EVALUATION OF ANTI-NNAV1.5 ANTIBODIES AS POTENTIAL THERAPEUTIC AGENT IN SUPPRESSING BREAST CANCER INVASION AND METASTASIS

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2022

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

SEPTEMBER 2022

ACKNOWLEDGEMENT

I would like to express my gratitude to Allah SWT for providing me with this chance and for his unending assistance in completing my Ph.D. The road to this Ph.D. project has been challenging. First and foremost, I want to express my heartfelt gratitude to my respected supervisor, Dr. Noor Fatmawati Mokhtar, for her sincere guidance and assistance in finishing this study. I would like to express gratitude to the Malaysian Ministry of Energy, Science, Technology, Environment, and Climate Change (MESTECC) through the Science Fund (Grant no: 06-01-05-SF0844), the Malaysian Ministry of Education through the Higher Institution Centre of Excellence (HICoE) Program (No. 311/CIPPM/4401005), and Majlis Amanah Rakyat (MARA).

This gratitude also extends to Universiti Sains Malaysia, namely the Institute for Research in Molecular Medicine (INFORMM), for providing me with the facilities and the precious chance to pursue a Doctor of Philosophy in Molecular Medicine. My heartfelt thanks also go to all USM employees, particularly those who worked in INFORMM, for guiding and assisting me in completing this project.

I am eternally grateful to my laboratory colleagues, Murtadha, Irfan, and Amira, for their endless encouragement and engaging discussion. I am really fortunate to have assistance from MLT Amy Amylda, who is always available to explain laboratory protocols and even assist me in performing laboratory work. Finally, I am grateful to my wonderful parents and family members for their unceasing inspiration, which has propelled me to this level. Moral support from my beloved husband, Nadzir Raman, as well as long-distance friends Nur Ainina Wahab, Husna Zakirah Hamdi, and Syarafina Adilah, is also priceless and unforgettable. Thank you to everyone.

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LIST OF SYMBOLS AND ABBREVIATIONS

®	Registered
^{o}c	Degree Celsius
x g	Times gravity
α	Alpha
α-fetoprotein	Alpha fetoprotein
β	Beta
β-actin	Beta actin
µ	Micro
µg	Microgram
µl	Microliter
µM	Micro molar
AC	Adriamycin and Cyclophosphamide
AC	Adenylate cyclase
ACK	Ammonium-Chloride-Potassium
ADCC	Antibody dependent cellular cytotoxicity
AI	Aromatase Inhibitor
AP	Ammonium Persulphate
APC	Antigen presenting cell
ARASC	Animal Research and Service Centre
ASE	Antibody signal enhancer
ATCC	American Type Cell Culture
BCS	Breast conserving surgery
BLAST	Basic Local Alignment Search Tool
BME	Basement Membrane
bp	Base pair
BRCA	Breast cancer gene
Bregs	Regulatory B cells
BSA	Bovine Serum Albumin
BSE	Breast self-examination
C57BL/6	C57 black 6
Ca^{2+}	Calcium ion

CAR	Chimeric antigen receptor
CBE	Clinical breast examination
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CDR	Complement determining region
CEA	Carcinoembryonic antigen
CMF	Cyclophosphamide, Methotrexate, and 5-fluorouracil
CNS	Central nervous system
CO^2	Carbon dioxide
ConA	Concanavalin A
CRM	Computed radiography mammography
CTC	Circulating tumor cells
CTL	Cytotoxic T-lymphocytes
CXCL	Chemokine ligand
CXCR	Chemokine receptor
D0	Day zero
D3	Domain 3
DC	Dendritic cells
DCIS	Ductal carcinoma in situ
DCregs	Regulatory dendritic cells
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBNA	Epstein-Barr virus nuclear antigen
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-mesenchymal transition
EMT-TFs	Epithelial-mesenchymal transition transcription factor

ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
gDNA	Genomic deoxyribonucleic acid
GLOBOCAN	Global Cancer Observatory
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCl	Hydrochloric acid
HDI	Human Development Index
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
H_2SO_4	Sulfuric Acid
HLA	Human leukocyte antigen
HMW	High molecular weight
HRP	Horse radish peroxidase
HT	Hormone therapy
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
IDT	Integrated DNA Technology, Inc
IDV	Integrated density values
IFA	Immunofluorescence assay
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
kb	Kilo base
kDa	Kilo Dalton
KLH	Keyhole limpet hemocyanin
L	Liter
LAS X	Leica Application Suites X
LCIS	Lobular carcinoma in situ
LOX	Lysyl oxidase
mAbs	Monoclonal antibody
mAb-nNav1.5	Monoclonal antibody of nNav1.5
MAPK	Mitogen-activated protein kinase
MDSCs	Myeloid-derived suppressor cells

mg	Milligram
ml	Milliliter
mm	Millimeter
MHC	Major histocompatibility complex
MMG	Mammography
MMP	Matrix metalloproteinases
MRI	magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
Na^+	Sodium ion
Nav	Voltage-gated sodium channel
NC	Nitrocellulose
NCX	Sodium-calcium exchanger
NCBI	National Center for Biotechnology Information
NESOpAb	Neonatal Nav1.5 polyclonal antibody
NGF	Nerve growth factor
NHE1	Sodium Hydrogen ions exchanger type 1
NK-cell	Natural killer cell
nm	Nanometer
nNav1.5	Neonatal Nav1.5
nSCN5A	Neonatal Nav1.5 gene
OA	Ovarian ablation
OD	Optical density
pAbs	Polyclonal antibody
pAb-nNav1.5	Polyclonal antibody of nNav1.5
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-L	Programmed death-ligand
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
PR	Progesterone receptor
PTEN	Phosphatase and Tensin

qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
ROI	Regions of interest
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcription
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S1	Segment 1
SCN5A	Sodium channel, Voltage-gated Type V, Alpha subunit
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard Error of Mean
SFM	Screen film mammography
siRNA	Small interfering RNA
shRNA	Short hairpin RNA or small hairpin RNA
SLN	Sentinel lymph node
TAAs	Tumor associated antigen
TAE buffer	Tris-acetate-EDTA buffer
TAMs	Tumor-associated macrophages
TCR	T cell receptor
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TGFβ	Transforming growth factor β
Th	T helper cells
TMB	Tetramethylbenzidine
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
TP53	Tumor protein 53
TREG	Regulatory T cells
TTP	Time to progression
TTX	Tetrodotoxin
TTX-R	Tetrodotoxin resistant
TTX-S	Tetrodotoxin sensitive
TSAs	Tumor specific antigen
UK	United Kingdom

ULA	Upper low attachment
uPA	Urokinase plasminogen activator
US	United States of America
USG	Ultrasonography
UV	Ultraviolet
V	Voltage
VEGF	Vascular endothelial growth factor
VGSCs	Voltage-gated sodium channel
Vm	Membrane potential
WHO	World Health Organization
X-ray	Energetic High-Frequency Electromagnetic Radiation

PENILAIAN ANTIBODI ANTI-NNAV1.5 SEBAGAI AGEN TERAPEUTIK TERHADAP INVASI DAN METASTASIS KANSER PAYUDARA

ABSTRAK

Barah payudara tahap lanjut mempunyai kadar kelangsungan hidup yang paling rendah dan menyumbang kepada 90% kematian yang kebanyakannya adalah akibat daripada metastasis, terhadnya molekul sasaran dan pilihan rawatan. Dalam kajian ini, agen terapeutik dari jenis antibodi mensasarkan varian pecahan Nav1.5, juga dikenali sebagai 'neonatal' Nav1.5 (nNav1.5), dari keluarga *voltage-gated sodium channels* (VGSCs) yang telah dibuktikan mempunyai hubungkait yang kukuh dengan barah payudara yang bermetastatik melalui peranannya yang besar dalam mengawal migrasi sel, pencerobohan/invasi *in vitro* dan perebakan/metastatik *in vivo* adalah dikaji. Monoklonal (mAb-nNav1.5) dan poliklonal antibodi, berdasarkan IgG tulen (pAb-nNav1.5) atau serum penuh terhadap nNav1.5 adalah diperoleh, yang mana potensi mereka sebagai agen terapeutik adalah dikaji, kajian kesan perebakan/invasi sel barah payudara *in vitro* dan kajian kesan anti-tumoral dan anti-metastatik di dalam model haiwan. Kajian ini adalah untuk menghasilkan antibodi monoklonal dan poliklonal terhadap epitop spesifik nNav1.5 dan menilai imunoreaktiviti antibodi berkenaan terhadap sel barah payudara yang mempunyai ekspresi nNav1.5. Seterusnya, untuk mengkaji kesan anti-perebakan/metastatik mAb-nNav1.5, pAb-nNav1.5 dan serum poliklonal nNav1.5 terhadap pencerobohan/invasi sel barah payudara dalam kultur sferoid 3D, dan untuk menilai kesan terapeutik rawatan mAb-nNav1.5 dan pAb-nNav1.5 pada tumor yang terhasil melalui inokulasi sel 4T1 dalam tikus betina BALB/c. Hasil kajian menunjukkan bahawa imunoreaktiviti mAb-nNav1.5, pAb-nNav1.5 dan serum haiwan terimunisasi dengan peptida pendek

nNav1.5 spesifik (imunogen) terhadap lisat sel yang mempunyai ekspresi nNav1.5, sel MDA-MB-231 dan 4T1 telah dibuktikan menggunakan ELISA, pemblotan *Western* dan ujian immunositokimia. Selain itu, mAb-nNav1.5, pAb-nNav1.5 dan serum poliklonal haiwan berjaya menghalang pencerobohan/invasi (masing-masing 30-58%, 30-50%, dan 10-55%) kultur sferoid 3D MDA-MB-231 dan 4T1 diikuti oleh pengurangan ekspresi gen (mRNA) (3-5 kali ganda) dan protein nNav1.5. Penurunan pencerobohan/invasi kultur sferoid 3D dan pengurangan ekspresi nNav1.5 ini didapati bersamaan dengan kesan tetrodotoxin (TTX), perencat spesifik VGSCs. Akhir sekali, rawatan dengan mAb-nNav1.5 dan pAb-nNav1.5 berjaya mengurangkan beban tumor tikus betina BALB/c yang diinokulasi dengan 4T1, antaranya; pengekalan berat badan sihat, ukuran tumor yang lebih kecil dan ketiadaan keradangan/ lesi metastatik makro pada organ utama (paru-paru, limpa, usus dan hati) dibandingkan dengan kumpulan tikus kawalan (PBS). Analisa gen (mRNA) pada tisu tumor menunjukkan ekspresi nNav1.5 menurun. Secara keseluruhannya, kajian ini berjaya mengesahkan kebaharuan pengawalan pencerobohan/invasi sel barah payudara dan metastasis dengan mensasarkan nNav1.5 tetapi paling penting, versi *humanized* mAb-nNav1.5 mungkin mewakili agen imunoterapi pasif yang berguna pada masa akan datang bagi mengawal barah payudara.

EVALUATION OF ANTI-NNAV1.5 ANTIBODIES AS POTENTIAL THERAPEUTIC AGENT IN SUPPRESSING BREAST CANCER INVASION AND METASTASIS

ABSTRACT

Advanced-stage breast cancer has the poorest survival rates and contributes to 90% of cancer mortality, primarily due to limited molecular targets and treatment options. In this study, a novel target for metastatic breast cancer, a splice variant of Nav1.5 named 'neonatal' Nav1.5 (nNav1.5), a member of the family of voltage-gated sodium channels (VGSCs), was evaluated. nNav1.5 has a strong association with breast cancer metastatic potential through its role in cell migration, invasion *in vitro*, and metastasis *in vivo*. Monoclonal (mAb-nNav1.5) and polyclonal antibodies based on purified IgG (pAb-nNav1.5) or whole serum against nNav1.5 were obtained, which were evaluated as potential immunotherapeutic agents, investigating their effects on the invasion of breast cancer cells *in vitro* and their anti-tumoral and anti-metastatic effects in an animal model. This present study is aimed to produce monoclonal and polyclonal antibodies against a specific epitope of nNav1.5 and characterize their reactivity against nNav1.5 expressing breast cancer cells. Next, to investigate the therapeutic effects and anti-metastatic potential of these agents on the invasion of the 3D-spheroids culture of breast cancer cell lines and to evaluate the therapeutic effect of the mAb-nNav1.5 and pAb-nNav1.5 administration in a model of tumour induction with 4T1 cell line in BALB/c female mice. Findings demonstrated that the immunoreactivity of mAb-nNav1.5, pAb-nNav1.5 and serum of immunized animals against the specific nNav1.5 epitope, was confirmed in cells and lysates of nNav1.5 expressing cells, MDA-MB-231 and 4T1 cells based on ELISA, Western blotting and immunocytochemistry. Moreover, mAb-nNav1.5, pAb-nNav1.5, and nNav1.5

polyclonal serum suppressed the invasion of MDA-MB-231-3D and 4T1 3D-spheroids (30-58%, 30-50%, and 10-55%, respectively) followed by reduced nNav1.5 gene (3-5 folds) and protein expression, similar to tetrodotoxin (TTX), a potent channel blocker which was used as control drug. Finally, treatment with mAb-nNav1.5 and pAb-nNav1.5 resulted in reduced tumour burden of 4T1-induced mammary tumour in BALB/c female mice, which maintained higher body weight, smaller tumour size, and absence of inflammation/macro metastatic lesions in organs (lungs, spleen, intestines, and liver) as compared to control group (PBS treated). Subsequent gene expression analysis revealed downregulation of nNav1.5 expression in the animals' primary tumour tissues. Collectively, this study confirms the novelty of targeting nNav1.5 in suppressing breast cancer invasion and prevention of metastasis but importantly, humanized versions of mAb-nNav1.5 may represent useful passive immunotherapeutic agents to target nNav1.5 in breast cancer.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Globally, about 2.3 million newly diagnosed female breast cancer cases were reported in 2020, represented by 1 in 4 cases among women, surpassing lung cancer as the leading cause of global cancer incidence (Sung et al., 2021). As gender-biased cancer, the disease ranks first for incidence in the vast majority of countries (159 of 185 countries) and the leading cause of cancer death in 110 countries worldwide, implying that effective solutions are urgently required (Sung et al., 2021). In Malaysia, breast cancer is the number one incidence of five common female cancers with 32.9%, followed by colorectum cancer (11.9%), ovary cancer (7.2%), cervix uteri cancer (6.8%), and corpus uteri cancer (5.5%) (GLOBOCAN, 2020). Like many other developing countries/low-middle income countries, the breast cancer mortality rate in Malaysia is alarming due to the high rate (23.3%-59.7%) of advanced stage (stage III & IV) at initial diagnosis (Ministry of Health, 2018). Due to this, Malaysia has low survival rate (67.3%) than other developed/high-income countries, with a survival rate of 80% (Ministry of Health, 2018). The spread of cancer represents the advanced stage to other organs or parts of the body or metastatic cancer.

In the disease pathophysiology, cancer becomes more heterogenous (in terms of variable target expressions, genomic instability, development of resistance to current therapies and tumour microenvironment with various mutational burdens) and presents with the worst prognosis when reaching the advanced stages (Cajal *et al.*, 2020). Tumour heterogeneity of advanced-stage breast cancer is a major cause of

acquired resistance to current therapies such as chemotherapy and targeted therapies (Lim and Ma, 2019). Hence, improvement on searching for reliable therapies is urgently required to manage advanced-stage breast cancer patients.

Immunotherapy emerges as a therapy that harnesses the immune system in killing the tumour. Immunotherapy in the last decade has been extensively studied with various antibody, cell-based, and virus-based therapies for treatments of various tumours (Jia *et al.*, 2017; El-Sayes, Vito and Mossman, 2021). It has a huge potential to exert strong responses against heterogeneity of cancers, e.g. by directly modulating the tumour environment (Murciano-Goroff, Warner and Wolchok, 2020; Baliu-Piqué, Pandiella and Ocana, 2020). Immunotherapy could also be an alternative option in treating breast cancer subtypes characterized by a lack of target receptors/molecules, e.g., the triple-negative (absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2).

Successful immunotherapy must first identify a novel cancer target or antigen, i.e., a tumour-associated antigen (TAAs) or tumour-specific antigen (TSAs). Tumour antigens can sometimes be present only by tumour cells and never by normal cells, typically resulting from viral antigens or tumour-specific mutation where this antigen falls under TSAs (Peres et al., 2015; Jia et al., 2017). In contrast, TAAs are molecules of non-mutated proteins but with atypical expression and can be overexpressed or re-expressed in tumours (Dermime et al., 2004).

TAAs, widely employed as biomarkers in various cancers, allow for the identification of cancer through changes in TAAs concentrations in serum/tissues. Hence, identifying ‘unique' antigens in malignancies gives essential pharmacological targets for developing unique cancer therapeutic methods and promotes the

development of antigen-specific cancer immunotherapies (such as vaccines and antibodies). Following this, TAAs can be utilized as reliable cancer detection markers and targeted cancer therapies, as seen in programmed death-ligand (PD-L1) being actively studied in breast cancer clinical trials as the protein is overexpressed in many types of cancer. The programmed cell death protein 1 (PD-1) and PD-L1-based cancer immunotherapy studies have emerged rapidly, with over 2000 ongoing trials investigating the effectiveness of various PD-1/PD-L1 targeted drugs (Tang et al., 2018). The transmembrane expression of the PD-L1 on the surface of tumour cells aids in targeting antagonists, primarily monoclonal antibodies, in binding to the target antigen (Wu et al., 2019). The anti-PD-1/PD-L1 demonstrates extremely durable and persistent effects, with some patients remaining cancer-free for many years (Pardoll, 2018). Additionally, atezolizumab (anti-PD-L1) treatment with nab-paclitaxel in triple-negative breast cancer (TNBC) led to a 40% reduction in the risk of progression or death, and the treatment improved patient survival with a favourable safety profile in phase 3 clinical trial (Schmid et al., 2018). Recently, atezolizumab and pembrolizumab have been approved by the Food and Drug Administration (FDA), USA, for metastatic TNBC patients. As a result of this encouraging progress with passive immunotherapy, we are eager to introduce a novel TAA as a target for advanced breast cancer immunotherapeutic agents.

The TAAs proposed in this study are derived from the voltage-gated sodium channel (VGSCs) family, which includes alpha (Nav1.1-Nav1.9) and beta (1-4) subunits. According to extensive *in vitro* and emerging *in vivo* evidence, functional upregulation of VGSCs occurs in various cancers, including breast cancer (Fraser *et al.*, 2005). A cardiac isoform of VGSCs, Nav1.5, contributes more than 80% of VGSCs expression in aggressive breast cancer cell lines, MDA-MB-231; however,

further sequence analysis indicated that the dominant version is the ‘neonatal' splice variant. The Nav1.5 undergoes alternative splicing, producing the neonatal form of Nav1.5, termed neonatal Nav1.5, nNav1.5. The nNav1.5 encoded by the *SCN5A* – a similar gene that encodes Nav1.5, which is prevalent in aggressive breast cancer cell lines/tissue biopsies as demonstrated in real-time PCR, sequencing, immunocyto/histochemistry, and siRNA (Chioni *et al.*, 2005; Fraser, Diss, Chioni, *et al.*, 2005; Brackenbury *et al.*, 2007). nNav1.5 was identified as a domain 1, segment 3 (D1:S3) 5’ splice variant characterized by a highly conserved aspartate residue at the extracellular end of D1:S3 that was replaced by a positively charged lysine. nNav1.5 differs from the adult exon by 31 nucleotides (equivalent to 7 amino acid changes) and is responsible for VGSCs-independent invasion. Cellular nNav1.5 mRNA expression is ~1800 times higher in metastatic MDA-MB-231 cells than in weakly metastatic MCF-7 cells (Fraser *et al.*, 2005). This upregulation is similar to increased protein expression. Tetrodotoxin (TTX), a VGSCs-specific inhibitor, suppressed nNav1.5 expression and several cellular activities such as directional motility and invasion (Brackenbury et al., 2007). Low detection of nNav1.5 protein in normal breast tissue has been reported, whereas its expression is undetectable in other tissues such as the colon, small intestine, stomach, and prostate (Yamaci et al., 2017). However, nNav1.5 is significantly higher in breast cancer tissues associated with lymph node metastasis, recurrence, or death within five years due to cancer (Fraser *et al.*, 2005). The unique sequence and tumour tissue expression of nNav1.5 highlight its potential as a novel tumour-associated marker against aggressive breast cancer.

1.2 Problem statement

It is predicted that breast cancer cases will keep on increasing, and by the year 2050, it is estimated that the incidence will reach approximately 3.2 million (Bhatia and Re, 2016). If the majority of the patients are diagnosed with an advanced stage (especially in the less developed and developing regions of the world), by that same year (2050), more women will die due to the disease.

Immunotherapy is now understood to help overcome tumour heterogeneity in advanced-stage breast cancer. Provided with a good/novel target, passive immunotherapy using antibodies has now revolutionized cancer treatment, e.g., PD-L1 of advanced-stage cancers. nNav1.5's potential in passive immunotherapy – the development of anti-nNav1.5 antibodies and their therapeutic potentials were explored after the molecule's uniqueness attracted our attention.

1.3 Scope of study

The TAAs molecule introduced in the present study is a member of the VGSCs family, nNav1.5, a splice variant of Nav1.5. Increased expression of nNav1.5 has been associated with the enhanced metastatic potential of human breast cancer cells *in vitro* and *in vivo* of metastatic animal models and patients' tumour tissues who are positive for lymph node metastasis (Fraser et al., 2005).

In this study, several mice were immunized with nNav1.5 specific peptide VSENIKLGNLSALRC and selected to proceed for the production of antibodies. nNav1.5 polyclonal and monoclonal antibodies (mAb-nNav1.5 and pAb-nNav1.5) and nNav1.5 polyclonal serum raised against a specific peptide VSENIKLGNLSALRC were successfully obtained. The antibodies and serum were validated for their

immunoreactivities in immunoassays- Enzyme-Linked Immunosorbent Assay (ELISA), Western Blotting, and Immunofluorescence assay (IFA). The assays answered whether the acquired antibodies and serum could yield immunoreactivity signals that determine the reliability of the antibodies for detection and therapeutic purposes. After successfully validating mAb-nNav1.5, pAb-nNav1.5, and nNav1.5 polyclonal serum by the mentioned assays, their evaluation as potential immunotherapeutic agents were carried out *in vitro* and *in vivo,* then proceeded with treatments for both assays. The therapeutic effects of both anti-nNav1.5 antibodies and nNav1.5 polyclonal serum were tested in monolayer cells (with abundant nNav1.5 expression), human breast cancer cell lines, MDA-MB-231 cells and mice mammary carcinoma cell lines, 4T1 cells and weakly nNav1.5 expressing cells, MCF-7. In addition, therapeutic effects of anti-nNav1.5 antibodies and nNav1.5 polyclonal serum as potential anti-metastatic agents were tested for their effects on the behaviour of cancer cells in the 3D-spheroid invasion assay. To further confirm the therapeutic effect of anti-nNav1.5 antibodies, an orthotopic animal model using BALB/c female mice was induced with 4T1 cell lines and treated with both pAb-nNav1.5 and mAb-nNav1.5. The nNav1.5 polyclonal serum was excluded from *in vivo* assays due to the similar results obtained with anti-nNav1.5 antibodies *in vitro* assays. Therapeutic effects *in vivo* were analyzed according to a physical assessment of the animal model, gross examination of excised organs, presence of inflamed/macro metastatic lesions in organs, immune system activation of the model, the persistence of the given antibodies, and expression of nNav1.5 mRNA (*nSCN5A*) in the extracted tumour.

Overall, the current work demonstrated the therapeutic potential of anti-nNav1.5 antibodies, pAb-nNav1.5, and mAb-nNav1.5 and/or nNav1.5 polyclonal

serum in breast cancer cell lines and an animal model of mammary tumours with high nNav1.5 expression, which was linked to enhancing metastatic potential.

1.4 Research objective

The present study aimed to obtain monoclonal, IgG polyclonal antibodies against a specific epitope of nNav1.5 and serum from immunized animals with the same epitope. According to a passive immunotherapeutic strategy, this present study investigated the effect of these agents on breast cancer cells *in vitro* and a mammary tumour-induced animal model. The specific objectives of this study were as follows:

1. To produce monoclonal and polyclonal antibodies against a specific epitope of nNav1.5 and characterize their reactivity against nNav1.5 expressing breast cancer cells.

2. To investigate the anti-metastatic potential of these agents on the invasion of the 3D-spheroids culture of breast cancer cell lines.

3. To evaluate the therapeutic effect of the mAb-nNav1.5 and pAb-nNav1.5 administration in an *in vivo* animal model of tumour induction with 4T1 cell lines in BALB/c female mice.

1.5 Research question

1. Is it possible to produce monoclonal and polyclonal antibodies against a specific epitope of nNav1.5 in mice?

2. How to characterize the reactivity of antibodies against nNav1.5 expressing breast cancer cells?

3. What are the effects of these agents on the nNav1.5 gene and protein expression of breast cancer cell lines?

4. What is the anti-metastatic effect of these agents on the invasion of the 3D-spheroids culture of breast cancer cell lines?

5. What are the therapeutic effects of the specific monoclonal and polyclonal administration in tumour-induced BALB/c female mice with 4T1 cell lines?

1.6 Hypothesis

1.6.1 Null hypothesis

1. There is no immunoreactivity detected from nNav1.5 monoclonal and polyclonal antibodies.

2. There are no therapeutic effects of these agents on the nNav1.5 gene and protein expression of breast cancer cell lines.

3. There is no anti-metastatic effect of these agents on the invasion of the 3D-spheroids culture of breast cancer cell lines.

4. There are no therapeutic effects of the mAb-nNav1.5 and pAb-nNav1.5 administration in tumour-induced BALB/c female mice with 4T1 cell lines.

1.6.2 Alternative hypothesis

1. There is immunoreactivity detected from nNav1.5 monoclonal and polyclonal antibodies.
2. There are therapeutic effects of these agents on the nNav1.5 gene and protein expression of breast cancer cell lines.
3. There is an anti-metastatic effect of these agents on the invasion of the 3D-spheroids culture of breast cancer cell lines.
4. There are therapeutic effects of the mAb-nNav1.5 and pAb-nNav1.5 administration in tumour-induced BALB/c female mice with 4T1 cell lines.

1.7 Significance of the study

In general, the findings of this study answered whether the immunotherapeutic agents against nNav1.5, mAb-nNav1.5, pAb-nNav1.5, and/or nNav1.5 polyclonal serum were able to control invasion and metastasis of breast cancer cells *in vitro* and in an animal model, respectively. Consequently, this study provided preliminary data on the therapeutic value of the treatment agents and initiated more extensive research in the future to evaluate the potential of these antibodies in treating patients with metastatic breast cancer.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

As shown in **Figure 2.1**, the breast is divided into three sections: lobules, ducts, and connective tissues. The lobule functions as a gland to produce milk, while the ducts function as a tube to carry milk to the nipple. Meanwhile, the connective tissue, which primarily consists of fibrous and fatty tissue, serves to hold the breast together.

Breast cancer is one of the most frequent malignancies that affect women worldwide, and it is the leading cause of cancer mortality in women (Scully et al., 2012). It is a disease in which cells in the breast proliferate uncontrollably and affect various parts of the breast (CDC, 2018). Cancer develops in the ducts or lobules of the breast and can spread to other organs *via* blood vessels and lymph nodes.

Not all patients with breast cancer present with the same symptoms; some appear with no symptoms. Symptom signatures of individual breast cancer patients can be divided into four groups; present with breast lump only (76%), non-lump breast symptoms (11%), both lump and non-lump symptoms (6%), and non-breast symptoms (5%) (Koo et al., 2017).

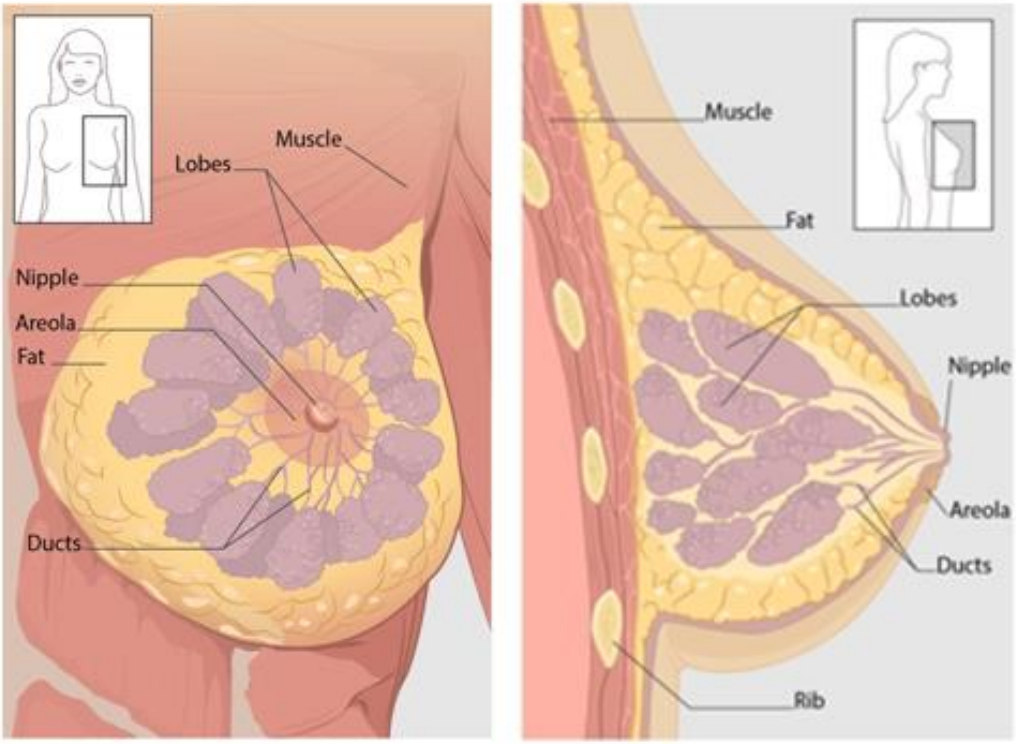


Figure 2.1 Anterior and cross-section view of the female breast. A) Anterior view B) Cross-section view of breast anatomy. The major components of the breast are lobes for milk production, ducts as tubes to transport milk to the nipple, and connective tissues (fat and fibrous tissues) to hold the structure together. Adapted from Centers for Disease Control and Prevention (CDC), 2018.

2.1.1 Epidemiology of breast cancer

2.1.1(a) Global statistics

Over 2.3 million newly diagnosed female breast cancer cases were recorded globally in 2020, accounting for 1 in every 4 cancer diagnoses among women (Sung et al., 2021). The disease is the most often diagnosed cancer in 159 of 185 countries worldwide, and it is the leading cause of cancer mortality in more than 100 countries (**Figure 2.2**) (Sung et al., 2021). Various factors have been thought to influence the distribution of cancer incidence worldwide, with 103 nations having a high incidence of female breast cancer while the rest of the countries have different leading cancers (colorectum, lung, cervix uteri and thyroid cancers) (**Figure 2.3**).

Human Development Index (HDI) (United Nations Development, 2020) is an indicator that is used to rank countries in terms of life expectancy, education, and income per capita; where these indicators determine the level of the countries into four tiers such as very high, high, medium and low HDI and recently, Malaysia is ranked in high HDI. Transitioned (developed) countries have extremely high and high HDI, while transitioning (developing) countries have middle/low HDI. Breast cancer vastly outnumbers other types of female cancer in both transitioned and transitioning countries, according to cancer incidence patterns, followed by colorectal cancer in transitioned countries (Europe and America) and cervix uteri in transitioning countries (Africa) (**Figure 2.4**) (Sung et al., 2021). In terms of mortality, statistics show that breast cancer cases are lower when compared to cancer incidence, although this cancer remains the most common in both transitioned and transitioning countries, followed

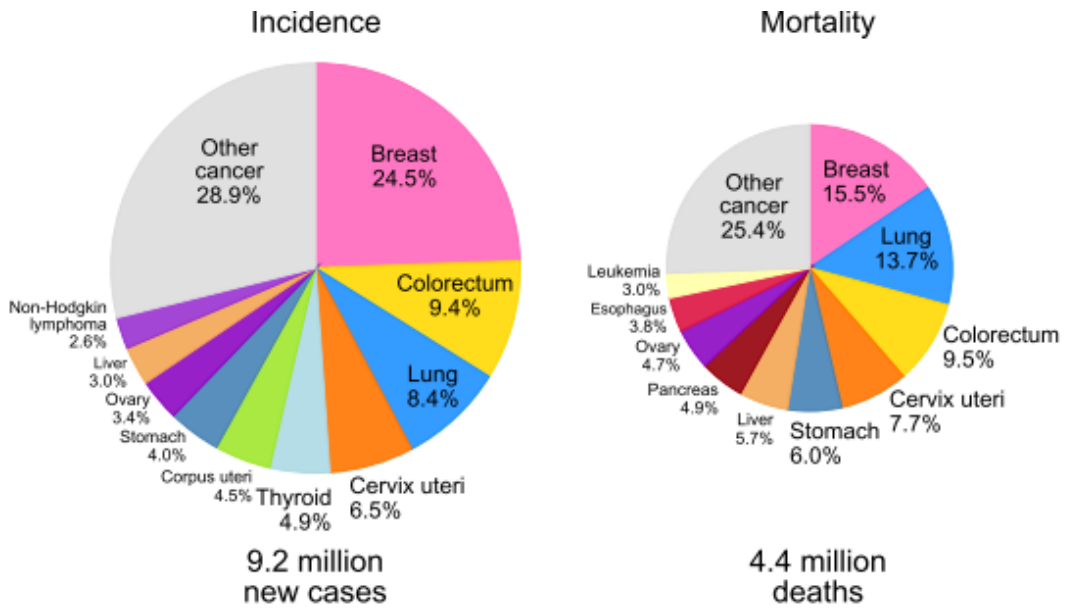


Figure 2.2 Pie charts depict the incidence and mortality rates of the most frequent female malignancies. Adapted from Sung et al., 2021.

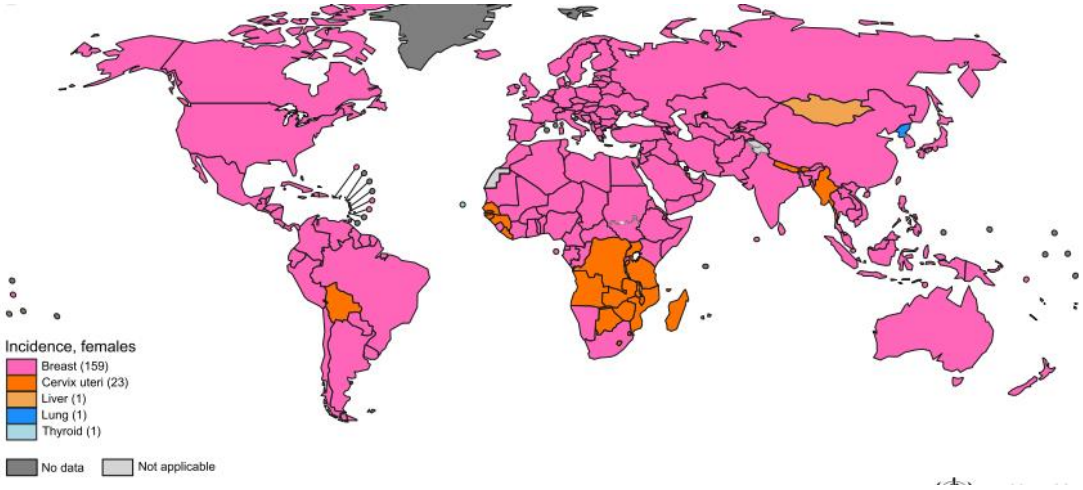


Figure 2.3 Global map shows the most common type of cancer in each country. Adapted from Sung et al., 2021.

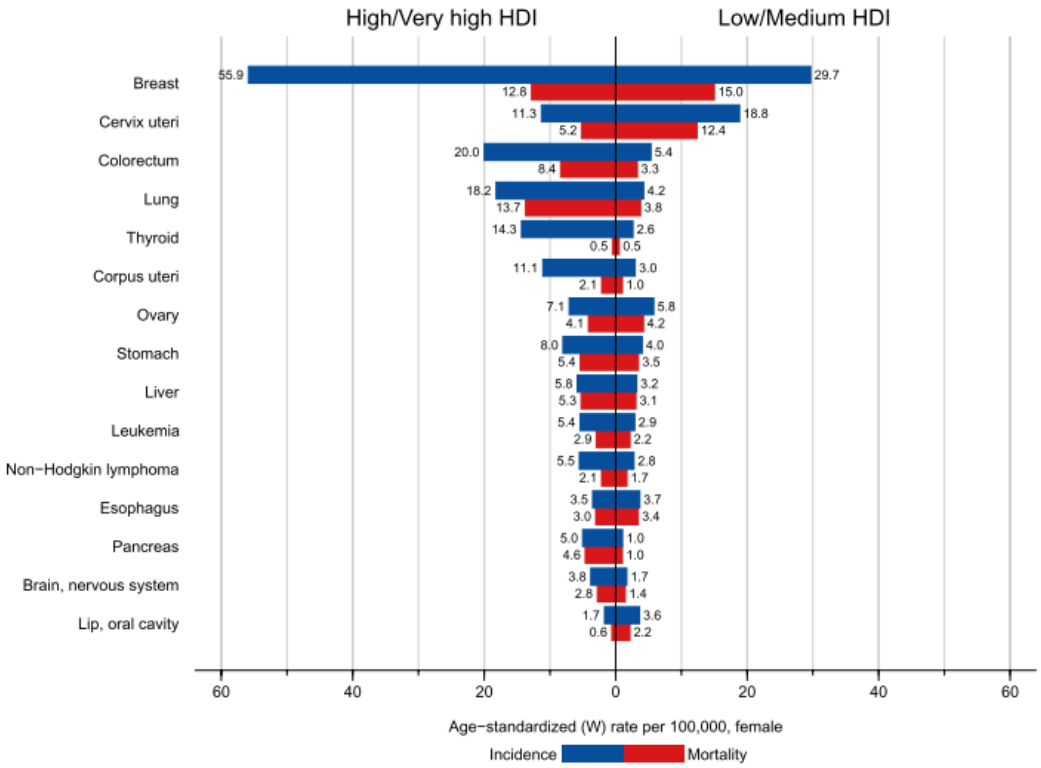


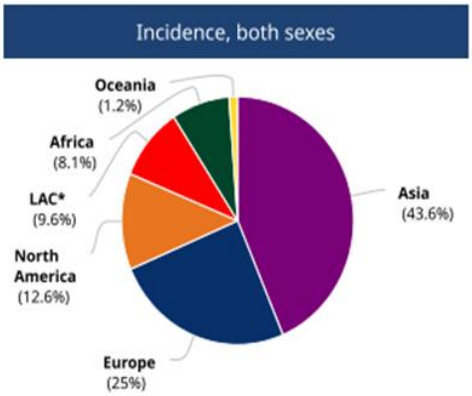
Figure 2.4 Bar charts of incidence and mortality rates in High/Very High Human Development Index (HDI) Regions Versus Low/Medium HDI regions among women. Adapted from Sung et al., 2021.

by colorectal cancer in transitioned countries and cervix cancer in transitioning countries (Sung et al., 2021). In short, breast cancer remains the most frequent disease in females regarding incidence and mortality, but rates are higher in transitioned countries than in transitioning countries.

Asia is the largest continent in the world as it comprises 4.64 billion of the population with 48 countries and is further divided into five regions: Central Asia, East Asia, South Asia, Southeast Asia, and Western Asia. For the past few decades, Asia has experienced rapid economic growth that contributes to increasing life expectancy, reducing mortality of communicable diseases, and westernising lifestyles. However, the incidence of breast cancer in Asia continues to rise year after year. According to GLOBOCAN 2018, Asia has the highest cancer incidence when compared to the rest of the world, including Europe, North America, Latin America and the Caribbean, Africa, and Oceania (GLOBOCAN, 2018). Regarding mortality, Asia has the highest percentage of cases, followed by Europe and Africa **(Figure 2.5)**. The rates of breast cancer in Asia are alarming, and it is hypothesized that changes in lifestyle from traditional to modern among Asian women led to this (Yip, 2016).

2.1.1(b) Breast cancer in Malaysia

Despite many health infrastructures and service improvements, Malaysia has a severe cancer incidence problem. The numbers for the most frequent cancers are concerning because cancer is the fourth highest cause of mortality, accounting for 39.3% of all deaths in government and private hospitals 2016 (Ministry of Health, 2018).



Population	Number
Asia	911 014
Europe	522 513
North America	262 347
*Latin America and the Carribean	199 734
Africa	168 690
Oceania	24 551
Total	2 088 849

Incidence, both sexes
Oceania
(1.2%)
Africa
(8.1%)
LAC*
(9.6%)
North
America
(12.6%)
Europe
(25%)
Asia
(43.6%)

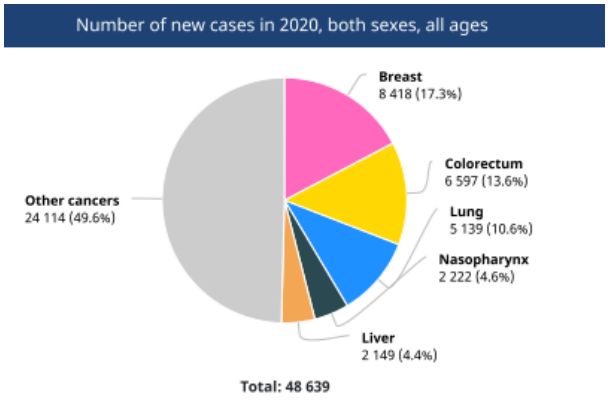
Population	Number
Asia	911 014
Europe	522 513
North America	262 347
*Latin America and the Carribean	199 734
Africa	168 690
Oceania	24 551
Total	2 088 849

Figure 2.5 Pie charts represent incidence and mortality rates of breast cancer cases in six regions worldwide (GLOBOCAN, 2018).

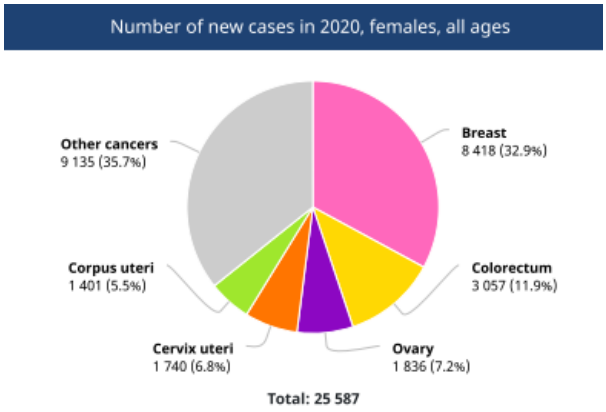
From 2007 to 2016, there has been an escalating trend from 11.3% (2007 to 12.6%) (2016) of cancer incidence (Ministry of Health Malaysia, 2017). According to the latest report by GLOBOCAN, out of five common cancers in Malaysia (breast cancer, colorectum cancer, lung cancer, nasopharynx cancer and liver cancer), breast cancer is the leading cancer type which contributed to 17.3% of reported cases in 2020, follows by colorectum cancer (13.6%), lung cancer (10.6%), nasopharynx cancer (4.6%), and liver cancer (4.4%) **(Figure 2.6 A)** (GLOBOCAN, 2020). For female cancers, again, breast cancer ranks as the first common cancer (32.9%) affecting females of all ages, followed by colorectum cancer (11.9%), ovary cancer (7.2%), cervix uteri (6.8%), and corpus uteri cancer (5.5%) **(Figure 2.6 B)** (GLOBOCAN, 2020). Data also shows that breast cancer has the highest mortality rate (20.7%) as compared to other cancers (**Figure 2.6 C**) (GLOBOCAN, 2020).

According to the summary of the Malaysian National Cancer Registry Report 2007-2011 (2017), the age-specific incidence rate for breast cancer is the highest between the ages of 55-59, followed by 50-54, 60-64, and 45-49 years old (Ministry of Health, 2017). Breast cancer usually starts to be actively diagnosed in females aged between 25-59 years old where this age group contributes to the highest statistics (40.7%) when compared to other cancers such as cervix uteri (8.9%), colorectal (7.6%), and ovarian cancer (6.9%). Among women aged between 60-74 years old, breast cancer ranks first (24.4%), followed by colorectal cancer (15.4%), trachea, bronchus and lung cancers (8.8%), and cervix uteri (7.1%).

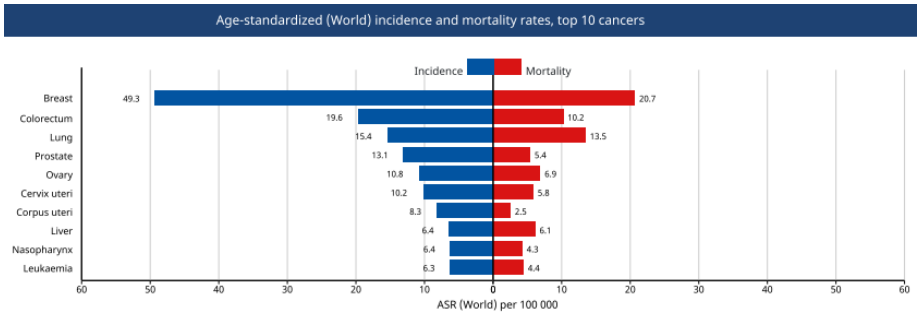
Breast cancer incidence decreases in older age groups due to differences in the presence of estrogen and progesterone hormones, as both hormones trigger the progression of cancer quickly and might present at a higher grade during the first diagnosis (Anders et al., 2008; Peng et al., 2011).



(A)



(B)



(C)

Figure 2.6 Incidence of cancers. (A) 5 common cancers (breast, colorectum, lung, nasopharynx, and liver) for both sexes of all ages in Malaysia, (B) 5 common cancers (breast, colorectum, ovary, cervix uteri and corpus uteri) for the female of all ages in Malaysia. (C) Incidence and mortality rates for top ten cancers in Malaysia. Breast cancer ranks the highest for both incidence and mortality rates than other cancers. Adapted from GLOBOCAN, 2020.

Among Malaysian citizens, Malay is the major ethnic group (55.6%), followed by Chinese (23.4%), indigenous peoples (examples, natives of Sarawak and Sabah, etc.) (13.0%), Indians (7%), and others (1%) (Ministry of Health, 2018). The incidence of breast cancer is the highest among Chinese (41.5%), followed by Indians (37.1%) and Malays (27.2%). However, the worst survival rate was reported in Malays (57.9%), followed by Indians (70.5%) and Chinese (76.5%) (Azizah et al., 2016). The survival rate in Malays is the worst, with many of them diagnosed at an advanced stage (stage III or IV) (Hisham and Yip, 2004) - delaying presentation influences the prognosis and the survival rate of cancer patients (Wan Mahiyuddin et al., 2014). This can be supported by a report which stated that many cancer patients (56%), including breast cancer patients, usually came to the hospital at late stages (stage III and IV) rather than at earlier stages, stage I (18%) and stage II (26%) (Ministry of Health, 2018). The quality of life and survival rate among Chinese was highest, followed by Malays and Indians, probably due to socioeconomic status and socio-cultural differences (Akhtari-Zavare et al., 2018).

2.1.2 Types of breast cancer

Breast cancer is a complex disease with clinical and genetic variations. It has been categorized in a variety of ways due to its uniqueness. Before the discovery of molecular subtypes of breast cancer (before 2000), the disease was diagnosed primarily through histology, which had numerous limitations. Accurate diagnosis is critical since it improves breast cancer patients' management and survival. Inadequate diagnosis, for example, due to limitations in disease detection information, will result in improper treatments for patients, as they will be under/over-treated with unspecific treatments such as chemotherapy, resulting in insufficient response or even resistance

to the treatments (Kittaneh and Montero, 2013). By clearly identifying the many types of breast cancer, cancer patients' treatments and survival will be considerably improved, as exact diagnosis leads to specific therapies being administered.

2.1.2(a) Histological classification of breast cancers

Breast cancer can be broadly classified as *in situ* carcinoma or invasive carcinoma under this histological categorization.

In situ carcinoma is further subdivided into ductal and lobular types, with ductal carcinoma *in situ* (DCIS) being more typically identified in breast cancer patients (90%) than lobular carcinoma *in situ* (LCIS), both of which are considered characters of increased breast cancer risk (Sharma et al., 2010). Both breast cancers are non-invasive because the cancer cells are restricted to the ducts and lobules and do not spread to other organs (Makki, 2015). Traditionally, DCIS has been separated into five subtypes based on architectural features of tumours: the comedo, cribriform, micropapillary, papillary, and solid (Malhotra et al., 2010; Eliyatkin et al., 2015; Makki, 2015). Other than morphological determination, DCIS is also graded into low grade, intermediate grade, and high-grade DCIS according to changes in tumour cell size, nucleus, presence of mitotic activity, and necrosis (Makki, 2015).

LCIS, on the other hand, is discovered when there is a significant rise in the number of cells within the breast milk gland (lobules) (Sharma et al., 2010). In general, this type of *in situ* tumour lacks distinguishing characteristics compared to DCIS, where LCIS is detected incidentally in breast specimens and is regarded as a risk factor for the development of invasive cancer. However, only a few markers have been used to distinguish DCIS from LCIS. LCIS lacks E-cadherin and B-catenin expression but

is positive for high molecular weight (HMW) keratin, whereas DCIS has both E-cadherin and B-catenin expression but is negative for HMW keratin (Makki, 2015).

Cancer cells that outgrow the duct and lobular walls and invade the surrounding fatty and connective tissue of the breast are invasive or infiltrating carcinoma. Invasive breast cancer is divided into non-specific ductal carcinoma, and specific subtypes, where the specific type has distinct characteristics and the non-specific type does not (Eliyatkin et al., 2015). Invasive carcinoma accounts for 20-25% of all breast cancer cases, the most prevalent being lobular, tubular, papillary, and mucinous tumours. **Figure 2.7** depicts the classification of invasive carcinomas as tubular, ductal lobular, invasive lobular, infiltrating ductal, mucinous, medullary, and infiltrating ductal (Malhotra et al., 2010).

2.1.2(b) Molecular classification of breast cancers

Breast cancer is a heterogeneous disease in which histological testing alone is insufficient in determining the precise characteristics of cancer cells. Due to its heterogeneity entity, Perou *et al* proposed a terminology, “Molecular Classification”, for the first time with an extensive gene expression profiling study that differentiates breast cancer into five molecular subtypes: luminal A, luminal B, basal-like, normal-like, and HER2-like (Cadenas, 2012; Perou et al., 2000). Breast cancer subtypes have their unique hallmark and luminal A is known to be positive ER/PR or HER2 negative. This kind accounts for half of all invasive breast cancers and comprises a variety of low-grade forms such as tubular and cribriform carcinoma. With a good prognosis, this tumour is sensitive to endocrine manipulation but less sensitive to cytotoxic agents. Luminal B breast cancer, on the other hand, has ER/PR positivity but variable

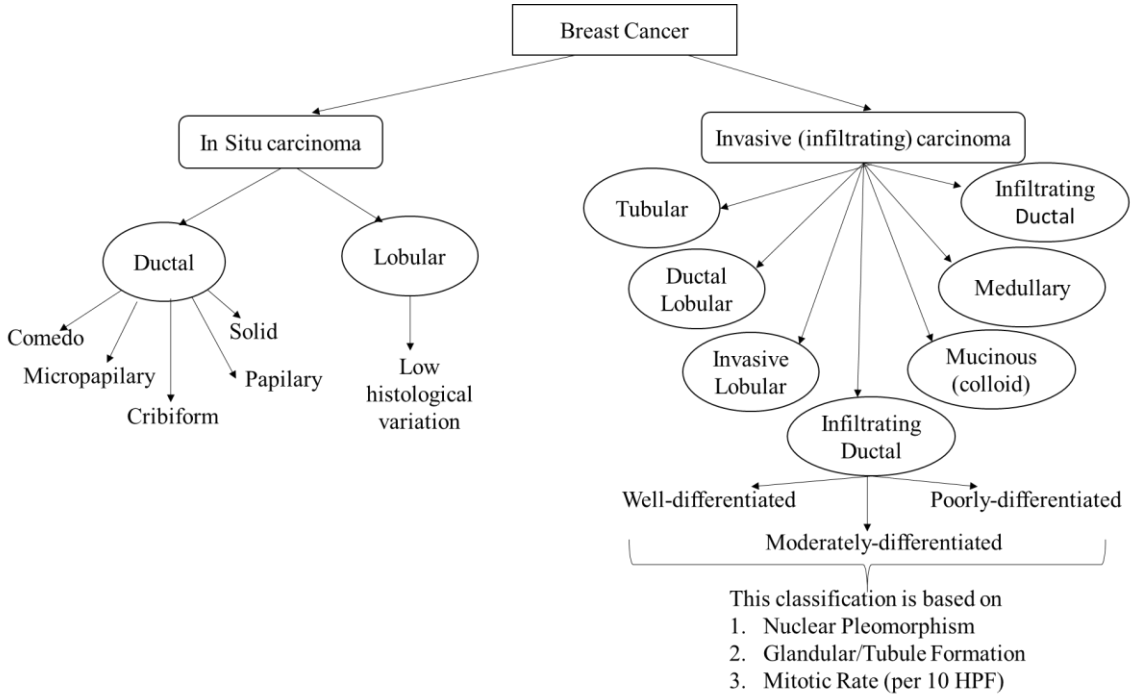


Figure 2.7 Histological classification of breast cancer subtypes. Adapted from Malhotra et al., 2010.

HER2 expression (positive or negative). This cancer subtype has a higher rate of proliferation than luminal A, which has a 14% expression of Ki-67. Luminal B has a worse prognosis than luminal A due to its high proliferation rate, and its response to chemotherapy can vary (Kittaneh & Montero, 2013; Makki, 2015).

Following that, basal-like tumour cells account for 15% of invasive ductal breast cancers. The name is derived from the expression pattern comparable to basal epithelial cells and normal myoepithelial cells in mammary tissues. These tumours are typically ER negative, PR negative, HER2 negative, and “triple-negative” due to the absence of all receptors; however, triple-negative and basal-like are not synonymous. Because all receptors are absent, these tumours do not respond to endocrine therapy but are responsive to platinum-based chemotherapy and have a poor prognosis. Similarly, because this type is poorly understood, normal-like breast cancer accounts for around 5-10% of all breast carcinomas. This kind is divided into intrinsic subtypes, including fibroadenomas and normal breast samples. Furthermore, this type of tumour cell expresses adipose tissue gene characteristics, providing an intermediate prognosis between luminal and basal-like malignancies, and does not usually respond to treatment. The same can be said for this type, called "triple-negative", since the cancer cells lack ER, PR, and HER2 expression. There have only been a few studies on this subtype, and the clinical significance of this cancer cell has yet to be determined. Some researchers assume this type is simply an artefact of excessive contamination with normal tissue during microarrays (Tang & Tse, 2016; Yersal & Barutca, 2014). Finally, HER2 overexpression accounts for 15% of all invasive carcinoma cases. By definition, HER2 expression is strongly positive, whereas ER/PR expression is often negative. Because of their strong HER2 expression, these cells are associated with high-grade tumours with lymph node metastases and typically have bad prognoses.

However, these cancer cells are more sensitive to trastuzumab (Herceptin) therapy (Makki, 2015; Yersal & Barutca, 2014). The molecular or intrinsic classification of breast cancer is illustrated in **Figure 2.8**.

2.1.3 Aetiological factors of breast cancer

The aetiology of breast cancer remains unknown until today, owing to the sporadic occurrence, despite studies indicating that 10-15% are genetically connected (Van Der Groep et al., 2006; Saslow et al., 2007). The study of breast cancer risk factors has been ongoing since 1950, as mortality rates among Japanese-Americans in the United States (US) continue to rise (Buell and Dunn, 1964; Vakil and Morgan, 1973). A myriad of interrelated factors has been associated with the increase or decrease of breast cancer risk, where the established risk factors include genetic mutations, age, family history, alcohol consumption, smoking, reproductive history, and postmenopausal hormone therapy (HT), etc.

2.1.3(a) Family history/genetics

Researchers once considered family history/genetics in triggering cancer incidence as one of the most potent cancer determinants. Women who have a first-degree relative with breast cancer, such as a mother or sister, have nearly twice the risk of developing breast cancer, and the risk is triple if both her mother and daughter have breast cancer (Mansfield, 1993; Sibio, Abriata, Buffa, Viniegra M Forman D, 2016). Furthermore, women with one, two, or three affected first-degree relatives had risk ratios of 1.80, 2.90, and 3.90, respectively, compared to women with no affected relatives (Shah et al., 2014).