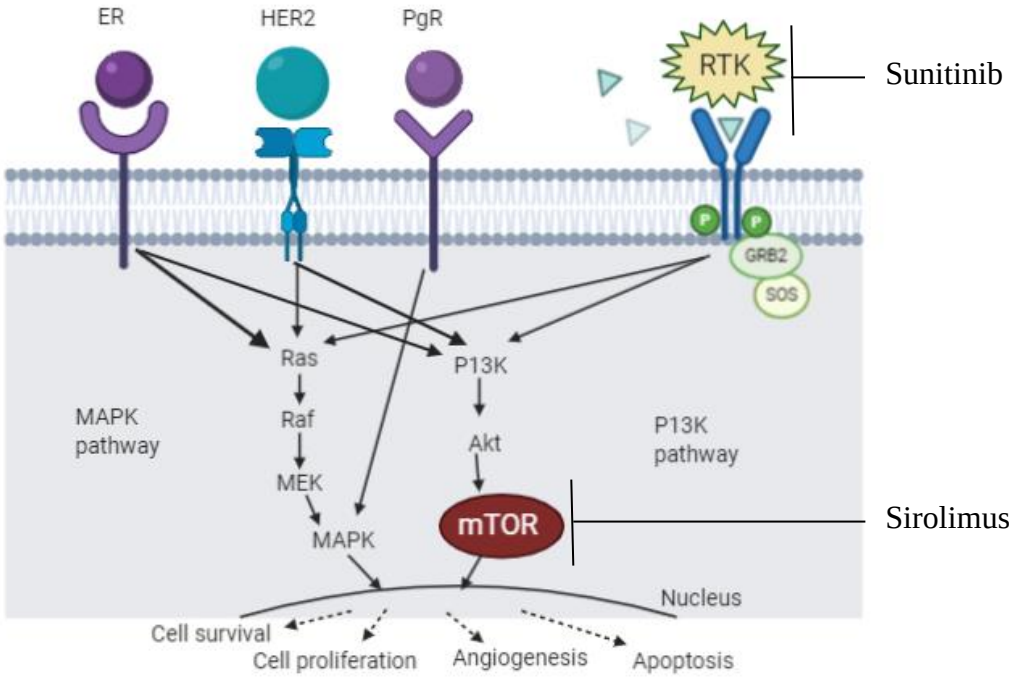

Figure 5.1 ER, PgR, HER2/neu and RTK play roles in mTOR signalling cascade

Breast cancer is dependent on estrogen and/or progesterone hormones for growth and this is mediated through estrogen receptors (ERs) and progesterone

receptors (PgRs). ER specifically ERα contributes to tumor aggressiveness (Muscat *et al.*, 2013) and the increasing histological grade and PgR functions as predictor of ER activity. As Booth (2006) reported that ER and PgR are localized in the nucleus of epithelial cells and will co-upregulated to label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands (Booth and Smith, 2006). In the study, it was hypothesized that anti-tumour effects of Sirolimus potentially lower the ER expression, inhibiting the estrogen-dependent mechanism thus blocking cancer cells progression.

Our study disclosed that all malignant breast carcinoma of control group are both overexpressed of ERs and PgRs (refer to Table 4.3 and 4.4). As expected, the ER and PgR expressions of Sirolimus-treated and Sirolimus + Sunitinib treated tumour are significantly lower compared to control group. In relation with tumour diameter, the Sirolimus-treated and Sirolimus + Sunitinib treated tumour noticeably reduced the tumour size of NMU-induced breast cancer. These suggest that the treatment reduces the growth of premalignant rat mammary lesions and inhibits the malignant transformation of mammary carcinoma.

Sunitinib was approved as anti-angiogenic receptor tyrosine kinase inhibitors (TKIs). Sunitinib can target multiple receptor sites simultaneously included vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and c-Kit. Sunitinib block the kinase activity of receptor and transduction of downstream signal involved in the proliferation, migration, and survival of cancer cell. During angiogenesis, estrogen was involved directly in mechanism of VEGF and PDGF transcription through the activation of ER (Rykala *et al.*, 2011).

Therefore, in this study, we predicted the possibility of inhibition of angiogenesis in suppressing ER and PgR expression.

However, Sunitinib did not significantly downregulate the expressions of ER and PgR expressions (refer to Table 4.3 and Table 4.4). This showed that inhibition of receptor tyrosine kinases (RTKs) by Sunitinib did not retard breast tumour growth in this NMU-breast cancer model. This suggested that the treatment reduced the growth of premalignant rat mammary lesions and inhibits the malignant transformation of mammary carcinoma. This result may be supported with previous research conducted by Miller (2010) that showed direct inhibition of mTOR pathway (Sirolimus) effectively suppressed the growth of both estrogen-independent and -dependent cells breast cancer cell growth associated with hyperactivation of the IGF IR/InsR/PI3K/mTOR pathway, but inhibition of nodes upstream (RTKs) and downstream (mTOR) of PI3K only partially blocked breast cancer cell growth (Miller *et al.*, 2010).

HER2/neu-positive breast cancer is a more aggressive type of breast cancer compared with HER2/neu-negative types. Our study showed all NMU-induced breast cancers were estrogen and progesterone receptors positive but HER2/neu negative (all luminal A subtype). In studies by Kinoshita *et al.* (2016) *in-vivo* study also reported that all induced mammary tumours in female rats were adenocarcinomas (luminal A subtype) based on the results of the ER and/or PgR positivity and HER2/neu negativity (Kinoshita *et al.*, 2016). The negative expressions of HER2/neu in our result may explained the decreasing grade in aggressiveness of Invasive Carcinoma of NST of treated groups. In addition, Sunitinib as tyrosine kinase inhibitor might not be able to work efficiently to inhibit

HER2/neu which involved in the tyrosine kinase mediated regulation of mammary gland since HER2/neu did not well expressed in NMU-induced breast cancer.

5.4 Effects of Sirolimus and Sunitinib on Gene Expressions of *ER, PgR,* and *HER2/neu* of NMU-induced Breast Carcinoma

Breast cancer is associated with distinct gene expression profiles involving a large number of genes. To gain more comprehensive understanding of breast cancer progression, it is critical to combine the protein expression pattern with mRNA expression due to proteins are the major effectors of most biological processes and are also the most suitable molecules for use as biomarkers, prognostic risk factors, and therapeutic targets (Rezaul *et al.*, 2010). The profiling of protein expression from pathological tissues provides a rough survey of the pathological, metabolic, oncogenic, and metastatic status.

In this study, the gene expression of *ER (Esr1), PgR and HER2/neu (Egfr)* were determined using quantitative Real-Time PCR and the relative changes in expressions of targeted genes compared to reference gene were analyzed by using $2^{-\Delta\Delta C_T}$ method. T-Test was performed to analyze the significant difference of $2^{-\Delta\Delta C_T}$ relative changes of targeted genes of treated groups compared to untreated control group (refer Table 4.8).

In Sirolimus treated group, it was assumed that anti-tumour effect of Sirolimus potentially downregulate the *ER, PgR,* and *HER2/neu* expressions, preventing the hormonal dependent and growth factor mechanisms towards breast cancer cells progression. In this study, it was interesting to show that the treatment of Sirolimus did not significantly downregulate *ER, PgR,* and *HER2/neu* compared to untreated control group at mRNA level. On the other hand, Sirolimus treated group

upregulate *PgR* and *HER2/neu* gene expressions not significantly even though tumour sizes were successfully regressed. However, Sirolimus treated breast cancer downregulate the hormonal expressions at protein level.

This might due to Sirolimus as anti-mTORC1 action of mechanism lead to increase the level of mRNA, and reduced rate of protein translation of ER, PgR, and HER2/neu. However, there are many complicated and varied post-transcriptional mechanisms involved in translate mRNA into protein that limit the ability to get a clear picture on how Sirolimus affecting breast cancer cells. The mechanistic target of rapamycin complex 1 (mTORC1) stimulates mRNA translation and other anabolic processes. In addition, mTORC1 controls mitochondrial activity and biogenesis by selectively promoting translation of nucleus-encoded mitochondria-related mRNAs via inhibition of the eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs). Stimulating the translation of nucleus-encoded mitochondria-related mRNAs engenders an increase in ATP production capacity, a required energy source for translation (Morita *et al.*, 2013). Studies conducted by Huo *et al.* (2011) reported that Sirolimus could be able to interfere with signaling from mTOR to 4EBP1 instead of a direct phosphorylation by mTOR (Huo *et al.*, 2011). Thus, Sirolimus might block mTORC1 and inhibiting the translation of mRNAs via promoting translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs).

ER, PgR, and *HER2/neu* gene expressions for Sunitinib (anti-VEGF) treated showed no significant difference compared to untreated control group in this NMU-model. An in-vitro study concluded that VEGF and VEGFR1 are more significantly expressed in ER-negative (MDA-MB-231) cells than ER-positive (MCF-7) cells (Young *et al.*, 2010). Thus, this Luminal A subtypes breast cancer NMU model

might have lower expression of VEGF, thus reduce the Sunitinib action as targeted therapy on this hormonal positive breast cancer.

CHAPTER 6

CONCLUSION

6.1 Summary of current study

In this *in-vivo* study, our present findings disclosed that NMU induced mammary carcinomas were malignant and highly aggressive. These were proven by the development of aggressive lesions of mammary phenotype at tumour size of 14.5 ± 0.5mm and more, indicating that the histology of tumours is invasive breast carcinoma.

Treatment with Sirolimus alone showed significant mammary tumour inhibition which presumably exerts its inhibitory effect through mTOR pathway. ER and PgR play a major role in breast cancer cell development and positively correlated with breast cancer cell proliferation. ER and PgR expressions of Sirolimus treated and combination treated tumour are significantly lower compared to control group, hence evidently reduced tumour size of NMU-induced breast cancer. However, gene expressions of ER and PgR at mRNA level were high with no significant differences with untreated control group, might due to Sirolimus cause post-transcriptional regulation in gene.

In contrast, treatment with Sunitinib shrinks the solid tumor after first treatment, but the diameter increases after second treatment. This might due to Sunitinib generate intratumoral hypoxia modulating the metastatic process and stimulating cancer stem cells in NMU-induced mammary tumor growth. Thus, present results suggested that Sirolimus is not synergistic or additive with Sunitinib.

Furthermore, Sunitinib might be an antagonist towards the Sirolimus activity as multi-targeted tyrosine kinase inhibitor.

6.2 Limitation of study

Sunitinib acts as multi-targeted tyrosine kinase inhibitors including VEGFR and PDGFR which play a role in both tumour angiogenesis and cell proliferation. HER2/neu or also known as Receptor tyrosine-protein kinase erbB-2 involved in the tyrosine kinase mediated regulation of mammary gland. Since HER2/neu does not express in this NMU-induced breast cancer model, the mechanism of action of Sunitinib on breast cancer was only explained for Luminal A subtypes breast cancer. Even though Sunitinib-treated breast cancer was failed to completely suppress tumour progression and subsequently failed to reduce tumour aggressiveness, the mechanism of action of Sunitinib in inhibit angiogenesis might be explained if we observed the effect of treatment on angiogenesis markers such as VEGF and PDGF.

6.3 Recommendation of future research

Research conducted by Conley et al. (2012) demonstrated that hypoxia-driven cancer stem cell stimulation limits the effectiveness of antiangiogenic agents, and suggest that to improve patient outcome, these agents might have to be combined with cancer stem cell-targeting drugs (Conley *et al.*, 2012). Hence, it was recommended to combine Sunitinib with drug targeting CSC-related signaling pathways such as Wnt, Notch, and Hedgehog pathways.

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APPENDICES

APPENDIX A TABLE OF TISSUE PROCESSING SCHEDULE USING AUTOMATED TISSUE-TEK® VIP

Number	Reagent	Time (Hour)	Process
1.	Formalin	1	Fixation
2.	70% ethanol	1	
3.	95% ethanol (1)	1	
4.	95% ethanol (2)	1	Dehydration
5.	100% ethanol (1)	1	
6.	100% ethanol (2)	1	
7.	100% ethanol (3)	1	
8.	Xylene (1)	1	
9.	Xylene (2)	1	Clearing
10.	Xylene (3)	1	
11.	Paraffin wax (1)	1	
12.	Paraffin wax (2)	1	Impregnation
13.	Paraffin wax (3)	1	
14.	Paraffin wax (4)	1	

APPENDIX B

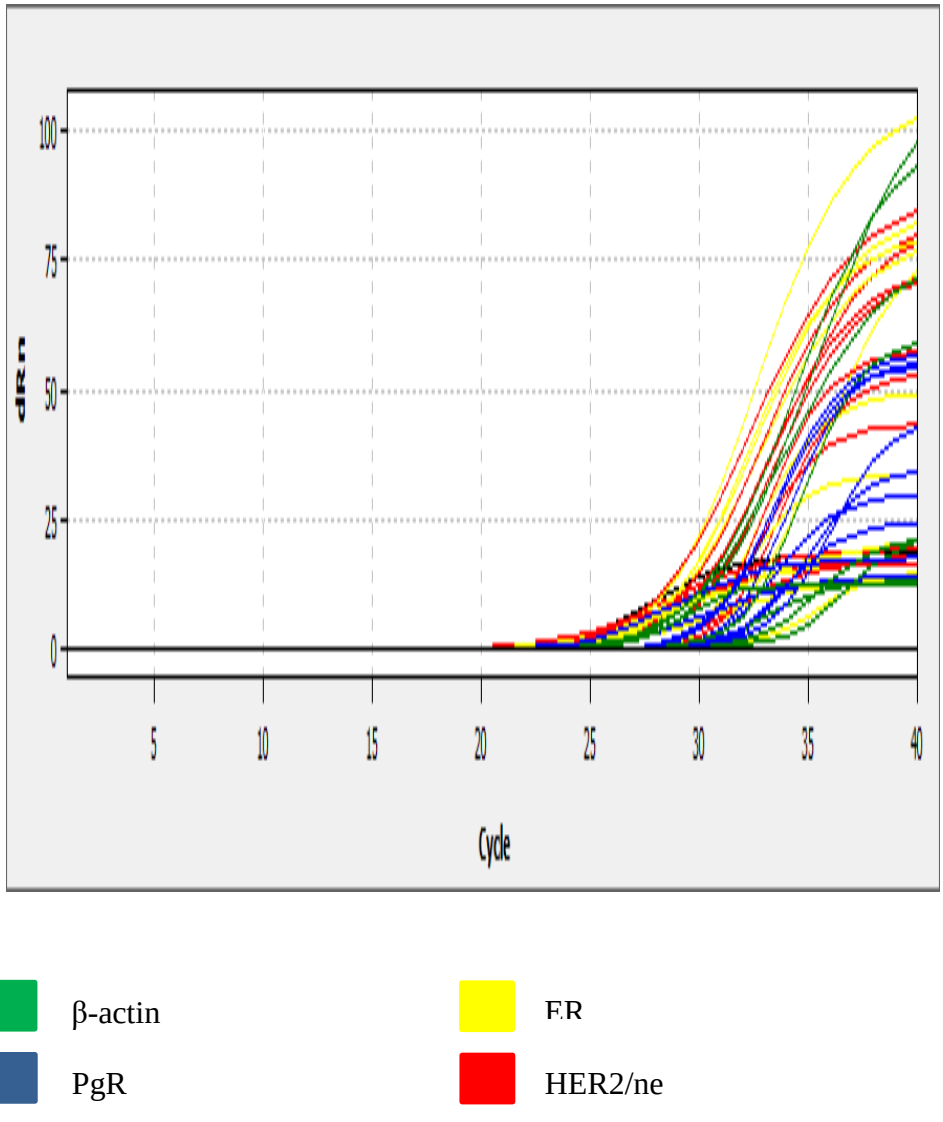


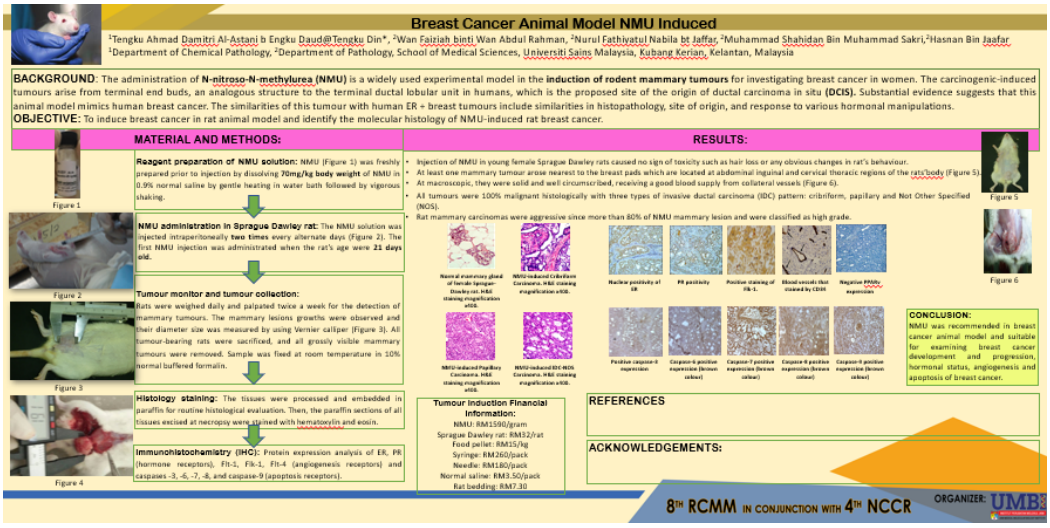
Figure 1 Amplification plot of β-actin, ER, PgR, and HER2/neu genes

APPENDIX C

Conference Presentation

8th Regional Conference on Molecular Medicine (RCMM) in Conjunction with 4rd National Conference for Cancer Research 2018 19-20th September 2018, Auditorium UMBI, Kuala Lumpur.

Title: Breast Cancer Animal Model NMU Induced



4th International Conference on Advances in Medical Science (ICAMS) 2019, 13 April 2019 (11.30 -12.50 AM), Waterfront Hotel, Kuching Sarawak
Tittle: Regression of Invasive breast carcinoma Treated With Sirolimus and Sunitinib in NMU- Induced Animal Cancer Model
Type of presentation: Young Investigator Presentation/Oral

UNIVERSITI KEBANGSAAN MALAYSIA
The National University of Malaysia

4th ICAMS 2019 | UKM Medical Centre

Reference : ICAMS/4/004
Date: 28 March 2019

MODE & SLOT OF PRESENTATION NOTICE

Dear Ms. Nurul Fathiyatul Nabila Jaffar

On behalf of the Organizing Committee of the 4th International Conference on Advances in Medical Science (ICAMS) 2019, we are pleased to inform you that your abstract has been accepted for:

Type of presentation	**: YOUNG INVESTIGATOR PRESENTATION/ORAL YIA 01**
Date	**: 13 April 2019 (11.30 -12.50 AM)**
Venue	**: Waterfront Hotel, Kuching Sarawak**
Tittle	**: Regression of Invasive Ductal Carcinoma Treated With Sirolimus and Sunitinib In Nmu- Induced Animal Cancer Model**
Presenter	**: Ms. Nurul Fathiyatul Nabila Jaffar**

PRESENTER REGISTRATION IMPORTANT INFORMATION:

Due to the session allocation and publication schedule for the program book, presenters are required to complete your registration for the meeting with full payment.

If you do not complete your registration with full payment by 1st April 2019, your name and title of your presentation shall not be included in the program book and your presentation will be cancelled automatically.

POSTER MOUNTING/ORAL PRESENTATION GUIDELINES:
The poster mounting/oral presentation guidelines will be posted on the conference website.

Time allocation for the oral presentation is 8 minutes' presentation + 2 minutes Q and A.

Please inform the secretariat if you need to use your own laptop during oral presentation. Please bring your own adaptor for your laptop.

All Power Point presentation slides need to be submitted to the secretariat on the morning of 13rd April 2019.

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SECRETARIAT INTERNATIONAL CONFERENCE ON ADVANCES IN MEDICAL SCIENCE (ICAMS) 2019
DEPARTMENT OF PHARMACOLOGY
Faculty of Medicine, Pusat Perubatan Universiti Kebangsaan Malaysia
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APPENDIX D

Publication (Accepted)

Journal: Asian Pacific Journal of Cancer Prevention (APJCP)

Title: Evaluation of NMU-induced Breast Cancer Treated with Sirolimus and Sunitinib on Breast Cancer Growth

Authors:
Nurul Fathiyatul Nabila Jaffar, Muhammad Shahidan Muhammad Sakri, Hasnan Jaafar, Wan Faiziah Wan Abdul Rahman, Tengku Ahmad Damitri Al-Astani Tengku Din