ELUCIDATING THE EFFECTS OF SIROLIMUS AND SUNITINIB ON NMU-INDUCED RAT BREAST CARCINOMA: *IN VITRO* AND *IN VIVO* STUDIES

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by

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LIST OF SYMBOLS

^{o}C	Degree Celsius
A	Alpha
B	Beta
μ	Micro
Γ	Gamma
®	Registered trademark
™	Trademark sign
V	Volt
Ω	Ohm
Π	Pi
Ca^{2+}	Calcium
Mg^{2+}	Magnesium

LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
4E-BP1	4E binding protein 1
ARASC	Animal Research and Service Centre
Ca^{2+}	calcium ions
CDK1	cyclin dependent kinase 1
DCIS	ductal carcinoma *in situ*
DMBA	7,12- dimethylbenzanthracene
EGF	epidermal growth factor
EGFRs	epidermal growth factor receptors
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FLK-1	fms-like kinase-1
GIST	imatinib-resistant gastrointestinal stromal tumour
GLOBOCAN	Global Cancer Observatory
H & E	haematoxylin & eosin
HIF-1α	Hypoxia inducible factor 1 alpha
HSP27	heat-shock protein-27
IDC-NST	infiltrative ductal carcinoma with no special type
IHC	Immunohistochemistry
IL	Interleukin
kDa	Kilodalton
KDR	kinase derived receptor
kg	Kilogram
L	Liter
LCIS	lobular carcinoma *in situ*
M	Molar
MAKNA	Majlis Kanser Negara
MAPK	mitogen-activated protein kinase
mg	Milligram
ml	Milliliter

mRNA	messenger ribonucleic acid
mTOR	mechanistic target of rapamycin
mTORC1	mechanistic target of rapamycin complex 1
mTORC2	mechanistic target of rapamycin complex 2
MVD	microvessel density
N:C	nuclear over cytoplasmic ratio
NBF	neutral buffered formalin
nm	Nanometer
NMU	N-nitroso-N-methylurea
NOS	nitric-oxide synthase
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDGFRs	platelet derived growth factor receptors
PEG-400	polyethylene glycol 400
PI3K	phosphoinositide 3-kinase
PLCγ	phospholipase C –gamma
qRT-PCR	quantitative reverse trancriptase polymerase chain reaction
RCC	renal cell carcinoma
REST	relative expression software tool
RET	rearranged during transfection
RNA	ribonucleic acid
RTK	receptor tyrosine kinases
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT3	Signal transducer and activator of transcription 3
TBS	tris buffer saline
TEB	Terminal End Bud
TGF	tumour-like growth factor
TGFRs	Tumour-like growth factor receptors
VEGF	vascular endothelial growth factor
VEGFRs	vascular endothelial growth factor receptors
w/v	weight per volume

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MENJELASKAN KESAN SIROLIMUS DAN SUNITINIB TERHADAP KARSINOMA PAYUDARA TIKUS YANG DIDORONG OLEH NMU: KAJIAN *IN VITRO* DAN *IN VIVO*

ABSTRAK

Kanser payudara merangkumi tisu yang mengalami perubahan atau pengubahsuaian genetik atau tisu yang bermutasi. Kini, terdapat pelbagai pilihan bagi rawatan kanser termasuklah pembedahan, kemoterapi, radioterapi dan rawatan imun-bersasar. Sirolimus juga dikenali sebagai rapamycin adalah perawatan anti-bakteria yang mengandungi kesan anti-fungus, imunosupresan dan merupakan agen anti-angiogenik dalam perawatan bersasar. Sunitinib merupakan perencat bermolekul kecil, pelbagai-sasaran receptor tyrosine kinases (RTKs). Dalam kajian ini, kesan sirolimus dan sunitinib terhadap kanser payudara dijelaskan. Faktor angiogenesis termasuklah VEGFR-2, PDGFR-β, mTOR, HIF-1α dan STAT dikaji. Sebagai tambahan, kajian mengenai korelasi antara MVD dengan ekspresi angiogenik dijalankan juga. Untuk model *in vivo*, sebanyak dua puluh empat (24) ekor Sprague Dawley betina berumur 20 hari diperoleh daripada ARASC USM Kubang Kerian. Segala eksperimen dan prosedur yang dilakukan terhadap haiwan tersebut dijalankan secara pengawalan ketat berpandukan Animal Ethics Committee (AEC) USM bagi panduan penjagaan dan kebajikan haiwan. Pembentukan kanser payudara diransang oleh bahan kimia NMU dan tikus yang mengalami kanser payudara dibahagikan kepada 4 kumpulan iaitu sirolimus, sunitinib, sirolimus+sunitinib dan tiada rawatan. Untuk model in vitro, titsan sel MCF-7 dan MDA-MB 231 digunakan bagi mewakili kanser payudara tahap agresif. Titisan sel tersebut dirawat dengan menggunakan ubat

yang dikaji dan dianalisis untuk pengenalpastian ekspresi protein dan apoptosis sel. Keputusan imunohistokimia terhadap keseluruhan kumpulan terawat mendapati pengurangan ekspresi faktor angiogenik yang signifikan (P<0.05) berbanding kumpulan kawalan. Analisis gen terhadap kumpulan terawat sirolimus dan sunitinib mendapati penurunan ekspresi yang signifikan terhadap faktor angiogenik berbanding kumpulan tidak terawat. Ini menunjukkan kebolehan ubat perawatan sirolimus dan sunitinib menekan proses angiogenik. Kajian MVD dengan menggunakan pewarnaan CD31 menunjukkan pengurangan bilangan vaskular yang signifikan (P<0.05) terhadap keseluruhan kumpulan terawat berbanding kumpulan tidak terawat. Kajian korelasi terhadap MVD dan ekspresi angiogenesis menunjukkan korelasi positif terhadap faktor angiogenesis (VEGFR-2, mTOR, HIF-1α and STAT3) dan bacaan MVD. Manakala bagi ujikaji *in vitro* terhadap titisan sel MCF-7 dan MDA-MB 231 mendapati perawatan dengan menggunakan sirolimus dan sunitinib menunjukkan pengurangan kadar tumbesaran sel dan kadar proliferasi sel yang signifikan (P<0.05) berbanding kumpulan tidak terawat dengan dos IC_{50}. Hasil kajian ini menunjukkan perawatan dengan sirolimus, sunitinib dan sirolimus+sunitinib adalah efektif bagi menghalang proliferasi sel MCF-7 dan MDA-MB 231 dalam pengaruh masa dan dos. Kajian apoptosis menunjukkan peningkatan yang signifikan terhadap bilangan sel apoptotik dalam kumpulan perawatan berbanding kumpulan kawalan. Kesimpulannya, sirolimus dan sunitinib sebagai agen persendirian mempunyai potensi sebagai ubat anti-kanser bagi perawatan kanser payudara dibuktikan dengan analisis di peringkat protein dan gen secara *in vivo* dan *in vitro* manakala perawatan sirolimus dan sunitinib secara kombinasi menunjukkan kesan sinergistik.

ELUCIDATING THE EFFECTS OF SIROLIMUS AND SUNITINIB ON NMU-INDUCED RAT BREAST CARCINOMA: *IN VITRO* AND *IN VIVO* STUDIES

ABSTRACT

Breast cancer tissue comprises genes altered, modified or/and mutated tissue. Nowadays, there are many options available for cancer remedy which includes surgery, chemotherapy, radiotherapy as well as immune-targeted therapy. A previous study found that combining drugs helps to improve tamoxifen effects towards breast cancer. Sirolimus also known as rapamycin, is a bacterial macrolide with anti-fungal properties, immunosuppressant and anti-angiogenic agent in targeted therapy. Sunitinib is a small molecule, multi-targeted receptor tyrosine kinases (RTKs) inhibitor. In this study, the effects of sirolimus and sunitinib on the breast cancer model were elucidated. Angiogenic factors which include VEGFR-2, PDGFR-β, mTOR, HIF-1α and STAT3 were studied. In addition, the correlation study of MVD with angiogenic expression was carried out as well. For *in vivo* model, a total number of twenty-four (24) female Sprague Dawley's rats were obtained from ARASC USM Kubang Kerian at day 20 of age. The experiments and procedures towards the animal were conducted strictly in accordance with approval from the Animal Ethics Committee of USM guidelines of animal care and welfare. The breast tumour was induced by NMU and grouped into 4 groups (sirolimus, sunitinib, sirolimus+sunitinib and untreated). For *in vitro* model, MCF-7 and MDA-MB 231 cell lines were used representing aggressive types of breast cancer. The cell lines were treated with drugs and analyzed for proteins expression as well as apoptosis. Immunohistochemistry

scoring for the entire treated groups showed a significant (P<0.05) reduction of angiogenic factors expressions compared to a control group. Gene analyses of sirolimus and sunitinib treated breast tumours showed significant (P<0.05) down-regulation of angiogenic factors compared to the untreated group. This indicated anti-angiogenesis properties of sirolimus and sunitinib suppressed angiogenic pathways. MVD study by using CD31 staining showed significantly reduced number of vascular obtained from the entire treated groups compared to the untreated group. A correlation study on MVD and angiogenic factors expression showed a positive correlation between angiogenic factors (VEGFR-2, mTOR, HIF-1α and STAT3) and MVD count. On the other hand, *in vitro* study of MCF-7 and MDA-MD 231 cell lines revealed that treatment with sirolimus and sunitinib significantly (P<0.05) reduced cell growth as well as proliferation rates compared to untreated at the IC_{50} dose. The result indicated sirolimus and sunitinib as well as sirolimus+sunitinib effectively inhibited proliferation of MCF-7 and MDA-MB 231 in the time and dose-dependent manner. Apoptosis study found a significant increase of apoptotic cells obtained in the entire treated groups. In conclusion, sirolimus and sunitinib as single agents represent potential effective anti-cancer drugs for breast cancer treatment which are proven at protein and gene analysis of *in vivo* and *in vitro* models while the combination of sirolimus and sunitinib showed a synergistic effect.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Breast cancer tissue comprises genes altered, modified or/and mutated tissue. The tissue undergoes several changes include hyperproliferation, disorganization and neglecting apoptosis process. There are many risk factors contribute to breast cancer development includes chemical exposure, radiation, smoking, female gender, food, gene inherited and many more (Ataollahi *et al.*, 2015). Breast carcinoma may arise *in situ* or invasive carcinoma which reflecting their nature and aggressiveness. According to tumour classification, *in situ* stage is categorized under benign stage or early stage of cancer development which commonly assemble within ducts or lobules (Feng *et al*., 2018). In contrast, malignant tumour is a late-stage tumour which appear to be disorganized, highly proliferated and metastases. Malignant tumour is very aggressive in nature and always resulting bad prognosis (Cooper, 2000). Benign and malignant tumour can be differentiating according to their phenotype. Malignant tumour always appears high nuclear over cytoplasmic ratio (N:C), nucleolus appearance, inorganized, irregular size and shape while benign tumour always appear conversely (Baba & Câtoi, 2007).

Signs and symptoms of breast cancer may include lump at the breast area, change of breast shape, dimpling skin, discharge, or a red scaly patch of skin. Higher stage of breast cancer may show few additional symptoms include swollen lymph nodes, shortness of breath, or yellowish skin (jaundice) (Kabel & Baali, 2015). Breast cancer was discovered since ancient time and was recorded as most common occurring cancer among woman and second most common cancer among solid tumour in 2018 (Ferlay *et al*., 2020). The incidence and mortality rate of breast cancer have been

reported skyrocketing every year in most of Asia countries. In Malaysia, breast cancer is one of the major concerns for Ministry of Health and several non-government organizations (NGO) has been formed to help the victims and funds the investigation for breast cancer treatment. Those organization includes Breast Cancer Welfare Association Malaysia, Breast Cancer Foundation, National Cancer Society of Malaysia and Majlis Kanser Negara (MAKNA).

Angiogenesis is an essential process for cell growth, development and wound healing. Angiogenesis is a process of new blood vessel formation arising from primary blood vessel (Bodnar, 2015). On the other hand, neo-angiogenesis is the formation of new blood vessel arises from existing primary vessel triggered by tumour cells. This mechanism is regulated by several proteins and genes known as angiogenic markers (Adair & Montani, 2010). When the tumour grew bigger in size, it demands for more supply of nutrients and oxygen to sustain (Siemann & Horsman, 2015). Several markers have been identified from vast of research which includes vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), tumour-like growth factor (TGF), interleukin (IL), nitric-oxide synthase (NOS) and epidermal growth factor (EGF). The process involves certain proteins released by tumour cells known as signalling process followed by detachment of endothelial cells from pericytes and sprouting process towards stimuli (Carmeliet & Jain, 2011). Endothelial cells undergone differentiation and proliferation processes and rapidly grow for maturation during sprouting process. The matured endothelial cells then assemble to form an interaction between cells. The cells then stick closely together forming immature vessel. Pericytes then recruited to the newly formed blood vessel and forming mature blood vessel (Bergers & Song, 2005). With the present of pericytes, blood vessel formed is more stable and less leaking network. The

antileakage of Ang1 requires activation of PDGF-dependent of pericytes to maintain the endothelial stability (Fuxe *et al.*, 2011).

There are several ways can be used for cancer remedy. These include chemotherapy, radiotherapy, surgery (amputation), targeted therapy as well as alternative medicine (herbs and traditional). Blocking angiogenic markers is not new in cancer targeted therapy (Masoud & Pagès, 2017). Targeted therapy is widely used nowadays instead of chemotherapy and radiotherapy option because the technique specifically targeting the roots of the disease which mainly the specific proteins or genes. This technique also been further developed to correct the genes in cancer disease and known as gene therapy (Das *et al*., 2015). By far, several countries have been given the authority to develop and licence to practice gene therapy and China was the first country to get since 2004. Targeted therapy is widely implemented nowadays to treat cancer disease, but the cost is a more expensive. The recent trend of research is to develop targeted therapy on cancer tissue (Falzone, Salomone & Libra, 2018). In this study, angiogenesis marker specifically vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth factor receptor beta-beta (PDGFR-ββ), mammalian target of rapamycin (mTOR), human inducible factor-1 alpha and signal transducer and activator of transcription-3 (STAT-3) were used as the target protein and gene to inhibit tumour angiogenesis. The expression of those markers indicating angiogenesis activity in both *in vitro* and *in vivo* models.

Sirolimus (Rapamune$^{(R)}$; Wyeth-Ayerst, PA, USA) also known as rapamycin, is a bacterial macrolide with anti-fungal properties, immunosuppressant and anti-angiogenic agent in targeted therapy. Sirolimus is produced by the bacterium *Streptomyces hygroscopicus*, found in Easter Island, Chile (Sehgal, 2003). It has the ability to inhibit the activation of B and T cells by reducing their sensitivity to

interleukin-2 receptor through blocking mTOR pathway (Thomson, Turnquist & Raimondi, 2005). Instead of mTOR, sirolimus down-regulates several angiogenic markers including vascular endothelial growth factor receptors (VEGFRs), fibroblast growth factor receptors (FGFRs), Platelet-derive growth factor receptors (PDGFRs), Tumour-like growth factor receptor (TGFR), nitric-oxide synthase (NOS) and epidermal growth factor receptors (EGFRs) which suggested to be one of the best multiple targeted anti-angiogenic drugs (Raica & Cimpean, 2010). Sirolimus is known to target the atypical Ser/Thr kinase mechanistic target of rapamycin (mTOR) and inhibit key mRNA translation of proteins required for cell cycle progression (Tee, 2018). In additional, sirolimus also modulate critical signal transduction pathways that link mitogenic stimuli to the synthesis of proteins required for cell cycle traverse from G_1 to S (Hidalgo & Rowinsky, 2000). Sirolimus has an impressive reaction on suppressing tumour activity but poor aqueous solubility and chemical stability restricted sirolimus to be widely use (Haeri *et al.,* 2018). However, a series of sirolimus analogues with improved aqueous solubility and stability have been synthesized and evaluated. CCI-779 (Wyeth Ayerst, PA, USA), a soluble ester analogue of rapamycin, was selected for development as an anti-cancer agent based on its prominent anti-tumour profile and favourable pharmaceutical and toxicological characteristics in preclinical studies.

Sunitinib (marketed as Sutent by Pfizer, and previously known as SU11248) is a small molecule, multi-targeted receptor tyrosine kinases (RTKs) inhibitor (Shukla *et al,* 2009). The effects of sunitinib can be seen on PDGFRs and VEGFRs markers which play a pivotal role in regulating cell proliferation and tumour angiogenesis (Niu & Chen, 2010). The inhibition of those receptors will reduce tumour vascularization and triggers cell apoptosis thus causing the tumour to suppress. The drug is approved

to be used to treat renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumour (GIST). Instead of those receptors, sunitinib also inhibit CD117 (c-KIT), RTK which activated by gene mutation. CD117 is the important ligand which drives the majority of gastrointestinal stromal cell tumour (Abbaspour Babaei *et al.,* 2016). Sunitinib has been named as second-line therapy for patients who develop mutations in C-KIT which have resistant to imatinib or not tolerated to the drug (Mulet-Margalef & Garcia-Del-Muro, 2016). In addition, sunitinib is well bind to other receptors includes RET, CD114 and CD 135. RET is an abbreviation for 'rearranged during transfection' which gene was originally found in lymphoma cells (Ancker *et al.,* 2017). Sunitinib is well-known anti-angiogenic drug which have multiple-targeted site on RTKs, it also has many side effects such as the classic hand-foot syndrome, stomatitis, and other dermatologic toxicities (Papaetis & Syrigos, 2009).

Many factors influence the development and survival of cancer. Breast cancer is angiogenesis-dependant type of cancer and highly depends on hormonal supply (Madu *et al.,* 2020). Evaluation on the regulation of associated angiogenic markers under tumour-angiogenesis suppressed environment is crucial in advanced stages of breast cancer treatment (Castañeda-Gill & Vishwanatha, 2016). The increased of incidence and mortality leads to need of improvement of breast cancer treatment technology and strategy. In this study, KDR and mTOR markers which plays a pivotal role in regulating neo-angiogenesis were blocked by using sunitinib, sirolimus and sirolimus+sunitinib. The aim of the study mainly to elucidate the effects of the drug(s) on specific angiogenic ligands; VEGFR-2, PDGFR-Beta, STAT-3, mTOR and HIF-1 alpha markers at protein and gene level. The expression of those markers was associated with microvessel density (MVD) expressed by CD31.

In this study, the breast cancer tissue was suppressed by using sirolimus and sunitinib drugs which are well known as anti-mTOR and anti-VEGF respectively. In many studies, those drugs were proven to show potent outcome to suppress angiogenesis in many types of cancer cell. Both drugs were combined in this study to determine the synergistic activity which will be useful for the development of new targeted therapy for treating breast carcinoma especially in high stage and triple negative breast cancer.

1.2 Literature review

1.2.1 Morphogenesis of normal mammary gland

The mammary gland of breasts is a dynamic structure. The gland will undergo continuous changes during pregnancy, lactation and involution (Brisken & O'Malley, 2010). The process of mammary development among primate is known as mammogenesis (Figure 1.1). It occurs across several phases includes prenatal, puberty, pregnancy and menopause. Development of mammary gland begins right after birth during the fourth week of gestation where only a few poorly branched mammary ducts are formed (Macias & Hinck, 2012). At puberty stage, oestrogen and progesterone hormones secreted stimulate the ductules elongation and branching which takes place at the Terminal End Bud (TEB). During pregnancy, estrogen and progesterone as well as prolactin are highly stimulated, promotes differentiation of existing ductules into lobules and alveoli (Arendt & Kuperwasser, 2015). At parturition, the lobuloalveolar epithelium is converted to a secretory phenotype. The structure is responsible to start synthesizing milk production (Akers, 2017). Involution of the lobuloalveolar system occurs in response to milk stasis at the end of lactation (Rezaei *et al*., 2016). At the

stage of menopause, the mammary start to cease and the breast undergo atrophy. This process occurs throughout life of female primate (Dewi & Cline, 2021).

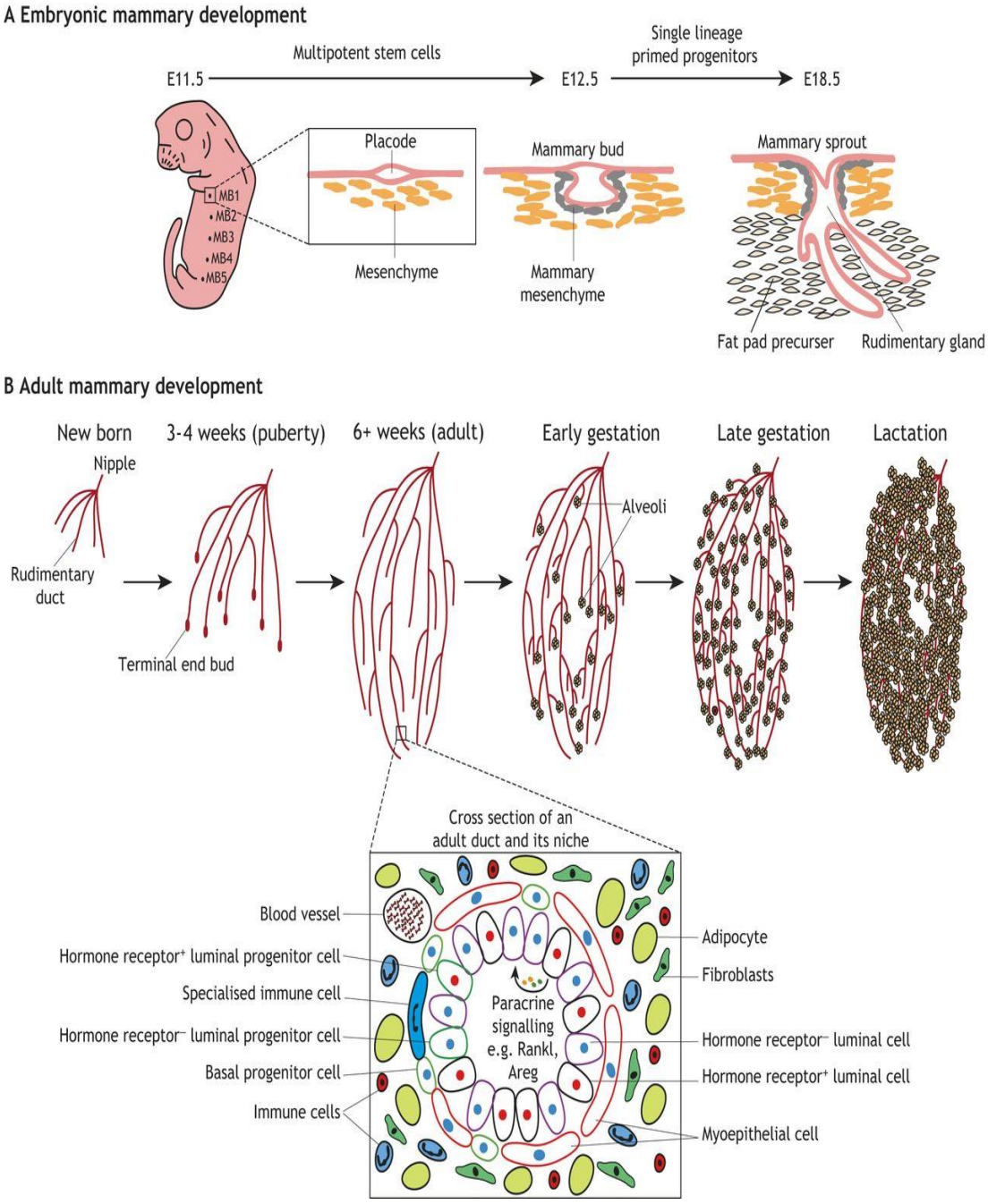

Figure 1.1 A shows a human embryonic mammary structure development. The mammary cells derived from multipotent stem cell and developed to become matured mammary cells. B shows an adult mammary structure development (Gjorevski & Nelson, 2011).

Hormonal regulatory plays an important role on the changes (Figure 1.2). Oestrogen hormone is highly influencing the changes of breast especially during period of breast feeding, pregnancy, menopausal, menses and weight loss/gain (Dall & Britt, 2017). Normal mammary gland is heterogeneously composed of glandular, adipose, ductal and connective tissue. Glandular structure of breast generally made of ducts and lobules. This structure is essential in synthesizing milk production which is literally controlled by several types of hormones including prolactin, somatotropin and growth hormone (Oakes, Hilton & Ormandy, 2006). The glandular tissues are embedded in connective tissues which composed of blood and lymphatic vessels, nerve, adipose, fibrous tissues and stromal. Connective tissue plays a crucial role to ensure essential supply of nutrition and oxygen as well as physical support (Zhu & Nelson, 2013).

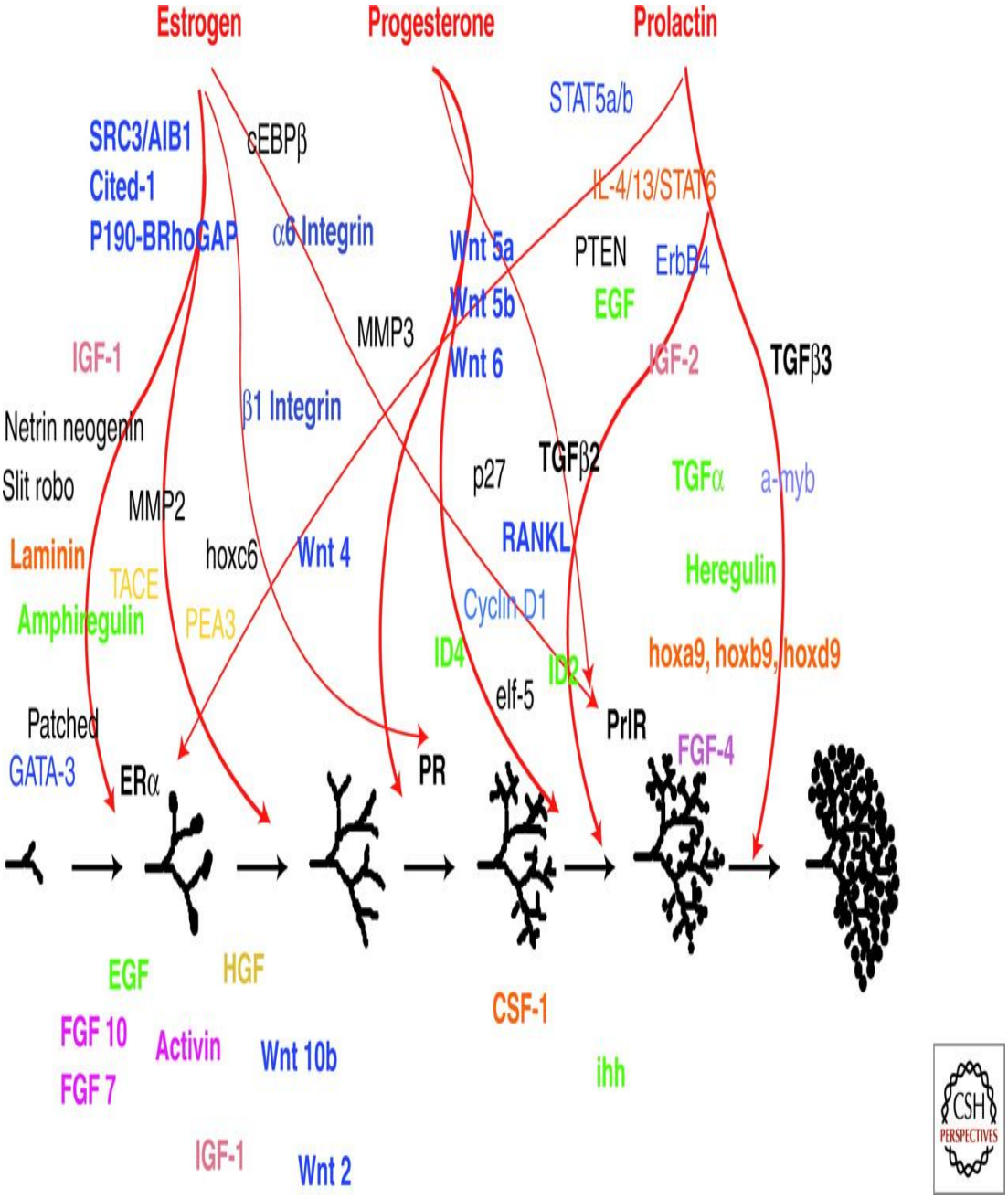

Figure 1.2 The illustrated diagram shows the hormonal influence on development of mammary structure. Progesterone, estrogen and prolactin appears to play a pivotal role in the human mammary development (Brisken & O'Malley, 2010).

1.2.2 Breast cancer

1.2.2(a) Breast cancer incidence and prevalence

Breast cancer is the most reported cancer in women with about 2 million new cases reported worldwide in 2018. Based on Figure 1.3, International Agency for Research on Cancer (IARC) has revealed that breast cancer incidence reported in Malaysia has climbed to second highest incidence across both sexes (male and female) in 2020 below other cancers type (exclude breast, lung, liver, nasopharynx and colorectum) with 49.6% (Ferlay *et al*., 2020). According to the data, women show high percentage of breast cancer incidence compared to other cancers (35.2%) which account for 32.9%. The statistic in 2020 shows breast cancer high mortality rate among women the incidence of developing cancer before the age of 75 is 15.2% (Table 1.1). Based on the data reported to the IARC in 2020 for Malaysia country, breast cancer leads the list of new cases with 8418 new cases which account for 17.3% and mortality rate at 11.9% of the cases reported (Table 1.2). According to Mun Seng Lee *et al.* (2019), 1 in every 19 women are at risk of getting breast cancer in Malaysia. According to the study conducted, low awareness among women leads to increase high stage breast cancer. Breast cancer is dominant among female sex diseases recorded worldwide. According to the record obtained by the agency, breast cancer has recorded second highest incidence among female sex which accounts for 32.9% just below other types of cancer with 35.7%. This report has shown that the incidence of breast cancer remained high since 2014. The report also highlighted that the mortality rate of breast cancer has been recorded the highest compared to other types of cancer reported with 20.7% (Ferlay *et al*., 2020). Interestingly, looking at the age statistic, young women showed a rapid increase of breast cancer cases from different parts of Asia which generally in advanced stages compared to their counterpart in developed countries.

The increased risk of breast cancer each year due to variety of causes leads to improvement of breast cancer treatment is a must and molecular-targeted combined therapy is found to give better outcome (Leora H. *et al*., 2014).

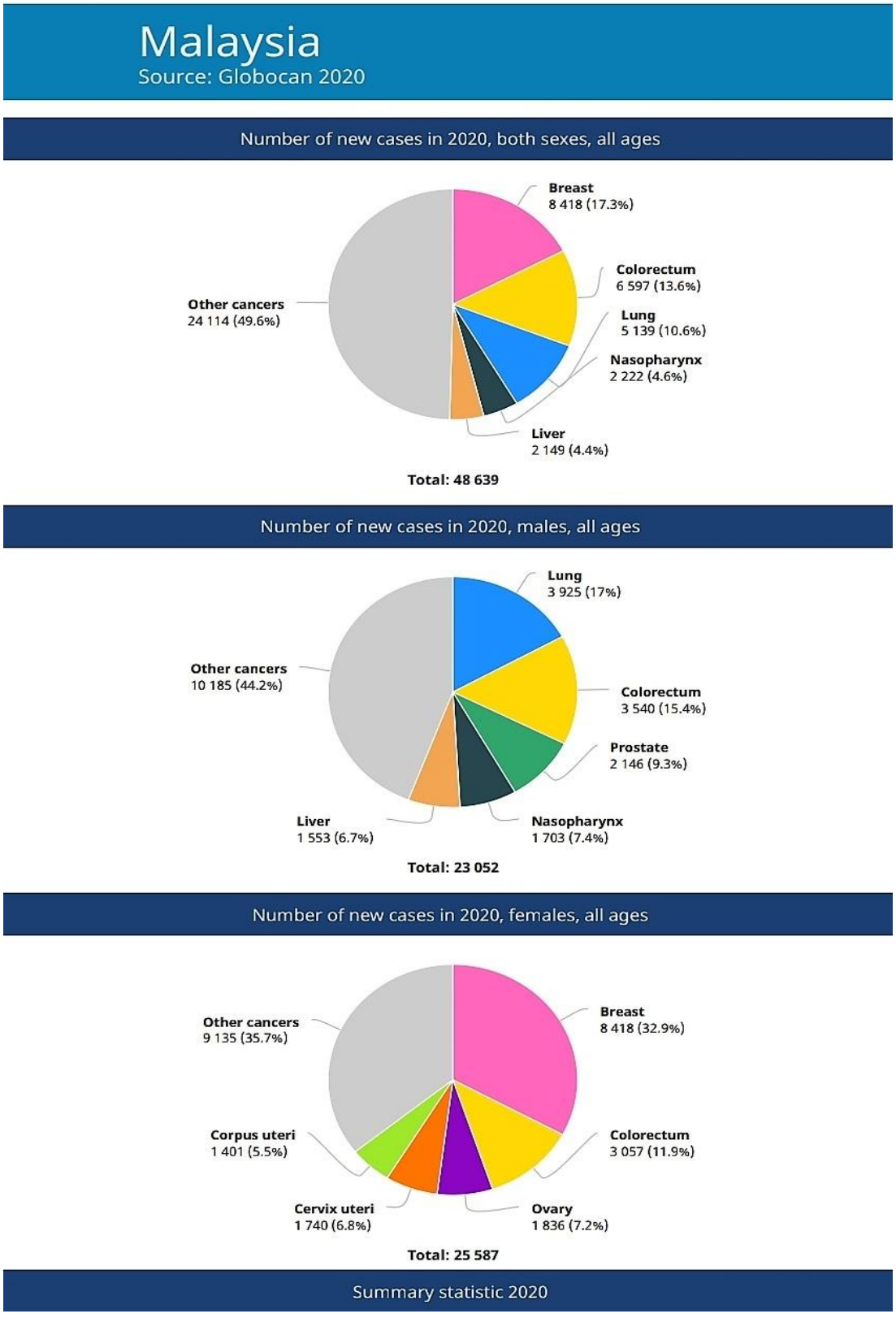

Figure 1.3 The percentage of new cancer cases reported in Malaysia according to gender for the year of 2020. Breast cancer accounts for the second highest incidence below other cancers with 17.3% and 32.9% for both sexes and female gender consecutively (Ferlay *et al*., 2020).

Table 1.1 The summary of new cancer excluding non-melanoma skin cancer reported for year 2020. Breast cancer is the highest case reported among female gender and both sexes above colorectum cancer (Ferlay *et al*., 2020).

Summary statistic 2020			
	Males	**Females**	**Both sexes**
Population	16 630 813	15 735 185	32 365 998
Number of new cancer cases	23 052	25 587	48 639
Age-standardized incidence rate (World)	137.8	151.4	143.9
Risk of developing cancer before the age of 75 years (%)	14.6	15.2	14.8
Number of cancer deaths	15 601	13 929	29 530
Age-standardized mortality rate (World)	92.9	82.2	87.3
Risk of dying from cancer before the age of 75 years (%)	9.7	8.5	9.1
5-year prevalent cases	54 044	73 974	128 018
Top 5 most frequent cancers excluding non-melanoma skin cancer (ranked by cases)	Lung Colorectum Prostate Nasopharynx Liver	Breast Colorectum Ovary Cervix uteri Corpus uteri	Breast Colorectum Lung Nasopharynx Liver

Table 1.2 The table of incidence, mortality rate and prevalence according to cancer site in the year 2020. Breast cancer accounts for the highest incidence and prevalence compared to other type of cancers and the second highest below lung cancer for the mortality rate (Ferlay *et al*, 2020).

Malaysia

Source: Globocan

Incidence, Mortality and Prevalence by cancer site

	New cases				Deaths				5-year prevalence (all ages)	
Cancer	Number	Rank	(%)	Cum.risk	Number	Rank	(%)	Cum.risk	Number	Prop. (per 100 000)
Breast	8 418	1	17.3	5.29	3 503	2	11.9	2.24	29 453	187.18
Lung	5 139	2	10.6	1.87	4 509	1	15.3	1.64	5 909	18.26
Colon	3 816	3	7.8	1.33	2 035	4	6.9	0.61	9 892	30.56
Rectum	2 690	4	5.5	0.94	1 385	7	4.7	0.43	7 622	23.55
Nasopharynx	2 222	5	4.6	0.69	1 450	6	4.9	0.50	6 985	21.58
Liver	2 149	6	4.4	0.77	2 050	3	6.9	0.73	2 267	7.00
Prostate	2 146	7	4.4	1.57	900	13	3.0	0.35	7 916	47.60
Non-Hodgkin lymphoma	1 940	8	4.0	0.62	1 104	10	3.7	0.35	5 933	18.33
Leukaemia	1 905	9	3.9	0.51	1 481	5	5.0	0.41	6 113	18.89
Ovary	1 836	10	3.8	1.16	1 175	8	4.0	0.73	4 989	31.71
Cervix uteri	1 740	11	3.6	1.12	991	12	3.4	0.67	4 962	31.53
Stomach	1 462	12	3.0	0.48	1 174	9	4.0	0.36	2 232	6.90
Corpus uteri	1 401	13	2.9	0.93	433	17	1.5	0.26	4 546	28.89
Pancreas	1 089	14	2.2	0.37	1 066	11	3.6	0.37	907	2.80
Kidney	1 009	15	2.1	0.34	532	15	1.8	0.15	2 712	8.38
Bladder	909	16	1.9	0.31	443	16	1.5	0.12	2 637	8.15
Brain, central nervous system	800	17	1.6	0.21	694	14	2.4	0.19	2 388	7.38
Thyroid	795	18	1.6	0.23	147	23	0.50	0.04	2 694	8.32
Lip, oral cavity	742	19	1.5	0.26	403	18	1.4	0.13	2 199	6.79
Larynx	594	20	1.2	0.21	363	20	1.2	0.11	1 774	5.48
Oesophagus	398	21	0.82	0.14	386	19	1.3	0.13	490	1.51
Multiple myeloma	344	22	0.71	0.12	287	21	0.97	0.10	883	2.73
Gallbladder	311	23	0.64	0.11	245	22	0.83	0.09	367	1.13
Hodgkin lymphoma	235	24	0.48	0.06	76	27	0.26	0.02	864	2.67
Salivary glands	233	25	0.48	0.08	98	24	0.33	0.03	725	2.24
Melanoma of skin	162	26	0.33	0.05	81	26	0.27	0.03	494	1.53
Hypopharynx	144	27	0.30	0.05	93	25	0.31	0.03	262	0.81
Oropharynx	140	28	0.29	0.05	76	28	0.26	0.03	361	1.12
Testis	119	29	0.24	0.05	17	33	0.06	0.01	436	2.62
Anus	91	30	0.19	0.03	42	29	0.14	0.01	266	0.82
Penis	66	31	0.14	0.05	23	32	0.08	0.01	255	1.53
Vagina	53	32	0.11	0.03	23	31	0.08	0.01	144	0.92
Vulva	50	33	0.10	0.03	15	34	0.05	0.01	158	1.00
Mesothelioma	27	34	0.06	0.01	24	30	0.08	0.01	27	0.08
Kaposi sarcoma	25	35	0.05	0.01	12	35	0.04	0.00	75	0.23
All cancer sites	48 639	-	-	14.85	29 530	-	-	9.05	128 018	395.5

1.2.2(b) Pathogenesis of breast carcinoma

Breast cancer accounts for one of the highest incidence and mortality rate among cancer disease worldwide (Sung *et al*., 2021). The aetiology largely influenced by many factors including genetic inheritance, carcinogen exposure, viruses, environment, food and metastases tumour. Breast cancer is the chronic type of disease which may take up more than years to develop (Parsa, 2012). Generally, cancer cells possessed several characteristics to sustain and survive; (1) self-sufficiency producing growth signals, (2) resistance to growth-inhibitory signals, (3) neglect apoptosis, (4) highly proliferate, (5) promote angiogenesis and (6) spread and metastases. One of the key hallmarks of breast carcinoma is the uncontrolled growth within the organized bilayer ducts (Folkman, 2003).

Normal mammary gland structure consists of single layer of epithelial cells lining up lumen while myoepithelial cells lining the basement membrane. The close arrangement between luminal and myoepithelial cells enables autocrine and paracrine interaction (Gudjonsson *et al*., 2005). The interaction potentially mediated by chemokines either between luminal and epithelial cells or between luminal epithelial cells and stromal cells including fibroblast, adipocytes, macrophages, eosinophil granulocytes, lymphocytes and endothelial cells. In breast cancer condition, abnormal proliferation of epithelial and myoepithelial cells results the formation of benign lesions (Cozzo, Fuller & Makowski, 2017). The diseases related to these benign changes include fibrocystic diseases, sclerosing lesions, epithelial hyperplasia, fibroadenomas, tubular adenomas as well as intraductal papillomas. Generally, these types of lesions are non-detrimental but may develop breast carcinoma *in situ* (CIS) if no proper treatment is taken immediately (Stachs *et al.*, 2019).

Carcinoma *in situ* (CIS) is a pre-malignant hyper-proliferative tissue of the breast which still confined within the basement membrane. The morphology of CIS may resemble carcinoma-like morphology (Carraro, Elias & Andrade, 2014). CIS is further classified into two sub-categories based on its original location; ductal carcinoma *in situ* (DCIS) which is originated from the ductal region and lobular carcinoma *in situ* (LCIS) from the lobular region (Alkabban & Ferguson, 2021). DCIS may exhibit in several histological subtypes including cribriform, comedo, solid and micropapillary. On the other hand, LCIS do not possess any histological subtype (Pinder, 2010). Both types of CIS are known to be good prognosis of invasive carcinoma. Invasive carcinoma retained the morphology similar to CIS but the cells invade to surrounding tissue and tumour cells also have breached the basement membrane (Jögi *et al.*, 2012).

There are several classifications of breast carcinomas based on the arrangement of the tissue. Commonly in human, mostly (>75%) reported was invasive ductal carcinoma with no special type (IDC-NST). IDC-NST are a special type of IDC which exhibit no special characteristic unlike other types (Badowska-Kozakiewicz *et al*., 2017). Other types of IDC have their own special characteristics such as tubular carcinoma (Figure 1.4) morphologically characterized by tube-shaped structure, cribriform carcinoma morphologically characterized by nest-like structure surrounded by ducts and lobules, solid papillary carcinoma characterized by round, well-defined nodules composed of low-grade ductal cells separated by fibrovascular cores and many more, rarely occur in breast carcinoma cases (Pal *et al*., 2010). The cancer cell will spread at distant places at late stage. The common places of metastasize are lungs, liver, brain, adrenal gland and bones through the nearest lymphatic and vascular invasion (Martin *et al*., 2013). There are numerous factors contribute to the

development of breast carcinoma. The commonest aetiology of breast carcinoma development is hormonal factors or endocrine. Commonly oestrogen and progesterone hormones account the common factor. Other than hormone, genetic factors, early menarche, late pregnancy, nulliparity and lifestyle factors for examples obesity and smoking, nutritional factors and reduced physical activity (Momenimovahed & Salehiniya, 2019).

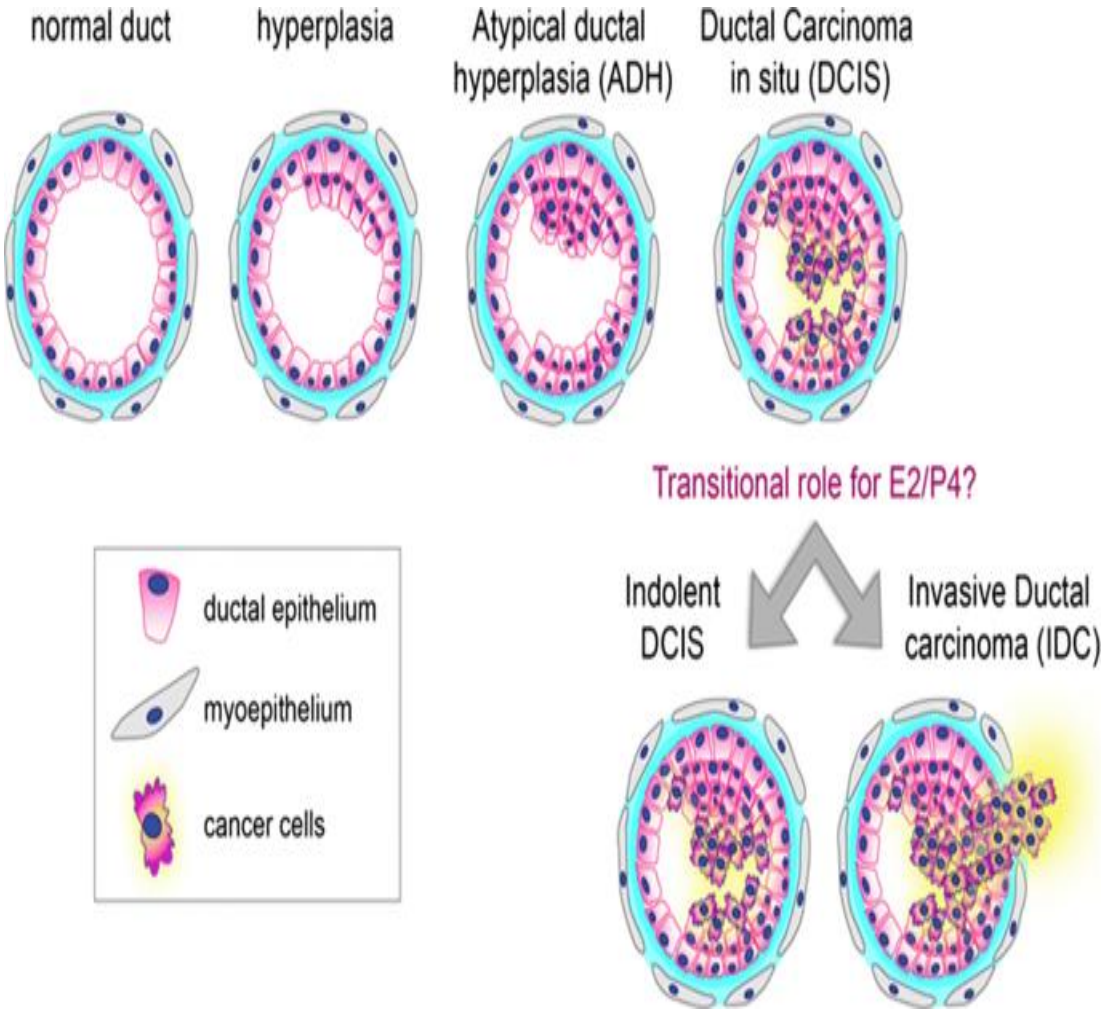

Figure 1.4 The illustration of normal duct, hyperplasia, atypical hyperplasia changes, ductal carcinoma *in situ* and invasive ductal carcinoma (Villanueva *et al.*, 2018)

1.2.2(c) N-nitroso-N-methylurea (NMU)-breast cancer induced in rat model.

There are numerous strategies has been underlined to fully understood grossly and molecular sites of the mammary carcinogenesis. Animal models notably from mammals seem to be the best option instead of others because the tumour model is grown in the natural environment and the biological activities are mimic the natural human biology. Furthermore, the characteristics of mammary carcinoma developed in rat models has a close resemblance to the one in human (Abdelmegeed & Mohammed, 2018). There are many ways to induce breast cancer in animal models for breast cancer study; however, chemically induced carcinogen has been widely used. Two most used carcinogens to induce breast tumour are NMU and 7,12- Dimethylbenzanthracene (DMBA) (Abba *et al.*, 2016, Chan *et al.,* 2005). NMU is an alkylating agent, exhibits its toxicity by transferring its methyl group to nuclei bases located in nucleic acids and leads to genetic mutations (Figure 1.5). NMU-induced breast carcinoma models have several advantages includes easy to prepare, the nature of the carcinogenic response help the tumour development within expected time and specific in location, the histological characteristics of the tumour induced easy to be identified and similar to human, the simplicity of the tumour induction methodology and the flexibility in the experimental design (Murray *et al*., 2009). NMU-induced breast cancer model has a number of superior advantages compare to DMBA. For instance, NMU can be used to demonstrate the development of histologically aggressive mammary carcinoma, has a higher proportion of malignant to benign tumour compared to DMBA as well as appearance of more oestrogen-dependent tumour (Alvarado *et al.*, 2017, Pula *et al*., 2013).

There are several suggested methods and routes of administration for NMU-induced mammary carcinoma of animal model. One of the best methods is

intraperitoneal injection at 21 days-old of female Sprague Dawley rats which known to be rapid induction method. The induction process is ideal at this period because the structure of terminal end bulb (TEB) is still in undifferentiated form and tend to be transformed due to mutation process or carcinogenesis activity (Garczyk *et al*., 2017). This situation will only occur in young or multiparous animals, but not in TEB-absent multiparous animals due to complete differentiation of the gland. Numerous *in vivo* studies of rat's model have proven that nulliparous rat is associated with higher risk of developing breast cancer compared to multiparous rat (Tsubura *et al.*, 2011). Other than TEB region, some studies have identified another two *in vivo* origin sites of mammary carcinoma which comprised of ducts and ductules. The similar pattern was observed with predominant human breast carcinoma originating from ductal in humans.

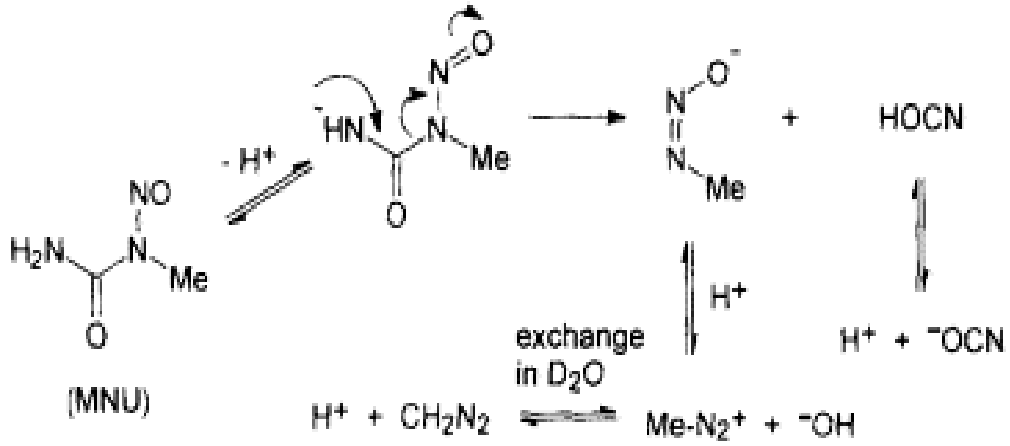

Figure 1.5 The chemical structure of N-methyl-N-nitrosourea (NMU) consist of $C_2H_5N_3O_2$. (Bernard TG *et al*., 1997). The NMU possess the ability to methylate DNA in aqueous environment. The key important process of NMU carcinogenesis involved base-induced hydrolysis of MNU which is initiated by deprotonation at the carbamoyl group.

1.2.2(d) Human breast cancer cell line

Human breast cancer cell line always be used in *in vitro* study substituted animal which straightly attached to the animal ethics guideline. The use of human breast cancer cell line in the study of human breast cancer will provide more precise and reliable outcome (Lacroix & Leclercq, 2004). There are more than 10 breast cancer cell lines available. Each cell line possess varies of properties and the selection must take place carefully based on the study conducted. The cell lines available includes 600MPE, AMJ13, AU565, BT-20, BT 474, EVSA-T, MDA-MB231, MCF-7, SkBr3, T-47D, ZR-75-1, Hs578T and BT-549 and their properties includes, estrogen and progesterone receptors, ERBB2 amplification, mutated TP53 and tumorigenic in mice (Lacroix & Leclercq, 2004). The selection of suitable breast cancer cell line is crucial for reliable result (Charise & Ludovic, 2017). Current technology has developed 3D cell culture which assembled human-like cellular environment providing more detail information and more reliable result. In this study, MCF-7 and MDA-MB231 were selected for in vitro study and the effects of sirolimus or/and sunitinib were accessed towards morphological, proteins expression and genes expression.

1.2.2(d)(i) MCF-7 cell line.

Michigan Cell Foundation-7 or also known as MCF-7 was found by Dr Soule from Michigan Cancer Foundation. The cell was isolated from a pleural effusion of 69-year-old Caucasian women who having metastatic adenocarcinoma of the breast (Moon *et al,* 2020). MCF-7 is a hormonal dependant breast cancer cells which exhibit as estrogen-dependant cancer cell specifically estrogen-receptor alpha (ER-α) for development and proliferation. However, MCF-7 cell line is less aggressive than MDA-MB231 cell line, slow growth rate and prone to bacterial contamination (Hegde

et al, 2016). The cell is mostly selected human breast cancer cell line in research world due to its well-characterized morphology and estrogen responsive (Comsa, Cimpean & Raica, 2015). Besides that, MCF-7 also maintain a number of characteristics similar to mammary epithelium and monolayers forming dome structures. This is due to fluid accumulation between the culture dish and cell monolayer (Soule *et al*, 1973).

1.2.2(d)(ii) MDA-MB231 cell line.

MDA-MB231 is an epithelial cancer cell isolated from pleural effusion of 51-year-old Caucasian women with metastatic mammary adenocarcinoma. MDA-MB231 is the commonest cancer cell used in laboratory for cancer research due to its properties (Cailleau, Olive & Cruciger, 1978). MDA-MB231 cell is a triple negative breast cancer cell line which absent of progesterone, estrogen and HER-2 amplification. In living human, triple negative breast cancer only found in very aggressive and late-stage breast cancer (Liu et al, 2003). The cell is highly aggressive, poorly differentiated and invasive. The cell is classified as claudin-low molecular subtype. The cell down-regulate claudin-3 and claudinin-4, low expression of Ki-67 proliferation marker. The cell exhibit as endothelial-like morphology and distinguished by its invasive phenotype (Chavez, Garimella & Lipkowitz, 2010).

1.2.3 Angiogenesis

The understanding of tumour development has been associated with a wide range of factors which neo-angiogenesis has been listed as one of the main factors (Lugano, Ramachandran & Dimberg, 2020). In 1960, Folkman and co-workers were initially discovered the important role played by angiogenesis in supporting tumour growth (Figure 1.6). Angiogenesis is a fundamental process of living cells which arise

from pre-existing vessel. During fetus, vasculogenesis made up the first blood vessel formation before angiogenesis take over to connect all cells throughout body to circulatory system (Ribatti & Pezzella, 2021).

Angiogenesis, which refers to the formation of new capillaries from the pre-existing vessels, plays a pivotal role in both physiological and pathological processes (Adair & Montani, 2010). In normal physiology, angiogenesis process is a highly ordered process under tight regulation of several angiogenic factors and inhibitors known as pro- and anti-angiogenic molecules, respectively (Ucuzian *et al.*, 2010). Pro-angiogenic molecules promote angiogenesis process, whereas anti-angiogenic molecules restrict the process. Both kinds of molecules are always exquisitely counterbalanced to compel changes in tissue mass and/or metabolic demands. This is very important to maintain essential nutrients and oxygens supply to cells throughout body (Zeng & Fu, 2020).

Angiogenesis is a pivotal process during embryonic development to ensure adequate vasculature to satisfy demand of growing and developing organs (Papetti & Herman, 2002). Besides that, the process is important for the physiological repair process in wound healing, mammary gland maturation and ovarian cycle during adult phase (Reynolds, Grazul-Bilska & Redmer, 2002). However, failure of angiogenesis process by overexpression or downregulation of angiogenic factors and/or inhibitors caused aberrant deployment of normal angiogenesis and resulted in various pathological conditions including vascular insufficiency (cerebral ischemia and myocardial infarction), atherosclerosis, psoriasis, rheumatoid arthritis and tumour growth as well as metastasis (Felmeden, Blann & Lip, 2003). Based on the study done by several researchers, the tumour demands for extremely amount of nutrients to grow

massively and rapidly as well as to metastases at distant location. Angiogenesis also works as the ideal pathway for metastases (Hillen & Griffioen, 2007).

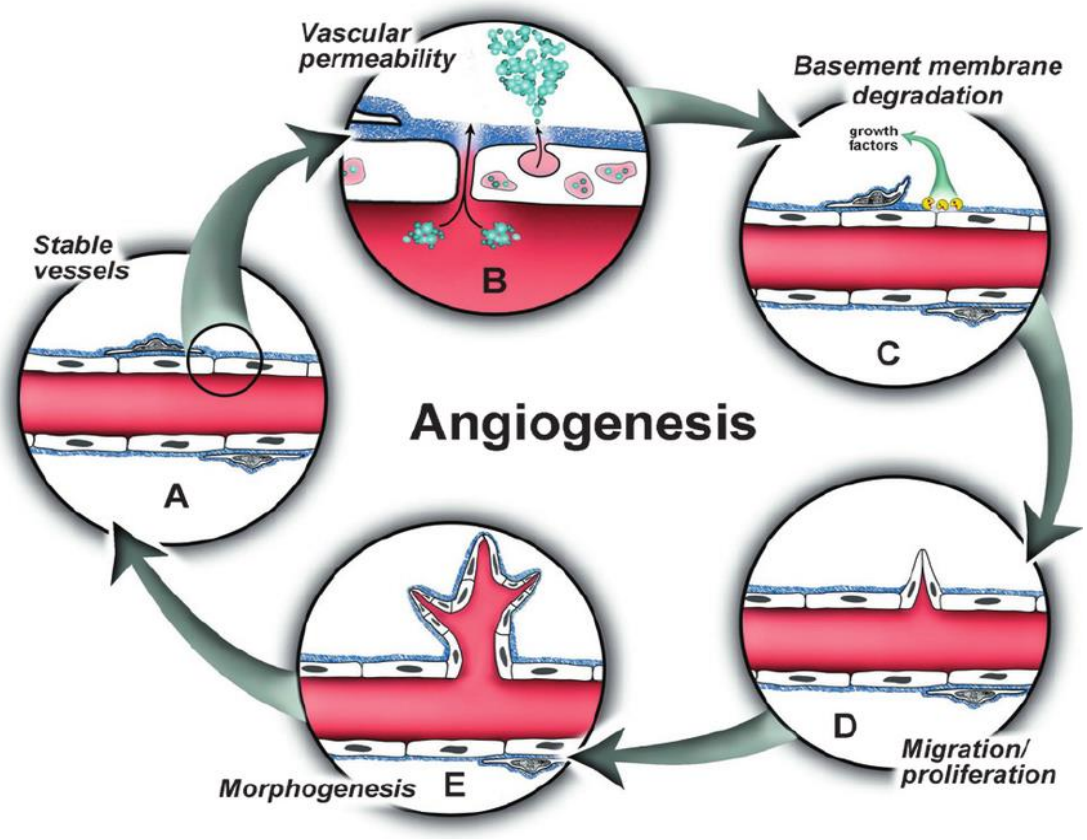

Figure 1.6 The illustrated figure shows 5 phases of angiogenesis process which consist of increase vascular permeability, basement membrane degradation, endothelial cells migration and proliferation, morphogenesis of vessel and stable vessel (Bryan BA & D'Amore, 2007).

1.2.3(a) Regulation of tumour angiogenesis in breast cancer

There are several types of cancer were identified as angiogenesis-dependant cancer. There are several factors influence the growth, mechanical structure, survival, apoptosis-escape and invasion (Figure 1.7). Those factors include vascular endothelial growth factor receptor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), tumour-like growth factor (TGF), interleukins (IL) and many more (Fouad & Aanei, 2017). Instead of angiogenesis-dependant, breast cancer also

known as endocrine-dependant type of cancer which largely depends on hormonal supply to sustain their growth and survival (Garcia-Martinez *et al*., 2021). Despite angiogenic factors and inhibitors, there are also other stimuli which have been noted to regulate breast tumour angiogenesis under direct or indirect manners including soluble growth factors, membrane-bound proteins, cell-matrix and cell-cell interactions, hypoxia, inflammation, mechanical factors (shear stress and stretch) and oxidative as well as glucose deprivation (Walker, Mojares & Del Río Hernández, 2018).

There are two common ways of neovascularization elicits their signalling mechanisms: either via angiogenic mechanisms (i.e. sprouting angiogenesis and lymphangiogenesis) or non-angiogenic mechanisms (i.e. intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option and vasculogenic mimicry) (Soda *et al*., 2013). Sprouting angiogenesis or angiogenesis has been described as the prominent signalling pathway in breast tumour neovascularisation. Thus, further details will mainly emphasize more on tumour-induced sprouting angiogenesis instead of the remaining mechanisms (Fox, Generali & Harris, 2007).