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

**NURUL FATHIYATUL NABILA BINTI JAFFAR**

**UNIVERSITI SAINS MALAYSIA**

**2020**

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

**by**

**NURUL FATHIYATUL NABILA BINTI JAFFAR**

**Thesis submitted in fulfilment of the requirements**
**for the degree of**
**Master of Science**

**October 2020**

## ACKNOWLEDGEMENT

First and foremost, I would like express my gratitude to the Almighty Allah for His countless blessing. A very special gratitude is to my supervisor, Dr. Tengku Ahmad Damitri Al-Astani Tengku Din. Deepest gratitude is to my co-supervisor Prof. Dr. Hasnan Jaafar and Dr. Wan Faiziah Wan Abdul Rahman who's without their knowledge and assistance, this study would not have been successful. Special thanked to my research grant partner for Muhammad Shahidan Muhammad Sakri in exchanging idea and skills. Thank you to all pathology graduate friends, especially, Putri, Syazana, Farhanah, Faliq, Ain, Rosmaizan, Syarah, Zulhelmi, Rasyid, Martina, Fatihah and Hussein for their invaluable assistances, insightful comments, exchanges of knowledge, skills, and venting of frustration during my graduate program, which helped enrich the experiences. Not forgetting to all staffs of Department of Pathology, USM especially Puan Ummi Atikah, En.Rosli and Puan Jamaliah who always been there. Not forgetable to INFORMM lecturer and staffs especially Dr. Noor Fatmawati Mokhtar, Elis Rosliza and Mawaddah Azlan, and Ms Norliana Ghazali from School of Dental Sciences who help me with molecular work.

A special thanks to my beloved family, for their understanding and endless love through the duration of my studies. Words cannot express how grateful I am to my parent (Hj Jaffar bin Bakar and Hjh Sarkiyah binti Yaakub) and my siblings (Ashraf Hafizi and Aqilah Nabilah) for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me this far. Last, but not lease I recognize that this research would not have been possible without the financial assistance of USM research grant (RUI/ 1001/ PPSP/ 8012222).

## TABLE OF CONTENTS

**ACKNOWLEDGEMENT** ..... **ii**

**TABLE OF CONTENTS** ..... **iii**

**LIST OF TABLES** ..... **viii**

**LIST OF FIGURES** ..... **ix**

**LIST OF ABBREVIATIONS AND SYMBOLS** ..... **xi**

**LIST OF APPENDICES** ..... **xvi**

**ABSTRAK** ..... **xvii**

**ABSTRACT** ..... **xix**

**CHAPTER 1 INTRODUCTION** ..... **1**

1.1 Background of the Study ..... 1

1.2 Problem Statement ..... 3

1.3 Objectives of the Study ..... 4

1.3.1 Specific Objectives ..... 4

**CHAPTER 2 LITERATURE REVIEW** ..... **5**

2.1 Overview on Breast Cancer ..... 5

2.1.1 Breast ..... 5

2.1.2 Breast Cancer Pathogenesis ..... 7

2.1.3 Aetiology of Breast Carcinoma ..... 8

2.1.3(a) Gene mutation ..... 8

2.1.3(b) Non-genetic aetiological factors ..... 9

2.1.4 Hormonal and growth receptors role in carcinogenesis of breast cancer ..... 11

2.1.4(a) Estrogen and Estrogen Receptor (ER) ..... 11

2.1.4(b) Progesterone and Progesterone Receptors (PgR) ..... 14

2.1.4(c) HER2 signalling and HER2-Positive breast cancer ..... 16

2.1.5 Breast Cancer Classification ..... 19

2.1.6 mTOR signalling pathway and cancer ..... 20

2.1.7 Angiogenesis in Breast Cancer ..... 22

2.1.8 Prevalence of Breast Cancer ..... 24

2.2 Sirolimus ..... 27

2.3 Sunitinib ..... 29

2.4 NMU induced mammary carcinoma ..... 31

2.5 Mammary carcinoma in rats model ..... 32

**CHAPTER 3 ..... 34**

**MATERIALS AND METHODS ..... 34**

3.1 Study Design ..... 34

3.2 Materials ..... 35

3.2.1 Materials for mammary tumour induction and interventions ..... 35

3.2.1(a) Preparation of NMU solution ..... 35

3.2.1(b) Preparation of Sirolimus solution ..... 35

3.2.1(c) Preparation of Sunitinib solution ..... 36

3.2.1(d) Preparation of 10% DMSO ..... 36

3.2.1(e) Preparation of 40% PEG300 ..... 36

3.2.1(f) Preparation of 5% PEG (80) ..... 36

3.2.2 Materials for Histology analysis ..... 36

3.2.2(a) Preparation of 10% Neutral Buffered Formalin (NBF) solution ..... 37

3.2.2(b) Preparation of Harris Hematoxylin working solution ..... 37

3.2.2(c) Preparation of Eosin working solution ..... 37

3.2.2(d) Preparation of different percentage of ethanol ..... 37

3.2.2(e) Preparation of 1% acid alcohol ..... 37

3.2.2(f) Preparation of 0.3% ammonia water ..... 37

3.2.3 Materials for protein expression analysis ..... 38

3.2.3(a) Preparation of washing buffer ..... 38

3.2.3(b) Preparation of different concentration of ethanol ..... 38

3.2.3(c) Preparation of 3% perhydrol ..... 38

3.2.3(d) Preparation of 1X Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) ..... 38

3.2.3 (e) Preparation of Tris-EDTA Buffer ..... 39

3.2.3(f) Preparation of primary antibodies ..... 39

3.2.3(g) Preparation of Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse ..... 39

3.3 Methodology ..... 40

3.3.1 *In-vivo* study ..... 40

3.3.1(a) Animal preparation ..... 40

3.3.1(b) Tumour Induction and Detection ..... 41

3.3.1(c) Experimental Design ..... 43

3.3.1(d) Tumour samples collection ..... 45

3.3.2 Histological study ..... 47

3.3.2 (a) Fixation, tissue grossing, and tissue processing ..... 47

3.3.2(b) Tissue embedding and sectioning ..... 47

3.3.2 (c) Harris Haematoxylin and eosin staining ..... 48

3.3.2 (d) Tumour classification ..... 49

3.3.3 Immunohistochemical staining ..... 49

3.3.3(a) Tissue preparation for immnohistochemistry ..... 50

3.3.3(b) Immunohistochemistry procedure ..... 50

3.3.3(c) Immunohistochemistry scoring ..... 52

3.3.4 Gene Expression Study ..... 53

3.3.4(a) RNA Extraction ..... 53

3.3.4(b) cDNA synthesis ..... 55

3.3.4(c) Primer design and quantitative Real-Time Polymerase Chain Reaction (q-PCR) ..... 56

3.4 Calculation of sample size ..... 59

3.5 Statistical analysis ..... 59

**CHAPTER 4 ..... 60**

**RESULTS ..... 60**

4.1 NMU induced rat mammary carcinoma *in-vivo* study ..... 60

4.1.1 Tumour incidence and latency of NMU induced mammary carcinoma ..... 60

4.1.2 Intervention of Sirolimus and Sunitinib ..... 60

4.2 Histopathological analysis ..... 61

4.2.1 Characterization of NMU induced mammary tumour in untreated group ..... 61

4.3 Protein expression analysis ..... 65

4.3.1 ER, PgR and HER2/neu expressions in control and treatment groups ..... 66

4.3.2 Association of prognostic markers expression within intervention group ..... 70

4.4 Gene expression analysis ..... 72

4.4.1 Relative changes in gene expression of ER, PgR and HER2/neu mRNAs in Sirolimus and/or Sunitinib- treated groups ..... 72

**CHAPTER 5 ..... 76**

**DISCUSSION ..... 76**

5.1 Effects of Sirolimus and Sunitinib on Histological Features of NMU-induced Breast Carcinoma ..... 76

5.2 Effects of Sirolimus and Sunitinib on NMU-induced Breast Carcinoma Growth ..... 78

5.3 Effects of Sirolimus and Sunitinib on Protein Expressions of ER, PgR, and HER2/neu of NMU-induced Breast Carcinoma ..... 81

5.4 Effects of Sirolimus and Sunitinib on Gene Expressions of ER, PgR, and HER2/neu of NMU-induced Breast Carcinoma ..... 84

**CHAPTER 6 ..... 87**

**CONCLUSION ..... 87**

6.1 Summary of current study ..... 87

6.2 Limitation of study ..... 88

6.3 Recommendation of future research ..... 88

**REFERENCES ..... 89**

**APPENDICES**

## LIST OF TABLES

**Page**


Table 2.1 Molecular subtypes of breast cancers .....20

Table 2.2 Number and percentage of cancers in Malaysia by age groups in adults .....26

Table 3. 1 Staining protocol of IHC .....51

Table 3.2 Guidelines of scoring ER and PgR by Allred scoring system .....52

Table 4.1 Tumour diameter (Mean ± S.D) of Intervention Groups after First Treatment and Five Days Post Second Treatment .....61

Table 4.2 The Tumour Types in the Intervention Groups.....65

Table 4.3 ER localization in control and treatments groups .....66

Table 4.4 PgR localization in control and treatments groups .....67

Table 4.5 HER2/neu localization in control and treatments groups .....67

Table 4.6 The Expressions of ER, PgR, and HER2/neu between Intervention and Control Groups .....70

Table 4.7 The Expressions of ER, PgR, and HER2/neu amongst the Intervention Groups .....71

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 $2^{-\Delta\Delta C_T}$ method .....74

## LIST OF FIGURES

**Page**


| | | |
|---|---|---|
| Figure 2.1 | Anatomy of the breast | 6 |
| Figure 2.2 | ER signalling pathway | 13 |
| Figure 2.3 | Signalling downstream of progesterone. | 15 |
| Figure 2. 4 | HER2/neu signalling pathway | 18 |
| Figure 2. 5 | mTOR signalling pathway | 22 |
| Figure 2. 6 | Global Maps Presenting the Most Common Type of Cancer Incidence in 2018 in Each Country Among Women. | 24 |
| 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. | 25 |
| Figure 2.8 | Structures of Sirolimus | 28 |
| Figure 2.9 | Sunitinib Chemical Structure | 29 |
| Figure 2.10 | Mechanism of action of Sunitinib in endothelial cells expressing the vascular endothelial growth factor receptors (VEGFRs) | 30 |
| Figure 3.1 | Flowchart of Study Design | 34 |
| Figure 3.2 | Rats in polycarbonate cages | 41 |
| Figure 3.3 | Intraperitoneal injection of NMU | 42 |
| Figure 3.4 | Measure tumour size by using vernier calliper | 42 |
| Figure 3. 5 | Anesthetize the rat by inhaled anaesthetics Isoflurane | 44 |
| Figure 3.6 | Intratumoral treatment injections. | 44 |
| Figure 3. 7 | Euthanize process through exposure to carbon dioxide gaseous in a closed plastic bag. | 46 |
| Figure 3. 8 | Measuring of the final diameters of the tumours | 46 |

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 ..... 62

Figure 4.2 Histology of NMU-induced Invasive Breast Carcinoma. ..... 63

Figure 4.3 NMU-induced Cribriform Invasive Carcinoma. H&E staining magnification X 400. Tumour cell (TC). Blood vessel (BV) ..... 63

Figure 4.4 NMU-induced Papillary Invasive Carcinoma. H&E staining magnification X 400. ..... 64

Figure 4.5 NMU-induced No Special Type Carcinoma. H&E staining magnification X 400. Tumour cell (TC), Blood vessel (BV), Mitotic Figures (MF) ..... 64

Figure 4.6 Representative of immunohistochemical nuclear expressions of ER on tumour specimens. 400X magnification ..... 68

Figure 4.7 Representative of immunohistochemical nuclear expressions of PgR on tumour specimens. 400X magnification ..... 68

Figure 4.8 Representative of immunohistochemical low expressions (scored 1) of HER2/neu on tumour specimens. 400X magnification ..... 69

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). ..... 75

Figure 5.1 ER, PgR, HER2/neu and RTK play roles in mTOR signalling cascade ..... 81

## 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 |

## LIST OF APPENDICES


| | |
|---|---|
| Appendix A | Table 1 Tissue processing schedule using automated Tissue-Tek® VIP |
| Appendix B | Figure 1 Amplification plot of β-actin, ER, PgR, and HER2/neu genes |
| Appendix C | List of presentations |
| Appendix D | Publication |

**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).

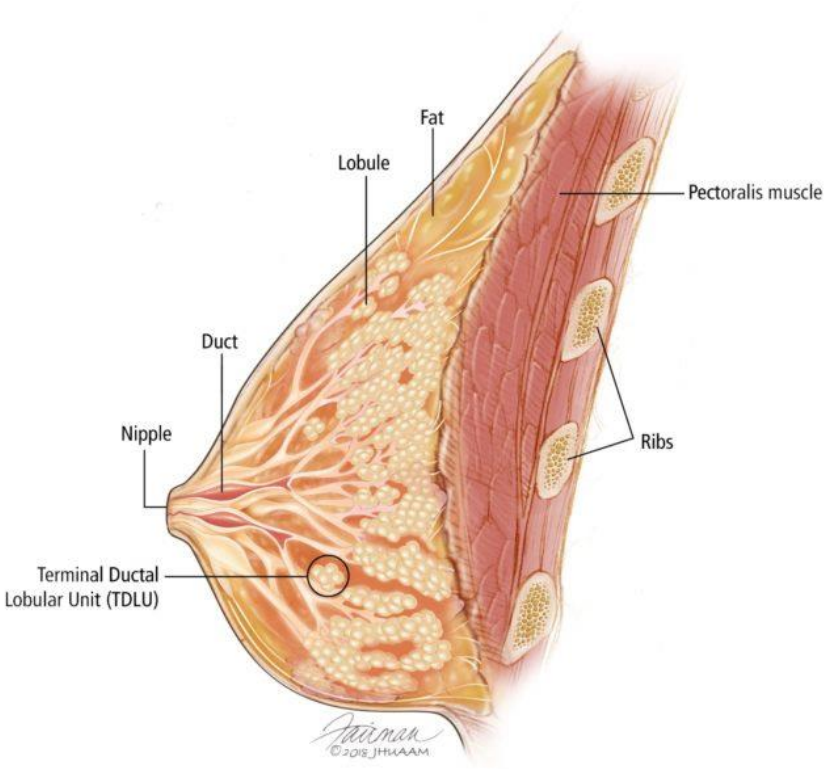


Figure 2.1 Anatomy of the breast

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).

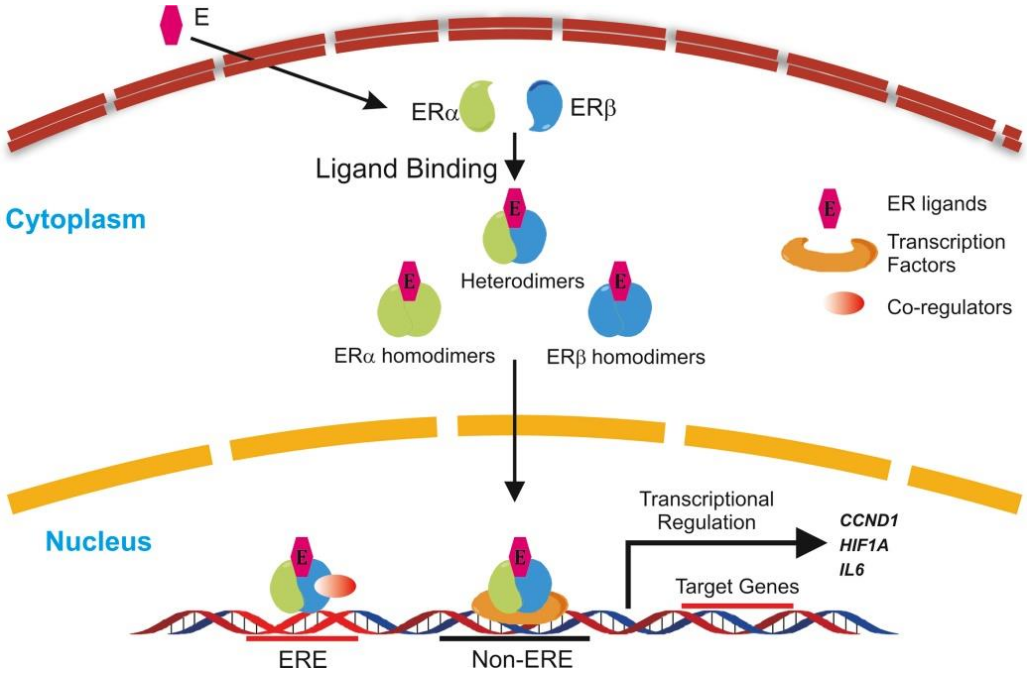


Figure 2.2 ER signalling pathway

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) .

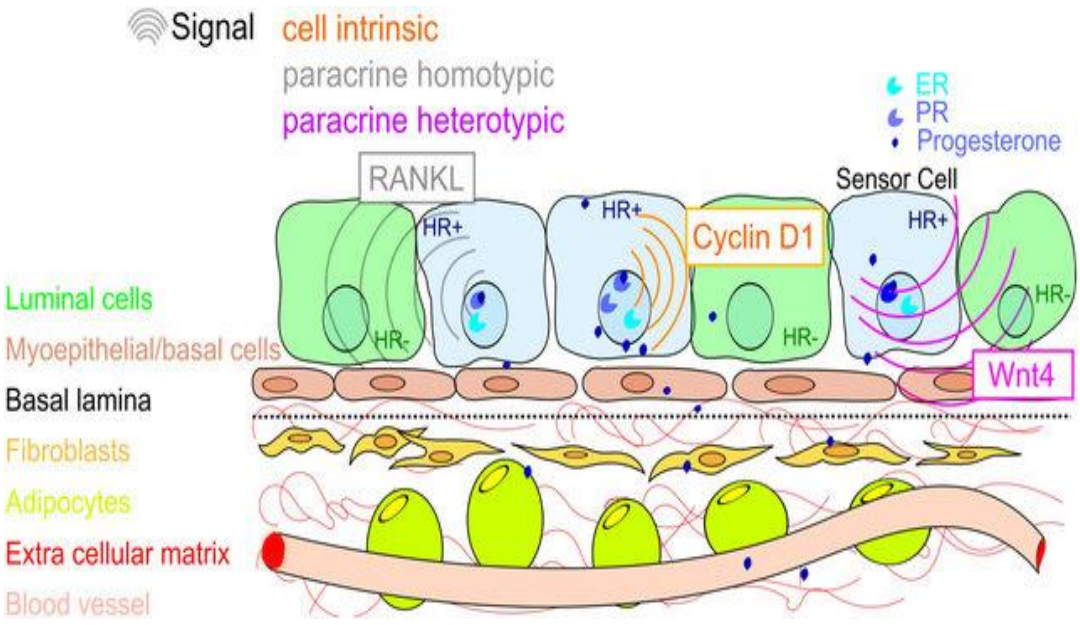


Figure 2.3 Signalling downstream of progesterone.

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).

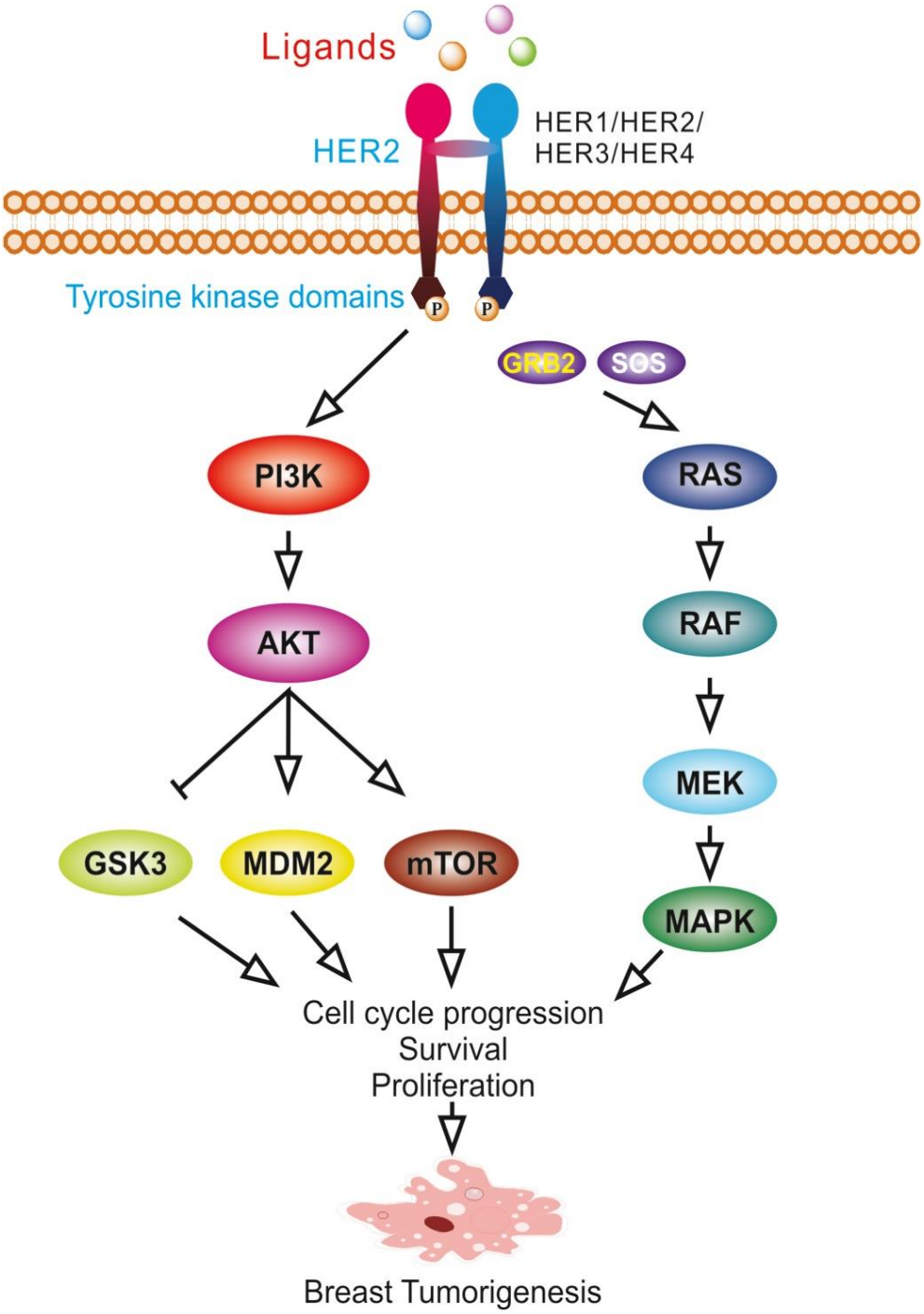


Figure 2. 4 HER2/neu signalling pathway

### 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<br>Best prognosis | Hormonal therapy<br>Targeted therapy |
| Luminal B | ER+, PR+, HER2/neu ±,<br>Ki67 ≥14% | 10-20%<br>Lower survival than Luminal A | Hormonal therapy<br>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<br>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.

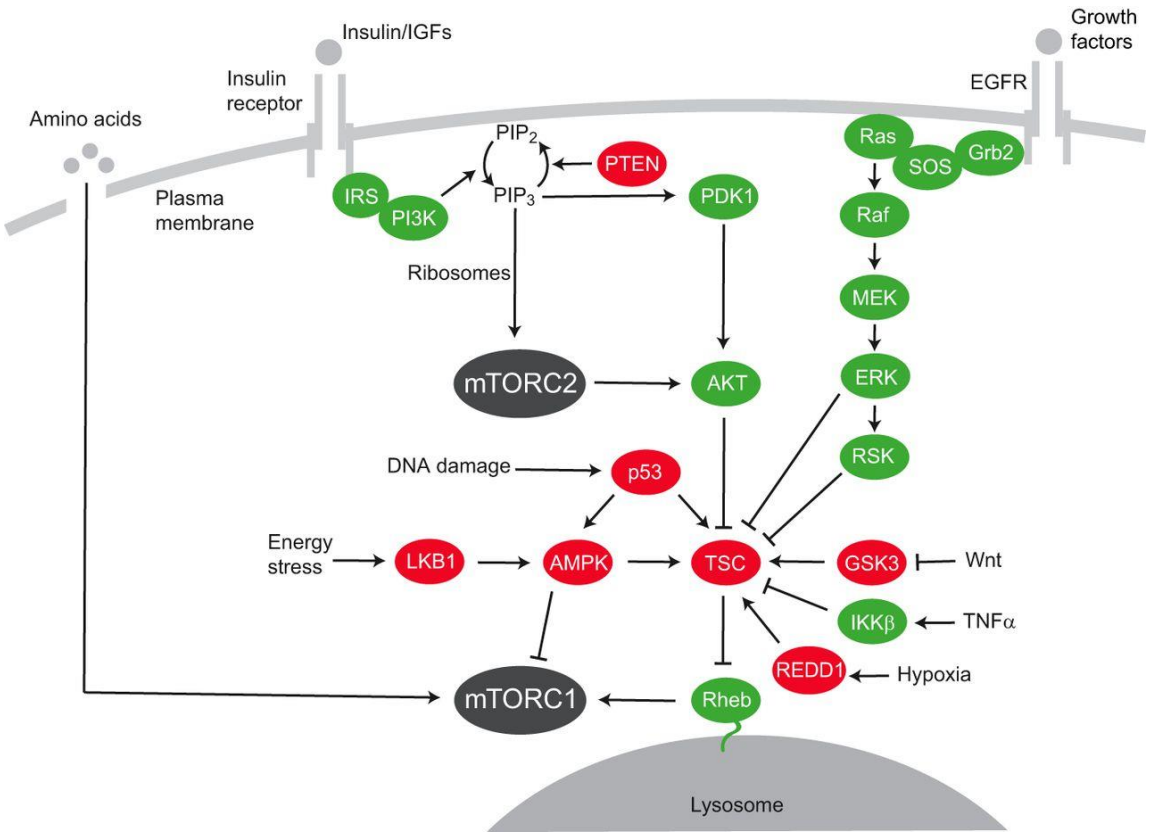


Figure 2. 5 mTOR signalling pathway

### 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).

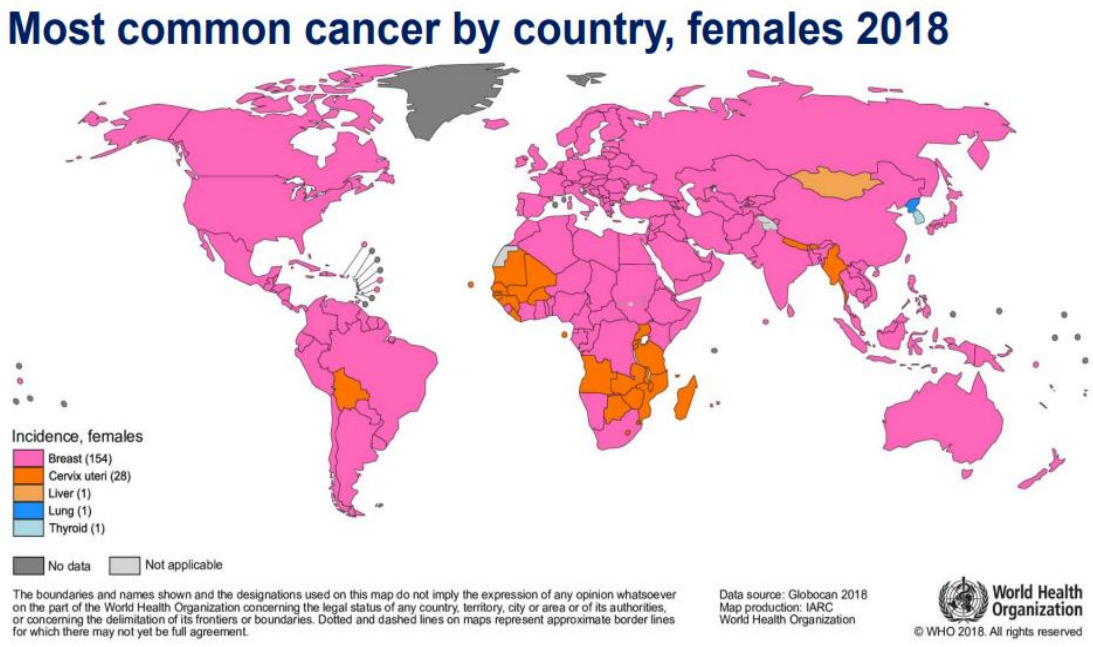


Figure 2. 6 Global Maps Presenting the Most Common Type of Cancer Incidence in 2018 in Each Country Among Women.