EFFECTS OF nNav1.5 EXPRESSION MODULATION ON MHC I ANTIGEN PROCESSING MACHINERY AND INVASION POTENTIAL OF BREAST CANCER CELLS

AHMAD HAFIZ MURTADHA BIN NOOR DIN

UNIVERSITI SAINS MALAYSIA

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by

AHMAD HAFIZ MURTADHA BIN NOOR DIN

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LIST OF SYMBOLS

%	Per cent
=	Equal to
CO_2	Carbon dioxide
Ca^{2+}	Calcium ion
Ct	Threshold cycle
H^+	Hydrogen ion
IC	Inhibitory concentration
K^+	Potassium ion
L	Liter
M	Molar
Na^+	Sodium ion
R^2	Coefficient of determination
™	Trademark
V	Volt
dp	Perimeter diameter
g	Gram
kDA	Kilodaltons
m	Meter
mL	Milliliter
n	Nano
®	Registered
°C	Degree Celsius
µ	Micro
× *g*	Times gravity
α	Alpha

β	Beta
γ	Gamma
Δ	Delta
$\leq$	Less than or equal to
$\geq$	More than or equal to

LIST OF ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
APC	Antigen presenting cells
APM	Antigen processing machinery
ATCC	American Type Culture Collection
ATP	Adenosine 5’-triphosphate
B2M	β2 microglobulin
BM-MSC	Bone marrow-derived mesenchymal stem cell
C	Cytosine
CoA	Co-activators
CDK	Cyclin-dependent kinase
COX	Cyclooxygenase
CRISPR	Clustered regularly interspaced short palindromic repeats
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELK-1	ETS like-1 protein
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
EREG	Epiregulin
ERK	Extracellular signal-regulated kinase
ETS	Erythroblast transformation specific
FGFR	Fibroblast growth factor receptor
FAK	Focal adhesion kinase
G	Guanine

HDAC	Histone deacetylase inhibitor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER	Human epidermal growth factor receptor
HNSCC	Head neck squamous cell carcinoma
HR	Hormone receptor
HUVEC	Human umbilical vein endothelial cell
ICI	Immune checkpoint inhibitor
IDC NST	Invasive ductal carcinoma of no special type
IFN	Interferon
ILC	Invasive lobular carcinoma
JAK	Janus kinase
L-Gln	L-glutamine
LCIS	Lobular carcinoma in situ
LMP	Large multifunctional peptidase
MAPK	Mitogen-activated protein kinase
MCC	Merkel cell carcinoma
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
NBD	Nucleotide-binding domain
NCBI	National Center for Biotechnology Information
NCX	Sodium calcium exchanger
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHE	Sodium hydrogen exchanger
OS	Overall survival
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCNA	Proliferating cell nuclear antigen
PD-L1	Programmed cell death 1 ligand 1
PD1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphoinositide 3-kinases
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor

RNA	Ribonucleic acid
RT	Reverse transcriptase
SAHA	Suberoylanilide hydroxamic acid
SEM	The standard error of the mean
SERD	Specific oestrogen receptor downregulator
SERM	Specific oestrogen receptor modulator
SHP	Small heterodimer partner
STAT	Signal transducers and activators of transcription
TAE	Tris-acetate buffer
TAP	Transporter of antigenic peptide
TCR	T cell receptor
TGF	Transforming growth factor
TIL	Tumour infiltrating lymphocyte
TMD	Transmembrane domain
TNBC	Triple-negative breast cancer
TNF	Tumour necrosis factor
TSA	Trichostatin A
TTX	Tetrodotoxin
UPS	Ubiquitin-proteasome system
USM	Universiti Sains Malaysia
UT	Untreated
UV	Ultraviolet
VEGFR	Vascular endothelial growth factor receptor
VGSC	Voltage-gated sodium channel
ZAP	Zeta chain of T cell-associated receptor protein
cAMP	Cyclin adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin kinase
pAb	Polyclonal antibody
qPCR	Quantitative real-time polymerase chain reaction
shRNA	Short hairpin RNA
siRNA	Small interfering RNA

LIST OF APPENDICES

KESAN MODULASI EKSPRESI nNav1.5 TERHADAP JENTERA PEMPROSESAN ANTIGEN MHC I DAN POTENSI INVASI SEL KANSER PAYUDARA

ABSTRAK

Peningkatan ekspresi dan aktiviti isoform *voltage-gated sodium channels* (VGSC), Nav1.5 neonatal (nNav1.5) dalam kanser payudara telah dikaitkan dengan peningkatan keupayaan metastatik. Oleh kerana kehilangan pengawasan imun membolehkan sel kanser bermetastatik, kajian ini direka untuk meneroka peranan nNav1.5 dalam mengawalatur ekspresi komponen imun, jentera pemprosesan antigen (APM) MHC I yang mengalami penyahkawalaturan dalam kanser payudara. Beberapa laporan telah menghubungkan VGSC dan APM dalam neuron, tetapi hubungan nNav1.5 dan APM dalam kanser payudara masih belum diketahui. Dalam kajian ini, ekspresi gen komponen imun APM (*PSMB8, PSMB9, PSMB10, TAP1, TAP2, B2M* dan MHC I) dan *CD274* diukur dan dibandingkan menggunakan *real-time PCR* dalam sel kanser payudara agresif, MDA-MB-231, sel kanser payudara kurang agresif, MCF-7 dan sel epitelium payudara bukan kanser, MCF 10A. Ekspresi gen nNav1.5 dan dua penanda metastatik, *MMP1* dan *FN1* turut diukur. Selepas *3D spheroid* MDA-MB-231 dan MCF-7 dibangunkan melalui kaedah tindanan cecair, ekspresi gen sasaran mereka dibandingkan dengan *monolayer*. Isipadu *spheroid* dianalisa menggunakan *SpheroidSizer*. Bagi menyekat nNav1.5, tetrodotoxin (TTX) dan siRNA digunakan pada *monolayer* dan *spheroid* MDA-MB-231, manakala Trichostatin A dan plasmid nNav1.5 digunakan untuk meningkatkan nNav1.5 dalam *monolayer* dan *spheroid* MCF-7. Kesan pemodulatan nNav1.5 dinilai dengan memprofilkan gen menggunakan *real-time PCR* dan mengukur tahap invasi *spheroid* menggunakan kit Cultrex. Dalam

monolayer, tahap gen nNav1.5 tertinggi dalam MDA-MB-231 > MCF-7, dan tiada dalam MCF 10A. MHC I, *B2M, PSMB8, PSMB9, TAP1* dan *TAP2* tertinggi dalam MCF 10A > MDA-MB-231 > MCF-7, kecuali *PSMB10*, dengan MCF-7 > MDA -MB-231. Untuk *CD274*, tahap tertinggi adalah dalam MDA-MB-231 > MCF 10A > MCF-7. Dalam *spheroid* MDA-MB-231 dan MCF-7, tahap nNav1.5, *MMP1* dan *FN1* meningkat manakala hampir semua APM tidak berubah (kecuali penurunan *TAP1*) berbanding *monolayer*. *CD274* menurun dalam *spheroid* MDA-MB-231 tetapi meningkat dalam *spheroid* MCF-7, berbanding *monolayer* masing-masing. Bagi eksperimen menyekat nNav1.5 model *monolayer*, TTX meningkatkan nNav1.5, *TAP2, B2M, MMP1* dan *FN1*, tetapi menurunkan *PSMB9*. siRNA mengurangkan nNav1.5 (73.22% selepas 48 jam, 10 nM siRNA) dalam MDA-MB-231 dan meningkatkan *B2M* dan MHC I, menurunkan *PSMB8, PSMB9, PSMB10, TAP2* dan *CD274*, manakala *MMP1* dan *FN1* tidak berubah. Dalam eksperimen menyekat nNav1.5 model *spheroid*, TTX dan siRNA gagal mendorong perubahan gen. Bagi keupayaan invasif, *spheroid* pra-rawatan siRNA gagal terbentuk. Dalam *monolayer* dan *spheroid* MCF-7, TSA meningkatkan nNav1.5, *PSMB9, B2M*, MHC I, *CD274* dan *MMP1*. Dalam MCF-7 *monolayer*, peningkatan nNav1.5 melalui transfeksi plasmid meningkatkan semua gen tetapi dalam *spheroid*, hanya nNav1.5 dan MHC I yang meningkat, manakala *PSMB10* dan *B2M* menurun. TSA meningkatkan keupayaan invasif *spheroid* tetapi sel pra-transfeksi plasmid nNav1.5 membentuk *spheroid* dengan diameter perimeter relatif lebih rendah. Ringkasnya, pemodulatan gen nNav1.5 memberi kesan terhadap MHC I APM, *CD274* dan tingkah laku sel sekaligus berpotensi menambahbaik imunoterapi kanser payudara dengan melawan mekanisma lepas sistem imun melalui peningkatan MHC I. Secara amnya, penemuan semasa kajian ini menyokong peranan nNav1.5 dalam pengawalaturan ekspresi molekul komponen imun dalam kanser payudara.

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ABSTRACT

The increase in expression and activity of voltage-gated sodium channel's (VGSC) isoform, neonatal Nav1.5 (nNav1.5) in breast cancer have been associated with enhanced metastatic ability. Loss of immune surveillance enables cancer cells to metastasize hence this study is to explore the possible role of nNav1.5 in regulating the expression of immune components, MHC I antigen processing machinery (APM), which is deregulated in breast cancer. Several reports link VGSC and APM in neurons, but the correlation between nNav1.5 and APM in breast cancer is unknown. In this study, the gene expression of APM components (*PSMB8, PSMB9, PSMB10, TAP1, TAP2*, *B2M* and MHC I) and *CD274* were measured and compared in the highly aggressive MDA-MB-231, less aggressive MCF-7 and control, non-cancerous breast epithelial, MCF 10A via real-time PCR. Gene expression of nNav1.5 and two metastatic markers, *MMP1* and *FN1*, were included. After 3D spheroids for MDA-MB-231 and MCF-7 were established via liquid overlay method, their target genes' expression was compared with the monolayer counterparts. The spheroid volumes were analysed using SpheroidSizer. To block nNav1.5 expression, tetrodotoxin (TTX) and siRNA were employed in MDA-MB-231 monolayer and spheroids, while Trichostatin A and nNav1.5-plasmid were utilised to increase nNav1.5 expression in MCF-7 monolayer and spheroids. The effects of modulating nNav1.5 were assessed by profiling all target genes using real-time PCR and on spheroid invasion ability using Cultrex kit. In monolayer model, highest nNav1.5 gene level was detected in MDA-

MB-231 > MCF-7 but absent in MCF 10A. In contrast, MHC I, *B2M, PSMB8, PSMB9, TAP1* and *TAP2* were at the highest level in MCF 10A > MDA-MB-231 > MCF-7, except for *PSMB10* with MCF-7 > MDA-MB-231. For *CD274*, the highest level was in MDA-MB-231 > MCF 10A > MCF-7. In spheroids of MDA-MB-231 and MCF-7, the level of nNav1.5, *MMP1,* and *FN1,* were upregulated but there were no changes in almost all APM genes (except *TAP1* downregulation), compared to their respective monolayer model. C*D274* is downregulated in MDA-MB-231 spheroid but upregulated in MCF-7 spheroid, compared to their monolayer model. In nNav1.5 blocking monolayer experiments, TTX upregulated nNav1.5, *TAP2, B2M, MMP1,* and *FN1* but downregulated *PSMB9*. Alternatively, siRNA knocked down nNav1.5 gene (73.22% after 48 hours, 10 nM siRNA) in MDA-MB-231, thus resulted in the upregulation of *B2M* and MHC I, and the downregulation of *PSMB8, PSMB9, PSMB10, TAP2*, and *CD274,* but unchanged *MMP1* and *FN1*. However, in MDA-MB-231 spheroid, both TTX and siRNA failed to induce expressional changes. On the invasion ability, siRNA pre-treated spheroid failed to form. In MCF-7 monolayer and spheroid model, TSA induced nNav1.5, *PSMB9, B2M*, MHC I, *CD274*, and *MMP1* expression. In monolayer MCF-7, nNav1.5 upregulation via plasmid transfection increased all target genes but in spheroid, only nNav1.5 and MHC I were upregulated, while *PSMB10* and *B2M* were downregulated. TSA showed increased spheroid invasion, but nNav1.5-plasmid pre-transfected cells formed spheroids with lower relative perimeter diameter. In summary, modulating nNav1.5 gene expression affected MHC I APM, *CD274*, and cell behaviour, hence can potentially improve breast cancer immunotherapies by countering tumour immune escape mechanism via rescuing MHC I. Overall, the study's current findings support the influence of nNav1.5 on the expression regulation of immune component molecules in breast cancer.

CHAPTER 1

INTRODUCTION

1.1 Rationale of study

Metastasis is the hallmark of cancer that is responsible for treatment failure in cancer patients and for cancer-related deaths. Continuous research efforts in cancer biology have revealed some of the molecular foundations in metastasis cascade. One of which is the failure of a patient's immune surveillance to efficiently monitor, detect, and destroy neoplastic-transformed cells (Sandoval-Valencia, 2019). Hence, the significant loss of immune surveillance, also stated as evading immune destruction, has been proposed as an emerging hallmark of cancer by Hanahan and Weinberg (2011), which is later firmly established as one of eight core hallmarks of cancer (Hanahan and Weinberg, 2011; Hanahan, 2022).

In breast cancer, failure of tumour immune surveillance is attributable to the immune escape mechanisms. The immune escape mechanism reduces cytotoxic T cells' activity against transformed cells (Spranger, 2016). This suppression of T cell activity can be achieved via several pathways, including downregulation of the antigen processing machinery (APM) components and overexpression of PD-L1 that suppresses the T cell activity (Kim and Chen, 2016). The central APM molecule, major histocompatibility complex class I (MHC I), is essential for cytotoxic T cell's immune responses, including against tumours. However, the APM is a multistep process involving several molecules that generate peptides to be loaded on MHC I. Initially, the bulk of peptides originate as proteasome degradation products of cytosolic proteins.

The immunoproteasome, which is responsible for the degradation process, differs from the normal proteasomes, in which it replaces three of the beta subunits with low molecular weight protein, LMP2, LMP7, and LMP10, resulting in a more efficient and dominant peptide for the MHC I epitope. The peptides will then be transported into the endoplasmic reticulum by the transporter of antigenic peptides (TAPs) 1 and 2. The peptides are then loaded onto the MHC I, forming the MHC I loading complexes, which are stabilised by the β2-microglobulin protein before the complexes migrate to the plasma membrane (via the Golgi apparatus) and present the MHC I-antigenic peptide to the T cell receptor (TCR) of CD8+ T cells (Leone et al., 2013). Hence, the downregulation of MHC I molecules in tumours leads to the decreasing antigenic peptide presentation to the TCR, allowing the transformed cells to thrive (Dhatchinamoorthy et al., 2021).

Another critical immune escape mechanism pathway is the immune checkpoint PD1/PD-L1 pathway. PD1 is a protein on the plasma membrane of T cells, and it binds with PD-L1, a protein on the surface of cancer cells. The binding hinders the activation and multiplication of T cells, enabling cancer cells to avoid the immune response (He and Xu, 2020). Generally, PD-L1 inhibits T cell activity by functioning as an immunological mechanism that moderates the signalling of the T cell receptor. In brain metastases with positive PD-L1 expression obtained from breast cancer patients, the highest occurrences were shown in the highly metastatic triple-negative breast cancer (TNBC) patients compared to the human epidermal growth factor receptor two positive (HER2+) and hormone receptor (HR) positive patients (Chehade et al., 2022).

Therefore, the loss of immune surveillance and metastasis were associated with the immune escape mechanism of cancers, developed from the downregulation of

MHC I APM and overexpression of immune checkpoint molecule, PD-L1 (Kallingal et al., 2023). In breast cancer patients, loss of immune surveillance was shown in the late chest wall metastases when compared to the temporally earlier chest wall tumour through the downregulation of immune signalling pathways (including MHC I and immune checkpoint molecule), which corresponds with the decline of CD8+ and CD4+ T cells pathology in late metastases (Blanco-Heredia et al., 2024). Therefore, it is thus crucial to understand the regulation of these molecules (i.e., MHC and PD-L1) that will shed light on immune surveillance and metastasis of breast cancer cells.

Voltage-gated sodium channels (VGSCs) are transmembrane ion channel proteins that generate and propagate action potentials in excitable cells such as neurons (Wang et al., 2017a). The alpha subunits of VGSCs are divided into nine isoforms, Nav1.1 to Nav1.9. These isoforms are localised differently, with Nav1.1, Nav1.2, Nav1.3, and Nav1.6 expressed in the central nervous system, Nav1.7, Nav1.8, and Nav1.9 in the peripheral nervous system, Nav1.4 in adult skeletal muscle, and Nav1.5 in cardiac muscle (Baroni and Moran, 2015). The Nav1.5 channel usually conducts an inward sodium ion current, which determines the sodium ion influx that depolarises the membrane potential during the upstroke of the cardiac action potential. Nav1.5 is critical in controlling proper cardiac development, keeping the heart rhythm stable, and preventing numerous cardiac disorders (Rajaratinam et al., 2022).

In an early study by Gustafson et al. (1993), VGSCα alternative mRNA splicing at domain 1: segment 3 (D1:S3) was shown to generate neonatal-specific isoforms and later on, neonatal Nav1.5 (nNav1.5) was discovered with its expression being prominent in neonatal cardiac mice muscle whereas Nav1.5 was expressed in adult mice cardiac muscle (Fraser et al., 2005). The alternative splicing converts a

conserved negative aspartate residue in the 'adult' isoform to a positive lysine. Because of the electrophysiological changes caused by Nav1.5 D1:S3 splicing, charge reversal in nNav1.5 affects the channel's kinetics, resulting in a prolonged resultant current and an increase in intracellular sodium ion (Na^+) influx (Onkal et al., 2008).

Interestingly, nNav1.5 was re-expressed in breast cancer, and the expression was higher in epithelial cells of breast cancer tissues compared to the routine breast tissue biopsy (Fraser et al., 2005; Yamaci et al., 2017). Currently, nNav1.5's enhanced expression and activity in metastatic breast cancer also makes it a potential biomarker for diagnostics and clinical therapies (Onkal and Djamgoz, 2009). The vital role of VGSCs expression and activity in cancers reached a different level when the adult Nav1.5 was shown to possess the ability to regulate gene expression in colon cancer, which is usually conducted by transcription factors (House et al., 2010). From these, a novel idea for the involvement of VGSCs in malignancies emerges: their enhanced expression and activity may govern metastatic cascades not only through Na^+ transport but also via gene expression.

In 1997, Neumann and colleagues showed that a specific VGSCs blocker, tetrodotoxin (TTX) treatment restores the expression of MHC I in Lewis rat neuron cells, in which the MHC I possess homology in sequence and function with the *Homo sapiens* counterparts (Neumann et al., 1995; Neumann et al., 1997). This further suggests that blocking VGSC activity might help restore MHC I expression, which is downregulated due to the immune escape mechanism of human tumour cells. Moreover, in neurons, PD1 activation via PD-L1-induced phosphorylation also seems to inhibit VGSCs, indicating a potential association between PD1/PD-L1 and VGSCs (Chen et al., 2017). However, the relationship between MHC I APM and PD-L1 gene

expression against VGSCs, particularly nNav1.5 expression in breast cancer cells with varied metastatic potential, has yet to be investigated.

1.2 Objectives of study

The general objective of this study is to investigate the effects of nNav1.5 expression modulation on MHC I APM and PD-L1 (*CD274*) in human breast cancer cells. The overall study design for this research is shown in **Figure 1.1** below. The specific objectives are as follows:

1. To compare the basal expression of VGSC (nNav1.5), MHC I APM components and *CD274* in *in vitro* models of human breast cancer cell lines.

2. To investigate the influence of VGSC (nNav1.5) using TTX and siRNA-*SCN5A* on mRNA expression of MHC I APM components, *CD274,* and metastatic markers, and on invasion properties of MDA-MB-231 cells

3. To study the influence of VGSCs' epigenetic and specific upregulation via an epigenetic regulator, Trichostatin A (TSA) and nNav1.5-plasmid, on mRNA expression of MHC I APM components, *CD274* and metastatic markers, and invasion properties of MCF-7 cells

The VGSC studied was the neonatal isoform of Nav1.5, nNav1.5, whereas the MHC I APM refers to the genes of *PSMB8* (LMP7), *PSMB9* (LMP2), *PSMB10* (LMP10), *TAP1, TAP2, B2M* and MHC I. *CD274,* is also included due to its role in tumour immune evasion. The *in vitro* culture models refer to the monolayer and 3D spheroid cultures. The metastatic markers were matrix metalloproteinases 1 (*MMP1*) and fibronectin 1 (*FN1*). In this thesis, the results and discussion for each specific objective were presented in separate chapters.

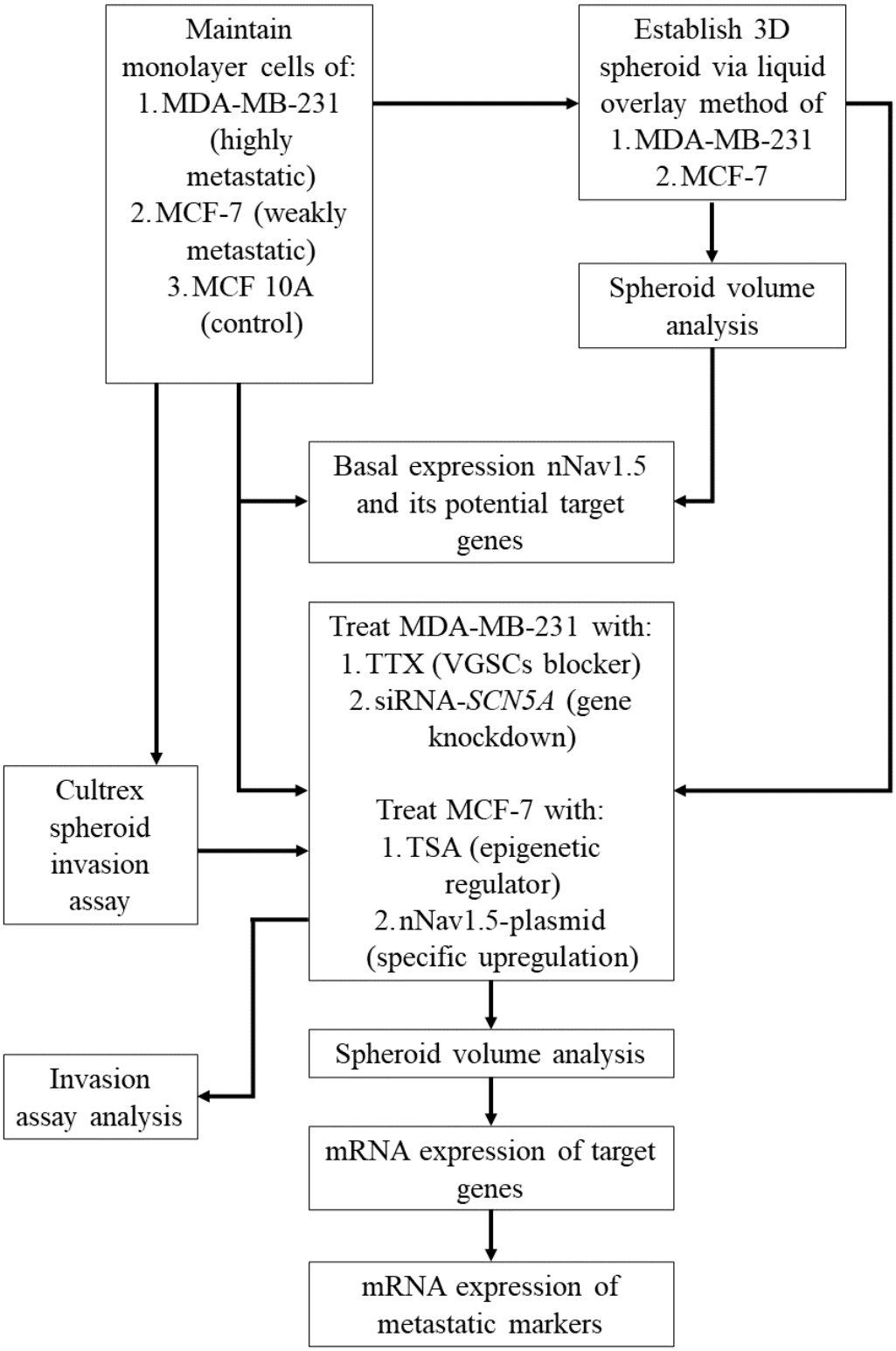


Figure 1.1 Overall flowchart of research design. The flowchart indicates the main objectives, which will be discussed in three chapters of this thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to breast cancer

Breast cancer originates in breast tissues where healthy cells undergo molecular changes, causing uncontrollable growth and the formation of tumours. Breast cancer is commonly associated with women, but among breast cancer cases, less than one per cent still affects men worldwide (Fox et al., 2022). Being women, ageing, oestrogen, family history, gene mutations and unhealthy lifestyle increase one's risks of breast cancer in their lifetime (Sun et al., 2017). Other risk factors include having dense breasts mainly occupied by connective tissues. The age demographic of breast cancer patients differs among populations worldwide, presumably affected by the variations of risk factors for individual populations or subpopulations (DeSantis et al., 2019). A worldwide synergistic statistical analysis signifies that the incidence rate of breast cancer increases with age. However, up until a certain age level (indicated as old age), the analysis data showed more apparent deaths caused by other factors such as lack of screenings and reduced detections (Howlader et al., 2017; Bidoli et al., 2019).

Localisation of breast cancer contributed significantly to the main classification procedure, with the main class referred to as either ductal carcinoma or lobular carcinoma (Malhotra et al., 2010). Apart from the two main classifications, breast cancer is also classified as either invasive or non-invasive based on the ability of the cancer cells to spread or invade the tissues of their surroundings. Invasive type of breast cancer is the major type with the highest mortality among patients worldwide, where the cancers metastasize to other areas of a distant body part, notably the bones, liver, lungs and brain (Cancer Facts & Figures, 2023).

There has not been a single or universal molecular pathway to explain the oncogenesis and progression of breast cancer. Still, the major cause of breast cancer is that healthy cells have their DNA mutated, allowing them to grow uncontrollably and undergo numerous modifications at multiple levels, resulting in the formation of breast cancer cell clones with distinct invasive capabilities. However, a multitude of research has been done involving cancer signalling pathways, tumour microenvironment, cell-to-cell interactions and immune-related causes to comprehend the basis of breast cancer, resulting in the emergence of potential treatments over time (Hanahan and Weinberg, 2011; Fouad and Aanei, 2017).

Breast cancer, in statistics, is often classified in terms of incidence rate or mortality rate. Breast cancer incidence rate is defined as the number of breast cancer cases happening during a year in a specified population, which is typically articulated as the number of cases per 100,000 population at risk (Cancer Incidence Statistics, 2022). The mortality rate is defined as the number of deaths caused by breast cancer in a specified population, expressed as a proportion of that population, over a given period (Cancer Mortality Statistics, 2022). In worldwide statistics, by the year 2022, breast cancer is ranked the top among other major cancer types in terms of both incidence and mortality rates affecting females of age ranging from 0 to 74, as shown in **Figure 2.1** (Bray et al., 2024).

In 2012, there were 14.1 million new total cancer cases, 32.6 million people who live with cancer (within five years of diagnosis) and 8.2 million deaths related to cancer, which amounts to 21.7% of total cases (2012; Mendis et al., 2014). Deaths cases by major cancer types show that breast cancer was fifth in rank (~522 000 deaths) in 2012 (Cancer Fact Sheets: Breast Cancer, 2012). In Europe and the United States,

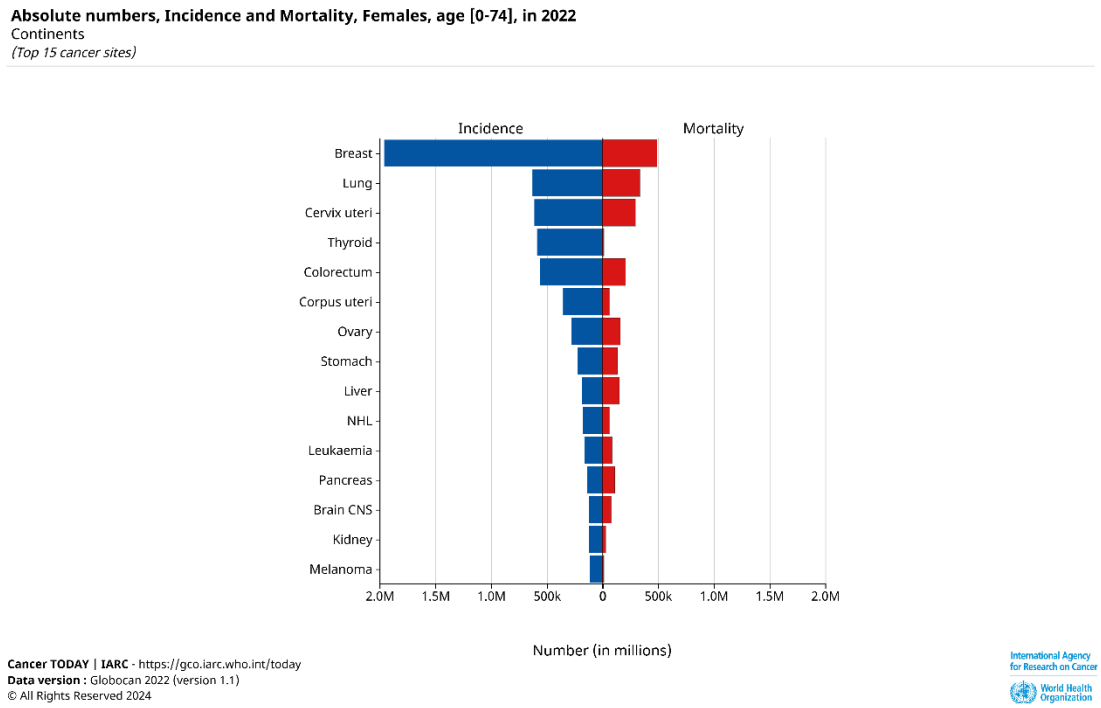


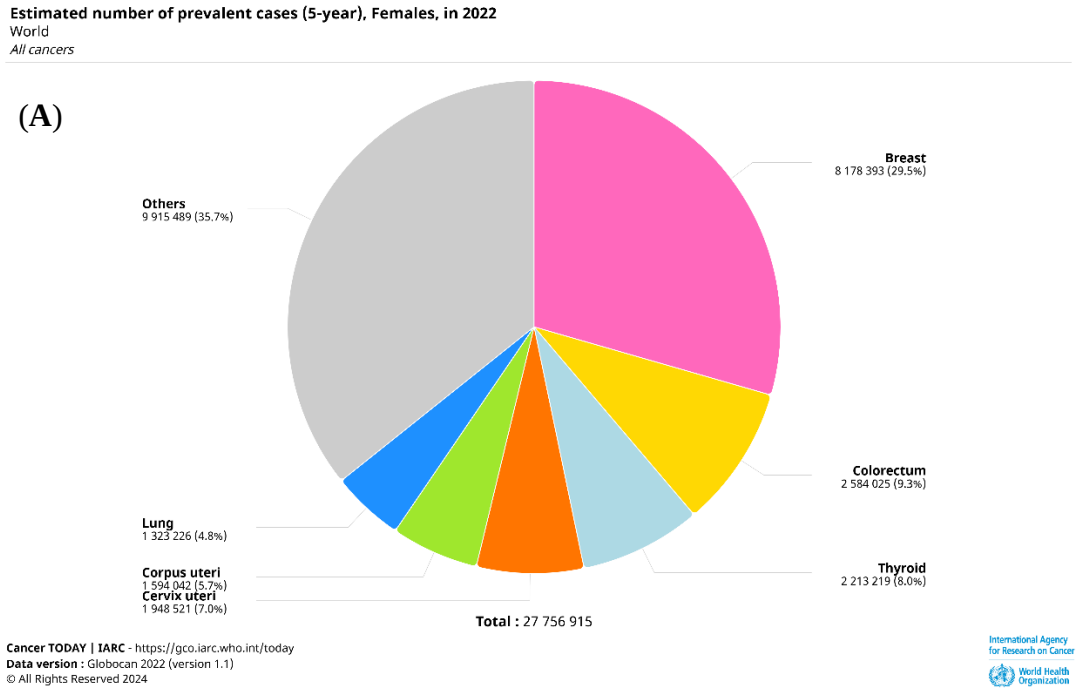
Figure 2.1 Worldwide cancer incident cases and mortality among females of ages 0 - 74. Breast cancer has the highest incidence and mortality rates among female cancer patients in the year 2022. Image adapted from Bray et al. (2024).

breast cancer is the most prominent cancer type affecting women, with 1 in 18 women at risk. However, in Malaysia, the risk of breast cancer affecting Malaysian women is at 1 in 19. Comparing the nine top cancer types that affect Malaysian women, breast cancer records the highest cases with 32.4%, 31.1% and 30% cases in Malay, Chinese and Indian women, respectively (Omar et al., 2006). As shown in **Figure 2.2**, in 2022, the 5-year most prevalent cancer cases in Malaysian females were of breast cancer at 39.8 per cent, slightly higher than the worldwide 5-year prevalent breast cancer cases at 29.5 per cent.

2.1.1 Breast cancer classification and treatments

The breast is composed of ducts and lobules, as shown in **Figure 2.3 (A)**. The ducts transport milk from the breast lobules towards the nipples, while the lobules are glands that produce milk. Breast cancer is classified as carcinoma by the histology analysis of the microscopic structure of breast tissue, depending on the location (ducts or lobules) and invasiveness (Vinay et al., 2010). Carcinomas in the breast can be categorised as in situ carcinomas (i.e., remain localised or preinvasive) or invasive carcinomas (i.e., spread to surrounding tissues). As summarised in **Figure 2.3 (B)**, ductal carcinomas that initiate in the breast ducts can be either in situ (ductal carcinoma in situ; DCIS) or invasive (invasive ductal carcinoma; IDC). Lobular carcinoma originating from the lobules is also categorised as in situ (lobular carcinoma in situ; LCIS) or invasive (invasive lobular carcinoma; ILC).

DCIS may affect only one part of the breast (unilateral) and deform the architecture of breast ducts, whereas LCIS does not deform the ducts and is bilateral (Harbeck et al., 2019). IDC of no special type accounts for 40 to 70 per cent of all mammary invasive carcinomas, and ILC is the second most invasive, with five to 15



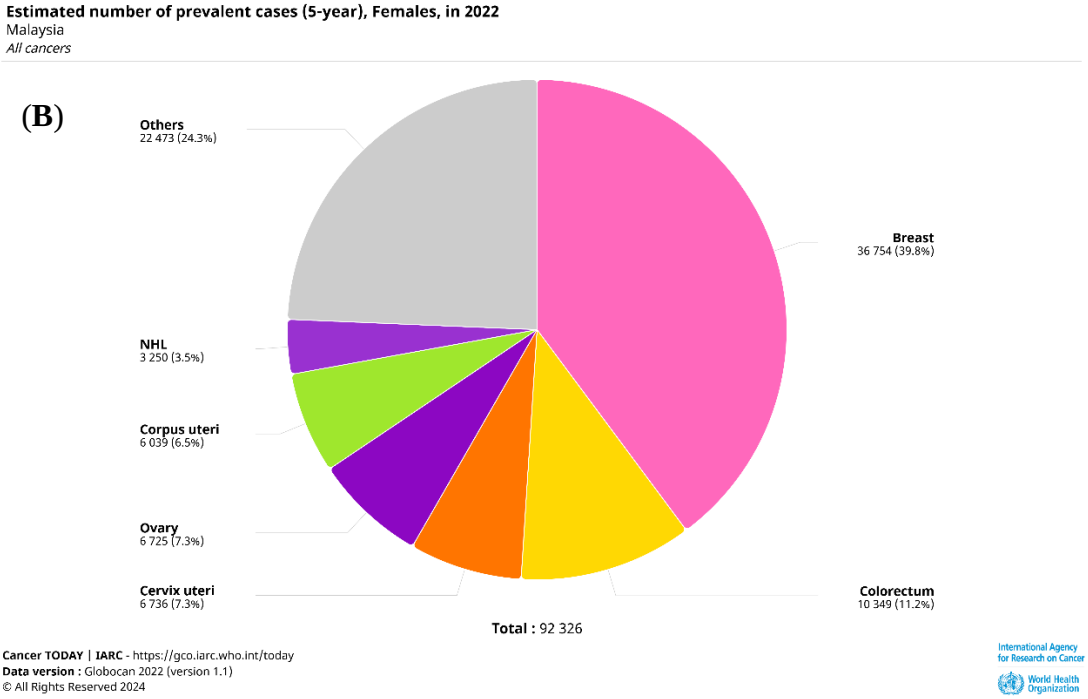
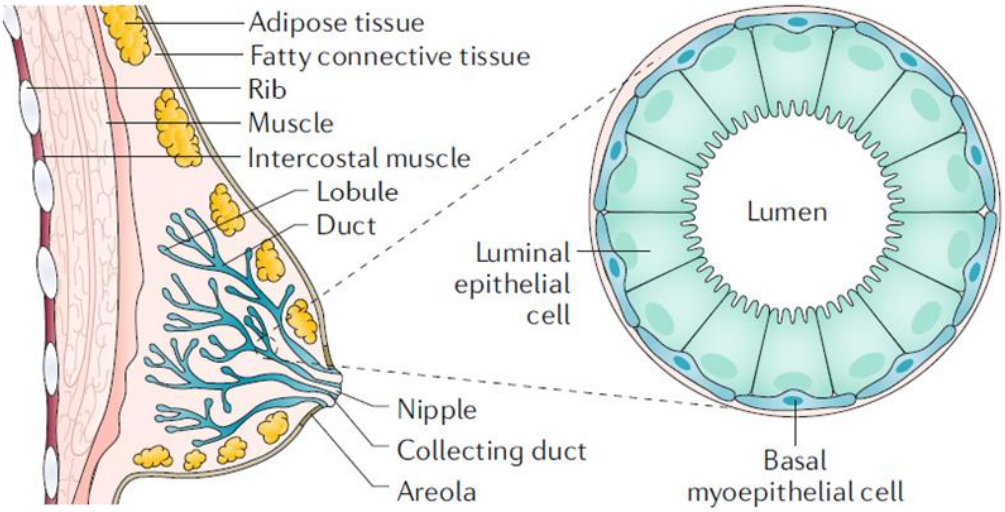


Figure 2.2 Statistics of prevalent cancer cases. (**A**) Worldwide 5-year prevalent cancer cases in females of all ages. (**B**) 5-year prevalent cancer cases in Malaysian females in 2022. Image adapted from Bray et al. (2024).

(A)



(B)

Histological subtypes

Preinvasive	**Invasive**
Ductal carcinoma in situ (DCIS) • Spreads through ducts and distorts ductal architecture; can progress to invasive cancer; unilateral	Ductal carcinoma no special type (NST) • Develops from DCIS; fibrous response to produce a mass; metastasizes via lymphatics and blood
Lobular carcinoma in situ (LCIS) • Does not distort ductal architecture; can be bilateral • Risk factor rather than precursor	Lobular carcinoma (ILC) • Isolated tumor cells (*CDH1* mutations) minimal fibrous response; metastasizes preferentially via viscera

Figure 2.3 Cross-section and breast cancer histological subtypes. (**A**) The cross-section showed the anatomical location of the breast lobule and duct, which can develop as lobular and ductal breast carcinomas. (**B**) The table summarises the histological subtypes of breast cancer. Images adapted from Harbeck et al. (2019).

per cent (Moinfar, 2007; Makki, 2015). However, diagnosis of ILC requires cytoarchitectural analysis and is challenging to detect via physical exam or imaging compared to IDC (Lakhani et al., 2012). More elaborated histological classifications are intensively summarised by Sinn and Kreipe (2013), revolving around the updates in the WHO Classification of Tumours of the Breast, 4th Edition, in 2012, compared to the 3rd edition in 2003. These categorisations are required for accurate diagnosis, therapeutic decisions, and patient management.

The molecular classification of breast cancer is based on distinct and repeatable changes in the cells' molecular characteristics or natural properties rather than their observable behaviour, which leads to carcinogenesis (Do Nascimento and Otoni, 2020; Johnson et al., 2020). Early global gene profiling data resulted in four molecular subtypes, Luminal A, Luminal B, HER2-enriched and basal-like, but molecular advancement also resulted in other subtypes such as Claudin-low (Perou et al., 2000; Prat et al., 2010). In clinical practice, immunohistochemistry analysis of hormone receptors are used such as oestrogen receptor (ER), progesterone receptor (PR), HER2, proliferation marker Ki-67 and cytokeratin 5, as reviewed by Ping Tang and Gary M. Tse (2016) and the references therein. Luminal A breast cancer is ER+/PR+ with HER2– and expresses less than 20 per cent of the Ki-67 marker (Orrantia-Borunda et al., 2022). Due to more treatment markers, Luminal A subtypes are showing better prognosis and the best 10-year overall survival (OS) rates compared to other subtypes (Van Maaren et al., 2019). The Luminal B subtype showed reduced PR expression, upregulation of Ki-67 and PCNA, greater p53 mutation, higher grade and less differentiated compared to Luminal A (Johnson et al., 2020). On the other hand, a special molecular subtype in which the breast cancer cells lack all ER, PR and HER2 expressions is defined as TNBC (Yin et al., 2020).

Breast cancer can be treated using a variety of clinical procedures, including surgery and radiation therapy, which is frequent for non-metastatic breast cancer in both local and systemic settings. Furthermore, treatments are largely dependent on the individual's condition, which clinicians will decide due to variances in histological features that increase the likelihood of carcinoma progression to the invasive form (Sinn and Kreipe, 2013). The therapeutic approach for DCIS is lumpectomy without radiation or surgical removal of the lesion, whereas, for more aggressive LCIS, full excision is common. IDC therapeutics method involves surgical excision of the tumour with vital dependencies on the tumour size and location, which may result in mastectomy or lumpectomy. Lumpectomy is often followed by adjuvant radiation treatment, whereas mastectomy is followed by postmastectomy radiation. As for ILC, localised treatments such as surgery and chemotherapy, radiation therapy, and targeted therapy are utilised. Prevention of possible recurrences may be done with long-term systemic treatment with hormonal therapy drugs such as tamoxifen.

In metastatic breast cancer, targeted therapy (i.e., hormone therapy, immunotherapy) showed the highest efficacies. Still, immunotherapies are developing more rapidly due to the utilisation of patients' immune systems to eradicate cancer cells or suppress cell proliferation, thus preventing relapse (Waks and Winer, 2019). Currently, advancements are also made for biological-based targeted therapy with antibodies (i.e., trastuzumab, pertuzumab) and immunotherapy involving checkpoint inhibitors such as anti-PDL1 antibodies (i.e., durvalumab, atezolizumab) and anti-CTLA4 (i.e., ipilimumab, tremelimumab) (Moo et al., 2018). For HER2+ breast cancer, anti-HER2 monoclonal antibodies were developed, as well as other methods involving adjuvants, cytokine activation and HER2-directed vaccines (Costa and

Czerniecki, 2020). In cases of TNBC, antibodies that target and block PD1, such as nivolumab and pembrolizumab, are developed (Mina et al., 2019).

Despite the advancement in treatment methods for breast cancer, the diversity and heterogeneity of breast cancer in TNBC and metastatic breast cancer will induce treatment resistance. Therefore, more biomolecules for breast cancer markers are required to develop more treatments against various breast cancer types. The heterogeneity of breast cancer ranges from intra-tumour and inter-tumour heterogeneity, even in genetic and phenotypic variations, including variations in the tumour microenvironment (Turashvili and Brogi, 2017; Januškevičienė and Petrikaitė, 2019). Cancer heterogeneity was closely related to cancer evolution, selection and clonal diversity, which eventually led to the resistance towards immune checkpoint inhibitors (ICIs) and targeted therapies, which indicates that multiple treatments and combinations are required to be tailored specifically for every individual patient (Turajlic et al., 2019).

2.1.1(a) Oestrogen receptor-positive breast cancer

ER+ breast cancer is a subtype of breast cancer characterised by the presence of oestrogen receptors on the surface of the cancer cells, also known as the luminal subtype. ER+ breast cancer is the most common, with statistics showing that among invasive breast cancer patients aged 20 and older in the United States (from 2015 to 2019), 80% of the cases are ER+ (Giaquinto et al., 2022). A wider study on 217,815 invasive breast cancer patients in the United States between the ages 20 to 49 years old, from the year 2000 until 2019, showed that ER+ cases are at 61.5% with increasing age-standardised incidence rate compared to the ER-types (Xu et al., 2024). In Malaysia, ER+ breast cancer affects 55.7% of patients, and in ER+/HER2+ patients, reduced survival outcomes are attributed to increasing age, tumour size and number of

lymph nodes (Yip et al., 2006; Lee et al., 2015c). Multiple breast cancer epidemiology research in Malaysia also showed that ER+ breast cancer cases comprise more than 53% of their sample sizes, and in a more recent study in Sarawak, ER+ breast cancer (inclusive as HR+ type) showed an increasing incidence rate by 4.46% per year (Yip et al., 2014; Sung et al., 2020).

ER+ breast cancer is often low in grade with smaller tumour sizes, and the patients tend to respond well to hormonal therapies, resulting in a long disease-free survival rate. The Kaplan-Meier OS graph from a study involving 2849 early-stage breast cancer patients showed that ER+ patients (75.4% of the total sample size) survive the longest compared to the ER− subtypes, with a mean survival of 5030.747 days. However, ER positivity did not show any significant association with the recurrence of the disease (Bulut and Altundag, 2015). In terms of mortality, ER+ breast cancer patients generated a lower incidence density mortality rate, with increasing mortality risk by 32% in the ER− counterparts (Belete et al., 2022). However, the median OS rate for ER+ breast cancer patients improved over the decade, from 33 months in patients from the year 1997 to 2006, to 42 months for patients from the year 2007 to 2017, potentially attributed to the advancement of diagnostic and therapeutic of breast cancer (Sánchez et al., 2020).

Currently, targeted therapy has shown significant benefits against ER+ breast cancer, for example, endocrine therapy, anti-HER2 treatment, and even a combination of both with chemotherapy. In endocrine therapy, drugs such as tamoxifen act as oestrogen-signalling inhibitors by competitively binding to ER and blocking the signalling effects, in which the pathways are shown in **Figure 2.4**. Other drugs that block oestrogen signalling include selective ER modulators (SERM) like aromatase

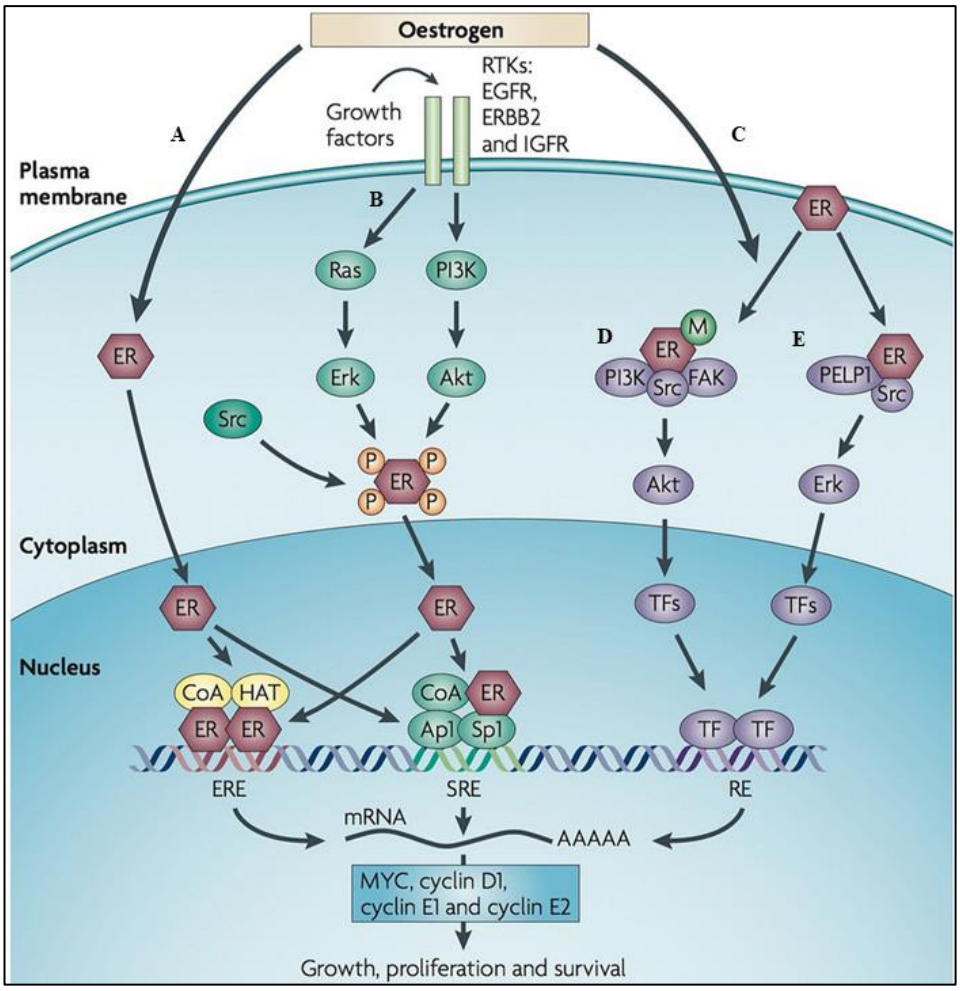


Figure 2.4 Oestrogen receptor (ER) signalling pathway. **(A)** Typical ER signalling regulates gene expression via ER dimerization, forming complexes with co-activators (CoA) and co-repressors. **(B)** Ligand-independent ER receptor activation via receptor tyrosine kinases (RTKs). **(C)** ER activates transcription factors without ER binding to DNA. **(D)** ER-phosphoinositide 3-kinase (PI3K)-Src-focal adhesion kinase (FAK) complex that activates AKT. **(E)** ER-Src-proline-, glutamate-, and leucine-rich protein 1 complexes that activate ERK. Image adapted from AlFakeeh and Brezden-Masley (2018).

inhibitors (i.e., letrozole, anastrozole) and selective ER downregulators (SERD) like fulvestrant (Rej et al., 2024). Regulatory approvals were also achieved for CDK4/6 inhibitors (i.e., abemaciclib, ribociclib), which function to block cell proliferation and are used against early and advanced-stage ER+ breast cancer (in addition to aromatase inhibitors and fulvestrant), resulting in better survival and tolerance (Gnant et al., 2023). However, tamoxifen treatment on ER+ breast cancer patients in a 25-year long-term study showed that the treatment is more beneficial for patients with PR+ status (Dar et al., 2021). Eventually, endocrine therapies were also resisted by some ER+ breast cancer via the downregulation of ER and alteration of the MAPK pathway, thus indicating the need to develop novel therapies against ER+ breast cancer (Raheem et al., 2023). This notion is also supported by the increasing cases of *de novo* metastatic breast cancer from ER+ patients, highlighting the importance of careful therapeutic selection and treatment regimes (Gombos et al., 2023).

2.1.1(b) Triple-negative breast cancer

TNBC was classified as ER−, PR−, HER2− and further categorised into six subtypes, which are basal-like 1, basal-like 2, immunomodulatory, mesenchymal-like, mesenchymal stem-like and luminal androgen receptor (Yin et al., 2020). TNBC also overexpresses multiple growth factor receptors such as VEGFR, EGFR, and FGFR, but targeting these molecules in clinical trials did not provide favourable outcomes for TNBC patients, probably due to the intrinsic tumour heterogeneity (Lehmann and Pietenpol, 2014). Multiple biological pathways have been identified as the regulator of TNBC, including p53 pathways, platelet-derived growth factor receptors, NF-κB, and angiotensinogen pathways. Furthermore, the increasing proliferation rate for TNBC was thought to be caused by several metabolism-related pathways, such as purine and pyrimidine metabolisms (Ossovskaya et al., 2011).

TNBC statistics for the Kaplan-Meier 5-year survival rate is 72%, with a median death period of 3.55 years, and 92% of the deaths occurred within the five-year period (James et al., 2019). Research also showed that TNBC has a lower 5-year OS rate than non-TNBC. Furthermore, metastatic TNBC from varying age groups also showed worse OS compared to non-metastatic TNBC (Hsu et al., 2022). In another study of 359 TNBC patients in Kuwait, the 10-year OS rate was 66%, reduced to 49% for stage III patients and zero per cent for stage IV patients (Fayaz et al., 2019). In Malaysia, 76 recruited samples that were TNBC-positive (confirmed via immunohistochemistry) showed a 5-year OS rate of 76.3%, and relapsed cases were associated with lymph node metastases (Abdul Aziz et al., 2020). However, in a population study, the most common sites of distant metastases from TNBC breast cancer patients are bone, followed by lungs, and the least common site is the brain (Gao et al., 2023).

Treatments for TNBC include surgery (i.e., mastectomy), radiation therapy, targeted therapy, and immunotherapy. However, mastectomy surgery is commonly performed on TNBC patients with larger tumour masses, higher pathologic stage, and higher frequencies of lymphovascular invasion (Adkins et al., 2011). Chemotherapy for TNBC cases includes cyclophosphamide, Epirubicin and Docetaxel, whereas neoadjuvant chemotherapy includes carboplatin plus Taxane and Trastuzumab. Interestingly, neoadjuvant chemotherapy showed a complete pathological response for 53.2% of TNBC patients, albeit with higher complications (Von Minckwitz et al., 2014). TNBC patients with eradicated tumours from both breast and lymph nodes showed better event rate survival and are associated with complete pathological response and long-term outcomes (Cortazar et al., 2014). Advancements in treatments against TNBC were also developed, such as PARP inhibitors and PD-L1

immunotherapy, which those treatments showed improved disease-free survival and OS in metastatic TNBC (Landry et al., 2022). However, due to the worse OS rate for TNBC compared to non-TNBC, the development of novel and efficient treatments, especially immunotherapy against TNBC and/or TNBC-specific cancer biomarkers, is essential to ensure a better prognosis for patients in the future (Li et al., 2017c).

2.1.2 Cell lines for *in vitro* breast cancer research

In 1951, the first immortalised tumour cell lines were successfully cultured from the cells of a cervical cancer patient, Henrietta Lacks, and since then, other cell lines have been developed from various cancer tissues (Adey et al., 2013). In 1958, the first breast cancer cell line, BT-20 cells, were developed from breast tumour of a 74-years old Caucasian woman via isolation and cultivation of cells spilling out of the tumour when it was cut into thin slices (Lasfargues and Ozzello, 1958). Throughout the years, multiple *in vitro* research was done using various breast cancer cell lines, including on cancer cell proliferation and signalling pathways, metabolic pathways, apoptosis, epigenetic regulation and the immune escape mechanisms (Lee et al., 2022; Xie et al., 2022; Zimmerli et al., 2022; Oliveira et al., 2023; Xu et al., 2023).

Due to the emergence of multiple breast cancer cell lines, validation of their characteristics and molecular subtypes is required to ensure accuracy for future research use. Various methods were used to characterise subtypes (i.e., Luminal A, Luminal B, TNBC) of breast cancer cell lines, such as immunoblotting, mRNA sequencing, quantitative PCR, SNP assays and transcriptomics approach to define mutations and transcript fusions in the cell lines (Smith et al., 2017; Pommerenke et al., 2024). Genomic profiling was also used to characterise breast cancer cell lines and compare them to actual breast cancer tumours (Liu et al., 2019). In a more advanced approach, single-cell transcriptomic profiling of breast cancer cell lines allows for a

deeper understanding of tumour heterogeneity and its impact on drug response (Gambardella et al., 2022). Undoubtedly, the use of breast cancer cell lines has reduced the cost of research and opened other opportunities, such as the generation of cell lines from patients' tumours to predict treatment response and even the development of mice breast cancer cell lines that imitate the tumour microenvironment profile for *in vivo* research (Chen et al., 2023; Perez-Lanzon et al., 2023). Today, the most used breast cancer cell lines are from the MD Anderson series, which were developed in the 1970s by the Michigan Cancer Foundation, including the ER+ MCF-7 cells and TNBC MDA-MB-231 cells, which were used extensively in *in vitro* and *in vivo* research (Witt and Tollefsbol, 2023).

2.1.2(a) MCF-7 as ER+ breast cancer cell line

Historically, in 1973, MCF-7 cells were cultured from epithelial cells of breast tissue from a 69-year-old metastatic adenocarcinoma patient via pleural effusion and have been used in research ever since (Brooks et al., 1973). Molecular subtyping via RNA expression data of 364 genes resulted in the MCF-7 cell line grouped as luminal-like, and hormone receptor characterisation showed that the cells lack HER2 receptors but express PR and ER, classifying them as Luminal A (Charafe-Jauffret et al., 2006; Dai et al., 2017). In the 1980s, due to the ER+ status, MCF-7 cells were used to elucidate the direct role of oestrogen on cellular tumour proliferation, in which the role was successfully confirmed *in vivo* (Huseby et al., 1984; Levenson and Jordan, 1997).

In breast cancer research, MCF-7 cells were utilised to investigate the impact of response from multiple endocrine therapy drugs such as tamoxifen, toremifene and DNA intercalating reagents such as doxorubicin (Altharawi et al., 2020). MCF-7 was also used to study ER signalling-related regulatory molecules and pathways in breast cancer, such as the impact of DNA damage on ER signalling, ER signalling regulation

by ubiquitin ligase TRIM56, the role of ER-Src signalling in bone metastases and reprogramming of ER signalling via DNA binding motifs (Chiu et al., 2017; Chi et al., 2019; Xue et al., 2019; Scherbakov et al., 2024). Furthermore, multiple studies on MCF-7 are associated with the testing of compounds on the ER signalling pathway, such as triptolide, tocotrienols, depsidone and benzophenone (Comitato et al., 2010; Li et al., 2015a; Darwati et al., 2021). High PR expression on MCF-7 cells also allowed for better anti-tumoral response against treatment such as progestin, which showed better anti-oestrogenic effects (Bajalovic et al., 2022).

In spheroid culture, MCF-7 was used for drug discovery study and was able to develop into multicellular cell aggregates with E-cadherin controlling the cell-cell adhesion molecules in the 3D structure (Dittmer et al., 2009; Chen et al., 2022c). Apart from E-cadherin, MCF-7 cells also express epithelial markers such as B-catenin but are negative for mesenchymal trait molecules such as vimentin (ComŞA et al., 2015). In terms of metastatic abilities, MCF-7 is weakly metastatic and possesses low invasion ability compared to the MDA-MB-231 cells, perhaps due to lower expression of pro-angiogenic factors (Lee and Kang, 2021).

2.1.2(b) MDA-MB-231 as TNBC cell line

Another widely used cell line in human breast cancer research is the MDA-MB-231, established from the breast tissue of an adenocarcinoma patient via the pleural effusion method at the MD Anderson Cancer Centre in the 1970s (Cailleau et al., 1974). Over the past years, MDA-MB-231 has been used as the *in vitro* model to represent aggressive breast cancer types. The characterisation of MDA-MB-231 later indicated that the cells do not express ER, PR and HER2 but possess mutated p53 and K-Ras molecules, thus classifying it as TNBC (Chavez et al., 2010). In *in vitro* studies, due to its TNBC subtype, MDA-MB-231 were used for multiple metastasis and

invasion-related research such as investigating the role of a transmembrane signalling molecule, role of novel miRNA and signalling transduction molecule, effects of invasion-related gene silencing (i.e., MMP-9, SIRT6) and the impact of antibiotic on metastasis and invasion (Zhang et al., 2019a; Wang et al., 2020c; Dong et al., 2021; Hong et al., 2022).

In *in vivo* studies, orthotopic transplantation of MDA-MB-231 instantly generates xenografts that metastasize to the lymph nodes (Cleris et al., 2019). The high metastatic abilities of MDA-MB-231 cells could be attributed to the expression of cytoskeletal proteins, vimentin and F-actin, in which simultaneous downregulation of both genes showed a reduction in cell survival and migration (Kwon et al., 2023). Currently, the use of MDA-MB-231 cells has also been extended via spheroid culture, in which the MDA-MB-231 spheroid was shown to possess elevated epithelial-mesenchymal transition (EMT) related protein expression, enhanced migrative behaviours and increased resistance towards antitumour compound (Huang et al., 2020).

2.1.2(c) MCF 10A normal breast epithelial cell line

In 1984, MCF 10A was generated via pleural effusion from the breast epithelial cells of a fibrocystic patient and, therefore, deemed as non-tumourigenic. Before the immortalisation process, MCF 10A was diploid cells karyotypically, but the immortalisation effects rearranged its karyotype into a near diploidy state (Soule et al., 1990). In terms of molecular characterisation, MCF 10A grown in 2D culture showed positive expression of both N-cadherin and E-cadherin, low expression of luminal markers such as Mucin 1 and no expression of oestrogen nor progesterone receptor, thus classifying them as basal-like (Qu et al., 2015). MCF 10A is widely utilised as a non-cancerous cell for comparison with other cell lines, such as MCF-7 and MDA-

MB-231, in *in vitro* cancer studies. For example, MCF 10A is used as a control comparison in breast cancer gene expression studies, determination of chromosomal instabilities, cell growth and proliferation assays, and invasion and metastasis assay (Yun et al., 2008; Gest et al., 2013; Kwon et al., 2023; Wang et al., 2024).

Furthermore, as a non-tumourigenic cell line, MCF 10A was also reprogrammed to study the regulation of EMT process (Antón-García et al., 2023). MCF 10A also serves as a platform to compare gene and protein expression involving real tissue analysis to allow for future alternative research on the cell line (Gurel et al., 2005). MCF 10A was also utilised as *in vitro* control against preinvasive and invasive primary breast tumours to confirm further the role of the HOXB13 gene in cell motility and invasion (Ma et al., 2004). Moreover, MCF 10A cells were successfully utilised in genetic research of driver mutations discovery in cancer, such as the consequences of PTEN and Tp53 deletion on MAPK signalling and chromosomal instability (Novikov et al., 2021). Other uses of MCF 10A in research include testing of biochemical compounds such as withaferin A against EMT-induced migration via inhibition of TNF-α and TGF-β (Lee et al., 2015b).

2.2 The Hallmarks of Cancer

The Hallmarks of Cancer by Hanahan and Weinberg (2000) is a comprehensive early review that revolves around devising principles from multiple cancer research areas (i.e., molecular and cell biology, physiology, biochemistry, tissue biology) that govern the alteration of normal cells into malignant, cancerous cells. As shown in **Figure 2.5 (A),** the first six hallmarks of cancers are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue