DEVELOPMENT OF ORTHOTOPIC BREAST CANCER AND DETECTION OF nNav1.5 SERUM ANTIBODY IN SYNGENEIC MOUSE MODEL

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by

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CERTIFICATE

This is to certify that the dissertation entitled "*Development of Orthotopic Breast Cancer and Detection of nNav1.5 Serum Antibody in Syngeneic Mouse Model"* is fide record of research work done by Ms Aina Akmal binti Mohd Noor during the period from February 2019 to July 2019 under my supervision.

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a while for any other masters at Universiti Sains Malaysia or other instituitions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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LIST OF SYMBOLS AND ABBREVIATION

α	Alpha
ANOVA	Analysis of variance
BALB/c	Bagg albino (inbred research mouse strain) genotype c
BALB/cfC3H	High-mammary-tumour-incidence mouse strain
β	Beta
CD	Cluster of differentiation
COO^-	Cobalt(II) oxide anion
ER	Oestrogen receptor
γ	Gamma
G	Gauge
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
HER-2/neu	Human epidermal growth factor receptor 2 protooncogene Neu
IACUC	Institutional Animal Care and Use Committee
IFM	Interfibrillar
kDa	Kilodaltons
LA-site	Local anaesthetic site
MCF-7	Michigan Cancer Foundation-7
MDA-MB-453	M.D. Anderson Metastasis Breast cancer (human breast cancer cell line
N	Population size
n	Sample size

NH_2	Azanide
NH_3^+	Ammonia cation
p	“petit” short arm of the chromosome
pH	Potential of hydrogen
p53	Tumour protein
SCN5A	Sodium voltage-gated channel α-subunit 5 gene
SD	Standard deviation
sp	Species
U.S.	United States
%	Percent
˚C	Degree celsius
cc	Cubic centimetre
g	Gram
L	Litre
µl	Microliter
mg/kg	Microgram per kilogram
mm	Millimetre
mm^3	Cubic millimetre
nm	Ultraviolet range
rpm	Revolutions per minute

PENGHASILAN KANSER PAYUDARA ORTOTOPIK DAN PENGESANAN SERUM ANTIBODI nNAV1.5 DALAM MODEL MENCIT SINGENIK

ABSTRAK

Fungsi "voltage-gated sodium channels" (VGSC) adalah untuk menghasilkan mekanisma potensi tindakan dalam sel aktif dan menggalakkan metastasis dalam sel kanser. Isoform VGSC, Nav1.5 dan varian sambungannya, neonatal Nav1.5 (nNav1.5) telah meningkat pada peringkat protein untuk membantu migrasi and pencerobohan sel kanser terutama dalam kanser payudara metastatik. nNav1.5 ialah gen kawalan pengembangan didapati meningkat dalam tisu neonatal tetapi tidak dalam tisu dewasa dan diklasifikasikan sebagai antigen berkaitan tumor (TAA). Namun, masih tiada kajian terhadap kewujudan antibodi terhadap nNav1.5 dalam kanser payudara; pesakit atau model tumor. Oleh itu, kajian ini bertujuan untuk menghasilkan kanser payudara daripada kultur sel 4T1 karsinoma payudara mencit dan mengesan serum antibodi nNav1.5 menggunakan ELISA. Sel 4T1 telah disuntik secara ortotopik ke dalam ruangan subkutaneus tisu lemak payudara mencit BALB/c untuk menghasilkan model singenik. Kejayaan perkembangan tumor payudara dapat dilihat bersama dengan penurunan berat badan, kelesuan dan keadaan bulu tidak normal secara signifikan oleh mencit. Disebabkan oleh pertambahan saiz tumor payudara, mencit yang teraruh ini telah mencapai titik akhir neoplasia pada hari ke 25 dan dikorbankan. Penilaian ELISA ke atas serum haiwan mendapati serum antibodi nNav1.5 tidak dapat dikesan dalam mencit normal dan teraruh kanser payudara. Ianya merupakan satu penemuan yang menarik di mana kedua serum tidak menunjukkan perbezaan signifikan walaupun peparu mencit teraruh kanser payudara terdapat tompok putih yang menandakan metastasis melalui hematogenous telah berlaku. Kajian terkini telah mencadangkan

bahawa serum antibodi nNav1.5 dalam sel tumor payudara berpotensi untuk menampilkan kesan penyamaran dan/atau mekanisma perlepasan. Oleh itu, hasil perolehan kajian telah menghasilkan pengetahuan awal yang berguna terhadap tingkah laku nNav1.5. Walaubagaimanapun, kajian masa hadapan perlu dilakukan bagi menghuraikan mekanisma tepat berikutan dapatan kajian terkini.

DEVELOPMENT OF ORTHOTOPIC BREAST CANCER AND DETECTION OF nNav1.5 SERUM ANTIBODY IN SYNGENEIC MOUSE MODEL

ABSTRACT

The functions of voltage-gated sodium channels (VGSCs) are to propagate action potential mechanism in excitable cells and promote metastasis in cancer cells. VGSCs isoform, Nav1.5 in its splice variants, neonatal Nav1.5 (nNav1.5) is upregulated in protein level to mediate cancer cell migration and invasion particularly in metastatic breast cancer. nNav1.5 is a developmentally regulated gene – shown to be abundant in neonatal tissue but not in adult tissue and is classified as tumour associated antigen (TAA). However, no study has investigated the presence of antibody against nNav1.5 in breast cancer; patients or tumour model. Hence, this present study aimed to develop breast cancer mouse model from cultured murine breast carcinoma 4T1 cells and to detect nNav1.5 serum antibody using ELISA. 4T1 cells were inoculated orthotopically onto the subcutaneous mammary fat pad of BALB/c mice to produce a syngeneic model. The successfully mammary tumour development could be observed concurrently with significant weight loss, lethargic response and abnormal coat condition of the mice. Due to the increased size of the mammary tumour, the breast cancer mice reached its neoplasia endpoint at day 25 and humanely euthanised. The ELISA assessment of the animal serum revealed that nNav1.5 serum antibody was not detected in both normal and breast cancer mice. It was an interesting finding that both tested sera showed no significant difference despite the lungs of the mammary tumour mice exhibited white patches suggesting that metastasis via haematogenous route had occurred. The current findings suggest that the nNav1.5 serum antibody within breast cancer cells potentially depicted a masking effect and/or an escape mechanism.

Therefore, the findings obtained could have generate useful preliminary knowledge in regards to the behaviour of nNav1.5. However, future investigations should be put forward to further delineate the exact mechanism that lies behind these current findings.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Breast cancer is a build-up of a tissue mass or a tumour acquired in a breast (National Breast Cancer Foundation, 2016). The disease is induced by various factors such as improper diet, genetic mutation, smoking and prolonged toxic or chemicals exposure (Seymour *et al*., 2013). Breast cancer incidence and mortality rate are rapidly escalating, affecting particularly women on a global scale. The World Health Organization (WHO) has estimated that breast cancer is responsible for about 11.6% of cancer cases and 6.6% of overall cancer deaths in 2018. Whereas in Malaysia, breast cancer in women is the top accounted for cancer at 17.3% from a total of 7593 new cancer cases (GLOBOCAN, 2018). The trend of breast cancer incidence is in ascending manner as the incidence rate observed from the year 2007 to 2011 is steadily increased. It ranks as the highest common cancer cases with 17.7% overall in Malaysian residents (Malaysian National Cancer Registry Report, 2011).

With all the data presented, it can be summarised that breast cancer can prove fatal due to its outstanding characteristics. Cancer can acquire capabilities in surviving itself within the host. Since it has maintained self-sufficiency in growth signals, it can achieve insensitivity towards anti-growth signals with limitless replicative potential, hence proceeding the mechanism of apoptosis. In the malignant state, the extensive build-up of cancer cells sustains angiogenesis which is the formation of new blood vessels for continuous oxygen and nutrients supplies for the cells (Hanahan and

Weinberg, 2000; Fouad and Aanei, 2017). Concurrently, these invasive tissues can enter the nearby blood vessels by breaking apart with its pseudopods from the primary site. It migrates to other tissues or organs *via* the bloodstream, hence potentially damaging the residential tissues as it is deposited. Through this mechanism, metastasis occurs which is the development of secondary malignant cancer cell (Chamber *et al*., 2002; Beheshti *et al*., 2017). However, once the cancer cells enter the blood stream, they are prone to cellular death due to several mechanical stress and through tumour immune surveillance (Mehlen and Puisieux, 2006). To overcome these limitations, cancer cells strategically can depict a masking effect and/or escape mechanism for self-survival within the blood vessels (Gout and Huot, 2008; Beatty and Gladney, 2015).

As evidenced by the all statistics collected, it is imperative to promptly establish multiple alternatives in developing therapeutic advancements in diagnosing and alleviating the breast cancer burden. Currently, the available treatments for breast cancer are surgery, radiotherapy, chemotherapy, endocrine (hormone) therapy and targeted therapy (Dhankhar *et al*., 2010). However, these therapy methods have certain limitations. As significantly seen in chemotherapy for instance, hair loss, gastrointestinal disturbances, neutropenia and depressed immune systems are some of the most common negative consequences (Lemieux *et al*., 2008). The constant drawbacks urgently demand the development of the selective drug-delivery system and novel treatment carriers towards safer and more effective breast cancer treatment strategies. Hence, immunotherapy of active targeting is further established with an idea of conjugating a targeting moiety such as a protein or an antibody to a nanoparticulate system. This approach is based on the specific interaction of ligand-receptor and antibody-antigen concepts (Dhankhar *et al*., 2010). There are four main types of

immunotherapies: monoclonal antibodies, immune checkpoint inhibitors, cancer vaccines and nonspecific immunotherapy (Stanculeanu *et al*., 2016). Hence, this preliminary study is intended to investigate the potential of sodium channels in regards to breast cancer immunotherapy.

Sodium channels are integral membrane proteins that form ion channels at the plasma membrane for action potential mechanism. Its superfamily of cation channel is named as voltage-gated sodium channels (VGSCs). They are found abundantly in excitable cells such as neurons, myocytes, and certain types of glia. However, VGSCs are still expressed poorly in non-excitable cells basically in progressing metastasis, in this case, breast cancer cells (Catterall, 2005; Black and Waxman, 2013).

VGSC is composed of an alpha (α) subunit and two beta (β) subunits with different functions (Kandel *et al*., 2000; Hille, 2001). In the α subunit structure, it has four repeated homologous transmembrane domains which are termed as D1 through D4. These transmembranes assembled symmetrically to form a pore. Each domain contains six membrane-spanning segments which are termed as S1 through S4. This formed sodium (Na) ion channel controls physiologically by voltage (V), hence the name "Nav" subscript. The genetic code of the subfamily using this subscript is named as Nav1 and its definite ion isoform as Nav1.1. Overall, the total of gene codes for VGSCα is named as Nav1.1 through Nav1.9 (Ertel *et al*., 2000; Goldin *et al*., 2000; Onkal, 2011). The gene encoded for Nav1.5 is SCN5A at chromosome 3p21 (Brackenbury *et al*., 2007). In breast cancer, Nav1.5 predominantly exists in its 'neonatal' splice variant known as nNav1.5. It is the post-translational modification product and has alternative splicing of exon 6 at D1:S3/S4. Subsequently, it produces

seven amino acid differences from its adult form, Nav1.5 at the extracellular structure (Diss *et al*., 2004; Chioni *et al*., 2005).

Specifically, in the late stage of human breast cancer, the VGSCs expression and activity of nNav1.5 are highly proliferated regardless the fact that nNav1.5 would normally be expressed only during foetal growth (Fraser *et al*., 2005; Ben-Porath *et al*., 2008). It has been found that nNav1.5 can promote breast cancer invasion *via* altering the pH regulation and cell signaling cascade since it is overexpressed by oncofoetal gene expression (Koltai, 2017; Rhana *et al*., 2017). Since Nav1.5 is present in both excitable and non-excitable (e.g. diseased) cells, it is considered as the tumour-associated antigen (TAA). The statement is further proven with the fact that breast cancer definitely can be caused by genetic mutation such as p53 gene mutation (Duffy *et al*., 2018). Therefore, by provoking the immune system, immunotherapy treatment is possible by defining the technique of targeting TAA to potentially attack or kill cancer cells.

Yamaci *et al*., (2017) stated that nNav1.5 antibody is a novel biomarker of metastatic breast cancer. However, it is important to take note of the possible background mechanism of the breast cancer cells to which can lead to the understanding of circulating nNav1.5 antibody in the serum, particularly in this current preliminary study.

1.2 Problem Statement

By a metastasis cascade, the breast cancer cells can invade to another distant area in the body to form the secondary tumour (Fouad *et al*., 2017). In proving that

nNav1.5 is a potential biomarker for the disease, previous studies had shown that nNav1.5 activity is highly expressed in the breast cancer tissues of the animal model (Fraser *et al*., 2005, Kocatürk and Versteeg, 2015). Hence, the blood circulatory system is one of the definite routes for these cancer cells to travel within the body. Therefore, this present study is intended to utilise the serum of the breast cancer of the living system instead of breast cancer tissues.

However, due to mechanical stress and immune surveillance system of the body, the cancer cells can be inevitably destroyed. This leaves approximately 0.01% of the overall survived cancer cells in the circulation (Mehlen *et al*, 2006; Bacac and Stamenkovic, 2008). These cancer cells can be very strategic to modify the cellular invasion and adapt itself to the different microenvironment. Therefore, multiple possible escape mechanisms and/or masking effects are delineated by the cancer cells (Gout *et al*., 2008; Beatty *et al*., 2015).

There are many strategic cascades for the cancer cells to survive in the body which is not fully discovered yet that is important for the successful tumour metastasis process. To date, no experiment has been reported in exploring the immunogenicity of nNav1.5 in the living system, which leads to the unknown possible exact escape mechanism of the breast cancer cells. Furthermore, the serum of the breast cancer of animal model is not yet being tested to confirm the presence of nNav1.5 despite having an ability to form metastasis in other organs.

1.3 Scope of the Study

Previously, Yamaci *et al*., (2017) had apprehended that nNav1.5 is the recent biomarker for breast cancer. To the best of our knowledge, there is no study reported in detecting the presence of nNav1.5 antibodies in the serum of the breast cancer animal model. Breast cancer cells from the primary site, which commonly is the mammary glands, can produce the secondary tumour elsewhere. Certainly, the blood circulation is one of the paths for these cancer cells to be metastasised (Hanahan *et al*., 2000; Feng *et al*., 2018). However, considering that the breast cancer cells can perform a masking effect and/or an escape mechanism, it is very likely that nNav1.5 is unable to be detected. Therefore, our present study is implementing the idea of detection of nNav1.5 in the serum of orthotopic breast cancer mouse model. Any possible mechanisms can be outlined when the absence of nNav1.5 in the serum is accurately confirmed.

In depicting this proposition, this study utilised an animal model; BALB/c mouse to mimic the late stage of breast cancer in a human by inoculating 4T1 cell lines onto the healthy mammary fat pad of the mouse. This cell line, which is an aggressive murine mammary carcinoma cell, was used since it is highly metastatic and easily transplanted orthotopically (Pulaski *et al*., 2001). The serum was further analysed once the blood was sampled from all mice. Based on the understanding of cancer cell metastasis and in blood circulation invasion, nNav1.5 antibody detection from serum in the current experiment was conducted using enzyme-linked immunosorbent assay (ELISA) technique.

Therefore, the results obtained from this study are expected to generate useful potential preliminary knowledge and information which could contribute to some advantageous in breast cancer research. The confirmation of nNav1.5 presence/absence in the serum can be precisely confirmed. Furthermore, a masking effect and/or escape mechanism can be taken into serious consideration by using the findings of this study.

1.4 Research Objectives

1.4.1 General Objective

To develop the orthotopic breast cancer and to detect nNav1.5 serum antibody in syngeneic mouse model.

1.4.2 Specific Objectives

1) To culture highly metastatic murine breast carcinoma 4T1 cell.
2) To induce orthotopic breast cancer using 4T1 cell line onto female BALB/c mice.
3) To assess the effects of breast cancer on general health, behaviours, body weight, morbidity and mortality counts on mice.
4) To examine breast cancer tissues and target organs from the breast cancer mice.
5) To detect the nNav1.5 antibodies in serum of normal and breast cancer mice using ELISA.

1.5 Hypothesis

1.5.1 Null Hypothesis

nNav1.5 serum antibodies could not be detected in the breast cancer mouse model.

1.5.2 Alternative Hypothesis

nNav1.5 serum antibodies could be detected in the breast cancer mouse model.

1.6 Significance of the Study

The findings of this study can provide preliminary knowledge of nNav1.5 antibodies in the serum of breast cancer mouse model. As it is a potent breast cancer metastasis marker, it can be implemented as a future guideline for rather advanced research in developing immunotherapy target in diagnosing and treating the disease. This approach is reasonable because nNav1.5 is a type of tumour-associated antigen (TAA) due to certain inherent conditions. Such novel therapeutic and prophylactic target can be established as one of the methods in reducing the progression of breast cancer invasion in clinical understanding.

Moreover, it is important to know the potential invisible masking effect and/or an escape mechanism of nNav1.5 in the serum if it is truly unable to be detected. It is interesting since the statement is contradicting to the fact that breast cancer cells can definitely travel in the blood to metastasise to the distant areas. In achieving this goal, it is vital to accurately confirm the presence/absence of nNav1.5 in the serum of the breast cancer animal model.

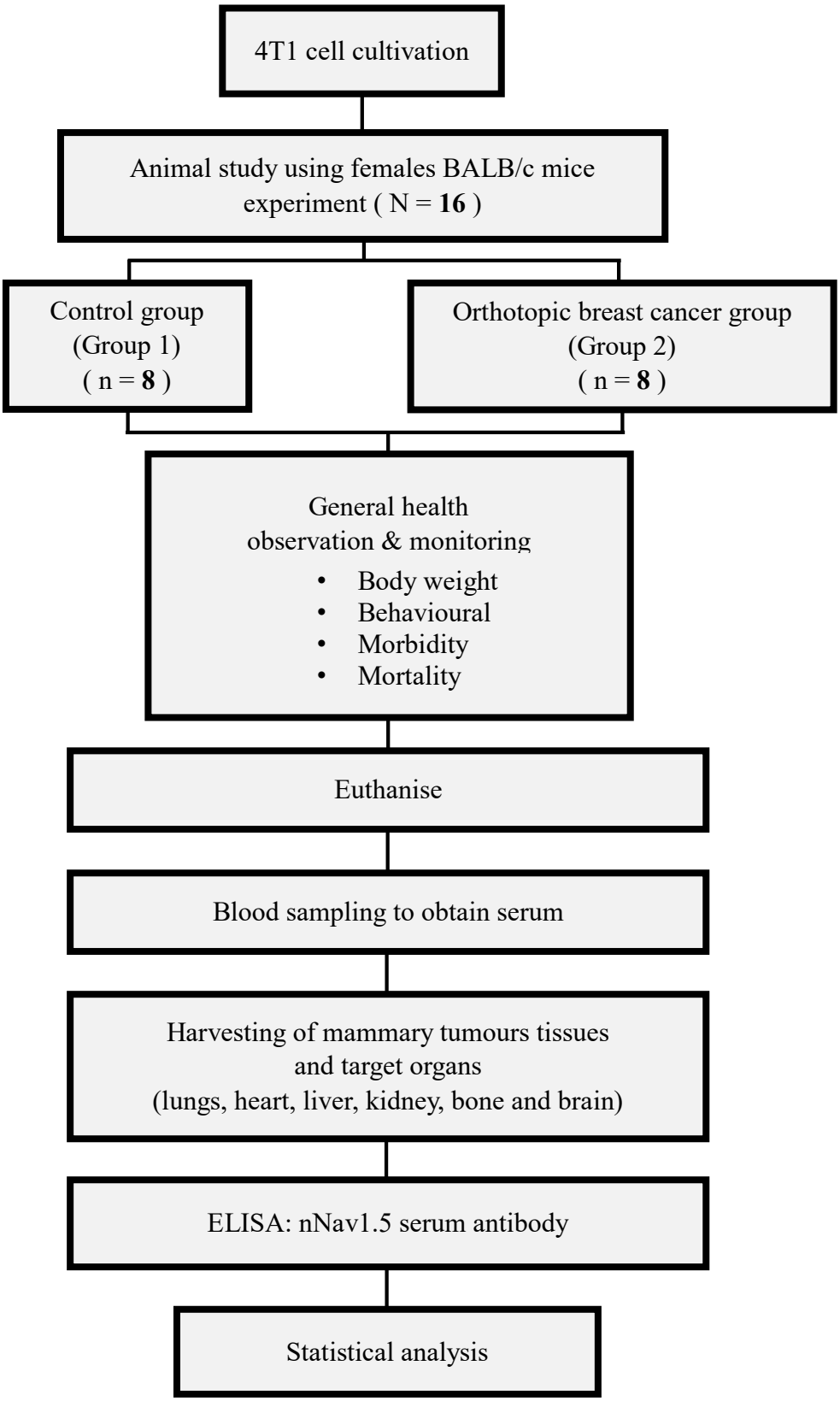

Figure 1.1 Experimental study design.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer

The mammalian breast is a collective of glandular and stromal tissues which is located in front of the chest wall as illustrated in Figure 2.1. The glandular tissues, which contain about 15 to 20 lobules, are circularly positioned for milk manufacturing and the ducts are responsible for the milk passage. The stromal tissues are made up of breast adipose and fibrous connective tissues. The lymphatic tissues intensively surround the breast primarily for removing cellular fluids and waste. The breast lies on the pectoralis major muscle supported by numerous ligaments attached. The breast size and shape depends on the collective deposition of fat which covers the lobes (Sharma *et al*., 2010; Alkabban and Ferguson, 2018).

Breast cancer is a disease arise from some neoplasm or abnormal growth of mutated cells from breast tissues which prone to self and unrestrained mitosis. This condition is usually initiated in one or two sites from the ducts in the breast (Feng *et al*., 2018). At the early stage upon development, the signs and symptoms are rather asymptomatic. Certain bodily hormones and chemicals are able to promote tumour growth. Hence, atypical appearances such as a lump or thickening of the breast, fluid dripping, pulled in nipple and changes to the skin or the nipple indicated that the breast cancer has further progressed (Koo *et al*., 2017). Meanwhile, the breast pain is considered as an unusual symptom which only occurs 5% of the time (Alkabban *et al*.,

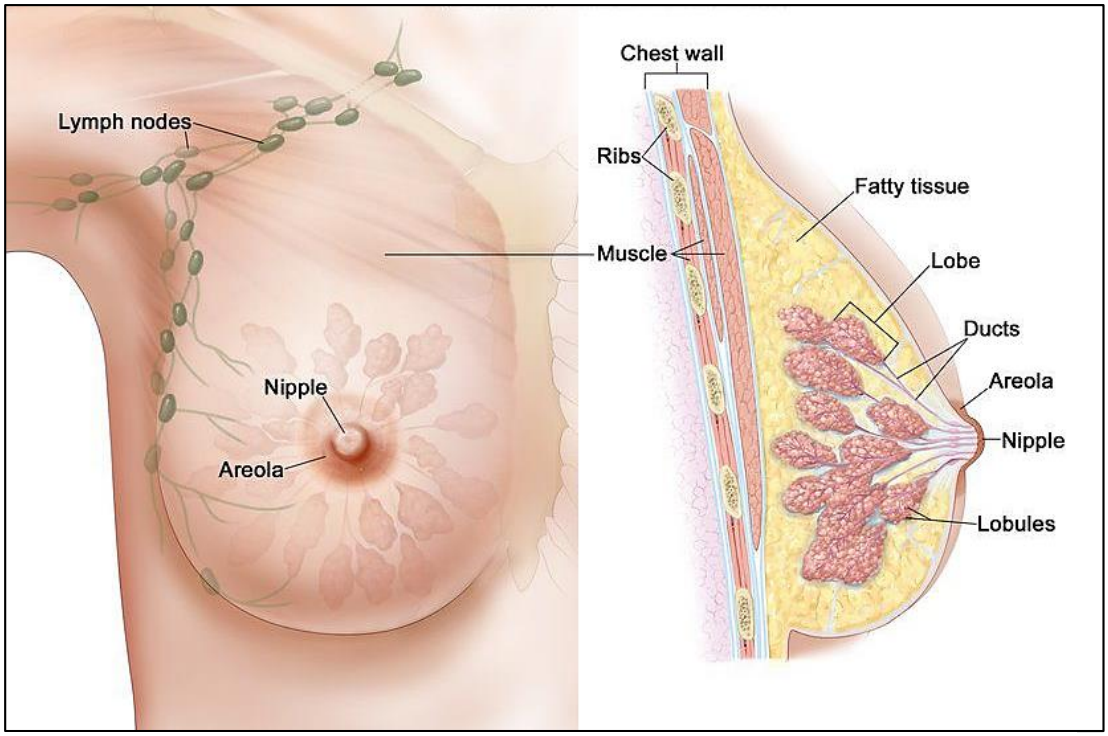

Figure 2.1 Anatomy of human breast. This image was adopted from Botesteanu *et al*., (2016).

2018). Anatomically, the adenocarcinoma of the cells is hyperproliferated often at the lining of the terminal duct lobular unit (Colditz *et al*., 2015). Breast cancer can appear in various types which are ductal carcinoma *in situ*, invasive ductal carcinoma, triple negative breast cancer, inflammatory breast cancer, metastatic breast cancer and breast cancer during pregnancy. Other rare types include medullary carcinoma, tubular carcinoma, mucinous carcinoma (colloid) and Paget disease of breast or nipple area. All the mentioned types differ in terms of the affected region, intensity and structural features (National Breast Cancer Foundation, 2016; Akram *et al*., 2017).

The exact etiology of breast cancer is unknown. However, studies have suggested that DNA damage and genetic mutations are associated to be the utmost causal factors in breast cancer development (Plantamura *et al*., 2018). The risk of having the disease is greater considering many factors such as age, early menstruation, family history, inherent histologic abnormalities, reproductive risk factors and the usage of exogenous hormones such as estrogens (Sun *et al*., 2017; Alkabban *et al*., 2018). Moreover, the lifestyle of smoking, drinking alcohol, obesity, breast implants, shift work factors, stress and diabetes mellitus are also contributed to high-risk factors of breast cancer. This is potentially due to extreme urbanization activity and western lifestyle (Dieterich *et al*., 2014). Consequently, carcinogenesis is stimulated when changes in epigenetic microenvironment in the tumour microenvironment has occurred as a result of mutated DNA methylation patterns (Bases and Arock, 2015).

Currently, breast cancer is routinely diagnosed by many methods such as breast self-examination (BSE), surveillance strategy, clinical assessment, mammography, sonography, ultrasound, magnetic resonance imaging (IMR), needle biopsy, HER-2/neu detection assay, blood-based assay and advanced technology (Roses, 2005; Nounou *et al*., 2015; Sun *et al*., 2017).

2.2 Breast cancer statistics

2.2.1 Worldwide statistics

Breast cancer is one of the leading causes of cancer worldwide. It is considered as a global burden since it can affect particularly women in both developed and less developed countries (Shah *et al*., 2014). Breast cancer can also occur in men, but it is 100 times more common in women than in men. About 1 in 1000 men will experience breast cancer during lifetime (Alkabban *et al*., 2018).

In 2018, breast cancer is ranked as the top three and fifth respectively in terms of incidence and mortality. Including lung and colorectal cancers, breast cancer encompasses one-third of the cancer incidence and mortality cases universally. Meanwhile, including lung cancer, breast cancer has caused approximately 2.1 million new cases which contributed up to 11.6% of the total cancer incidences. Due to the strong susceptibility of breast cancer occurrence especially in developed countries, this disease ranks at fifth from overall cancer-causing death at nearly 627 000 cases (6.6%).

If the statistics are observed from the female only, breast cancer is the most diagnosed cancer at 24.2% which accounts 1 in 4 of all new cases in 154 out of 185 countries worldwide. From overall cancer death, breast cancer ranks first at 15.0% (World Health Organization, 2018).

In a meta-analysis study done by Kaur (2018) based on GLOBOCAN 2018 database, the statistics for breast cancer calculated for the last 4 years had summarised that 31 out of 52 countries sampled have increased number of breast cancer incidences.

2.2.2 Malaysia statistics

Considering both genders in the year 2018, breast cancer ranks the first and second respectively in terms of overall new cancer cases (7593 cases; 17.3%) and mortality (2894 deaths; 11.82%). In the female only, the number of new breast cancer cases is the highest at 7593 (32.7%) of all ages (GLOBOCAN, 2018).

The increasing trend of breast cancer incidence can be seen from the incidence rate observed from the year 2007 to 2011. It ranks as the topmost cancer cases with 17.7% overall in Malaysian residents. The percentage of breast cancer detected at stage I and II are 56.9% and 17.7% respectively. Among female residents, it accounts 32.1% of all cancer cases in Malaysia (Malaysian National Cancer Registry Report, 2011).

2.3 Metastasis as the hallmark of cancer

With all the data observed, it can be concluded that breast cancer can lead to death due to its unique characteristics. Collectively, these characteristics are summarised as the hallmarks of cancer. Initial findings suggested that there are six main hallmarks; self-sufficiency in growth signals, insensitivity towards anti-growth signals, apoptosis evasion, angiogenesis sustainability, unlimited replicative potential and tissue invasion and metastasis (Hanahan *et al*., 2000). Exploration of new findings leads to the revision of another emerging hallmarks of cancer. The additional hallmarks of cancers are tumour promoting inflammation, genome instability and mutation, deregulation of cellular energetics, immune destruction avoidance and invasion and metastasis activation (Lhomond *et al*., 2015).

In this context, the highlighted hallmark of cancer is the metastasis of the cancer cells. It is the defining feature of malignancy whereby the cancer cells have potentials to invade surrounding tissues and travel to distant sites, hence developing secondary growth (Fouad *et al*., 2017). Cancer cells must complete a series of events called a metastasis cascade. This cascade involves six major mechanisms; invasion of extracellular matrix, intravasation into tumour vasculature, surviving transport in circulation, extravasation at parenchyma of distant organs, adaptation in changed microenvironment and colonisation (Valastyan and Weinberg, 2011; Massagué and Obenauf, 2016).

However, individual cancer cells which enter the circulation are inevitably vulnerable to harsh selective conditions which lead to cellular death. The most significant conditions are celullar mechanical stress and tumour immune surveillance (Mehlen and Puisieux, 2006; Alix-Panabières and Pantel, 2016). Consequently, only a minority of approximately 0.01% circulating cancer cells are able to survive in the vascular circulation to complete the metastasis cascade (Chambers *et al*., 2002; Bacac *et al*., 2008).

To resist the cellular death, strategic circulating cancer cells are proficient to intercorrelate between invasion approach and adaptation to the changed microenvironment. In devising adaptive techniques within the blood circulation, cancer cells are able to exhibit a masking effect and/or escape mechanism (Gout and Huot, 2008; Beatty and Gladney, 2015). Through these mechanisms, the cancer cells can remain intact within the circulation for extravasation pathways in developing secondary malignant growths (Fouad *et al*., 2017).

2.4 Current treatment of breast cancer

Although the risk reduction approaches of breast cancer are continuously ongoing, the latest reports have indicated that there is an increment of global statistics in the overall number of newly diagnosed cases. Immediate treatments are required in reducing this cancer burden. Fortunately, considering breast cancer as the most prevalent cancer, hence increase in survival rate is conceivable (Nounou *et al*., 2015).

Breast cancer grading is one of the significant strategies to determine the treatment planning and establish the likely prognosis (Chand *et al*., 2013). Grading the tumours help to determine the most effective treatment in general as a hypothesis depicts the lower the tumour grade, the higher the potential recovery. Nevertheless, full recovery is still achievable at each stage and even at the highest grade of aggressive tumours (Margolese *et al*., 2003). A TNM staging system is a superior method in diagnosing the tumour stage whereby tumour, nodes and metastasis intensities are assessed (Cserni *et al*., 2018).

Surgery is commonly preferable especially during the early stage of breast cancer development (Roses, 2005). Nonetheless, when the malignant cells are disseminated *via* extravasation, it produces new tumours as a secondary tumour which demand rather advanced treatments. Thus, higher breast tumour burden requires other available treatments such as radiation therapy (RT), chemotherapy (CT), endocrine (hormone) therapy (ET), and targeted therapy (Dhankhar *et al*., 2010).

However, some of the mentioned treatments can cause many harmful side effects under certain circumstances. Surgery for instance is only effective for a local removal of the breast tumour and the patient is obliged to undergo an adjuvant therapy

afterward as well as an optional breast reconstructive surgery. Patients are vulnerable to haematomas, wound dehiscence and necrosis post-surgery (Fraser *et al*., 2016). CT on the other hand has disadvantages whereby it leads to cardiotoxicity, hair loss, gastrointestinal disturbances, neutropenia and a depressed immune system in patients after treatment (Lemieux *et al*., 2008). Meanwhile, RT can provoke a decreased sensation in the affected region, local skin problems (soreness, itching, peeling, and/or redness) and moist skin condition at the end of the therapy (Akram and Siddiqui, 2012). Moreover, ET can elevate the risk of thromboembolic complications, endometrial hyperplasia and endometrial cancer in patients (Hirsimäki *et al*., 2002).

These continuous limitations urgently require the advancement in the selective drug-delivery system and new treatment with minimum negative consequences such as cancer immunotherapy. Immunotherapy is a treatment to artificially stimulate the immune system to fight cancer. Hence, the immune system can enhance its natural capability to treat cancer. This treatment is further improved in active targeting with a concept of associating a targeting moiety (such as a protein or an antibody) to a nanoparticulate system. The idea depends on the specific interaction of ligand-receptor and antibody-antigen concepts (Dhankhar *et al*., 2010). In normal settings, antibodies are bound to the pathogen antigens, hence eliminating the foreign substances in the body. Cancer cells can plausibly trick the immune cells by a certain mechanism to avoid immune surveillance. Therefore, modified immunotherapy antibodies can bind to this tumour antigen marking for the immune system to recognise and fight the cancer cells (Davis, 2000). The classification of immunotherapies are monoclonal antibodies, immune checkpoint inhibitors, cancer vaccines and nonspecific immunotherapy (Stanculeanu *et al*., 2016). Therefore, targeting the voltage-gated sodium channel

specific structures associated with breast cancer in immunotherapy is an alternative method in alleviating the burden of this disease.

2.5 Voltage-gated sodium channel

The cation channel at the plasma membrane of the cell is also known as VGSCs. It is majorly made up of one α subunit and two β subunits which function respectively as the ion conduction pore and channel gating modulation (Kandel *et al*., 2000; Hille, 2001). The α subunit as shown in Figure 2.2 is the main processing site with 260 kDa which connected to the peripheral β subunit with 33 to 39 kDa (Catterall, 2000). Sodium channels belong to this superfamily of cation channels along with other channels; voltage-gated potassium and calcium channels. Due to its outstanding recognition as a type of protein with an established amino acid sequence determination, only sodium channels are considered as the utmost functional member of its superfamily (Yu and Catterall, 2004). Sodium channels form integral membrane proteins which are assembled to build ion channels located within the plasma membrane of the cells. The overall function of voltage-gated sodium channels is for the action potential mechanism in which initiation and propagation occur in excitable cells such as myocytes, neurons and certain types of glia (Hille, 2001). However, VGSCs are still expressed poorly in non-excitable cells with amorphous physiological functions (Black *et al*.,, 2013).

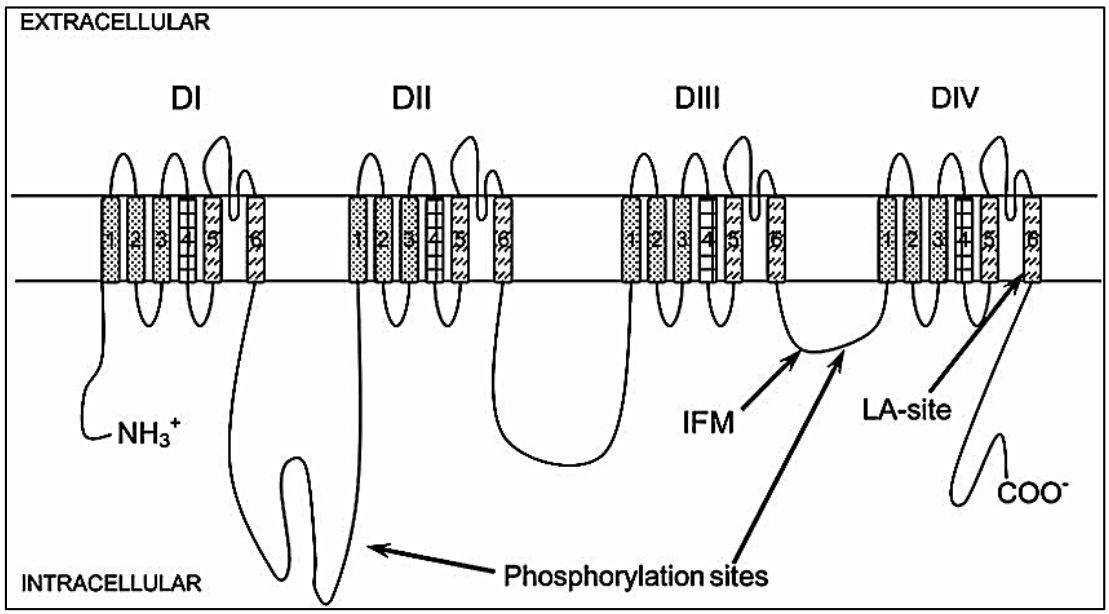

Figure 2.2 The α subunit structure of VGSC (Bölcskei *et al*., 2008).

When the VGSCα structure is observed from the collateral to the cell membrane surface, about 47% and 24% of its mass are located respectively at the intracellular and extracellular membranes. These collective structures take a shape like a bell when viewed in 3-dimensional (3D) system using helium cooled cryogenic electron microscopy and single-particle image analysis (Catterall, 2000; Sato *et al*., 2001). As the main processing site, the α subunit of the sodium channel contains four repeated homologous transmembrane domains which are named as D1 through D4. All domains are located in a symmetry revolving the middle hydrophilic pore which influenced the maximum efficiency of VSCSαs functionality (Yu and Catterall, 2003; Long *et al*., 2007). Moreover, each mentioned domain has six membrane-spanning segments which are named as S1 through S6. These segments are able to propagate outward the extracellular side of the plasma membrane when it is stimulated due to voltage exchange in the transmembrane. This condition allows the channel to be permeable to ions (Onkall, 2011). S1 to S3 of negatively-charged acidic residues are required in voltage-sensing to complement the charge changing of positively-charged S4 residues (Tombola *et al*., 2006; DeCaen *et al*., 2009; Catterall, 2010). Therefore, focusing on VGSCαs, it can be concluded that it has transmembrane regions of two segments repeated four times; a voltage sensing module (S1 to S4) and a pore-forming module (S5 and S6) (Swartz, 2008; Catterall, 2010).

Sodium channels indeed have a functional similarity with potassium and calcium channels. However, only the sodium channels are initially named without a fixed nomenclature system according to isoforms differences. Therefore, to overcome the uncertainty, researchers had established a standard nomenclature specially for VGSCs. This nomenclature has made the voltage-gated potassium channel nomenclature as its main reference (Chandy and Gutman, 1993; Goldin *et al*., 2000).

Hence, a numerical system is created to describe all the subfamilies with their subtypes according to the same amino acid sequences between the channels. This system states that the combination of a channel with its chemical symbol of the permeating ion and the primary physiological controller is labelled as one subscript. In this case, the channel with its ion is "Na" and the physiological controller is the "voltage". Collectively, it is named as a "Nav" subscript. Furthermore, this subscript can specify the genetic code of the subfamily such as Nav 1, hence its definite channel isoform according to its genetic code is Nav1.1. For the splice variants, it is written in lower-case letters after the subscript and numbers such that Nav1.1a (Ertel *et al*., 2000; Onkal, 2011).

Anyhow, by observing from the whole exposition, VGSCα genes have a total of nine genes named Nav1.1 through Nav1.9 which are located at chromosomes number 2, 3, 12 and 17 (Goldin *et al*., 2000). The genes related are named as SCN1A through SCN11A in which SCN6/7A genes have an unknown purpose. Exclusively for Nav1.5, it is encoded by gene SCN5A located at chromosome 3p21 (Brackenbury *et al*., 2007). In breast cancer, VGSCs can undergo operative upregulation as evidenced in both *in vitro* and *in vivo* studies (Brackenbury *et al*., 2007; Yamaci *et al*., 2017).

2.6 Nav1.5 and nNav1.5 variant in breast cancer

Normal excitable cells such as heart, brain and gastrointestinal smooth muscle express Nav1.5 significantly for the systemic action potential mechanism (Hille, 2001). However, Nav1.5 is also expressed in non-excitable cells practically in progressing

metastasis, in this case, breast cancer cells (Catterall, 2005; Mazzone *et al*., 2008; Gillet *et al*., 2009; Black *et al*., 2013). Due to these characteristics, Nav1.5 can be categorised as a tumour-associated antigen (TAA) since the antigens are expressed at both normal and tumour cells (Criscitiello, 2012). This statement is also supported by the fact that the antigen expression of the breast cancer cells is a result of mutated cellular genetic codes e.g. p53 (Duffy *et al*., 2018). Therefore, by stimulating the immune system, immunotherapy treatment is conceivable by outlining the technique of targeting TAA to promote the attack of immune cells to kill cancer cells.

In breast cancer, adult Nav1.5 sustains post-translational modification which produces its "neonatal" splice variant as its residue, hence the name; nNav1.5 (Marionneau and Abriel, 2015). This event mainly occurs in D1:S3/S4 structure with an alternative splicing site 1 of exon 6. The type of post-translational modification occurs is the aspartate substitution in Nav1.5 which produces either neutral or positive residues of nNav1.5 (Copley, 2004). Exon 6 is encoded by SCN5A gene as illustrated in Figure 2.3(a). In nNav1.5, exon 6 has 31 different nucleotides from a total of 91 nucleotides in an analog exon 6 of Nav1.5. As a result, the negatively-charged aspartate in Nav1.5 has changed to a neutral amino acid sequence (Plummer and Meisler, 1999; Diss *et al*., 2004). Hence, this event gives rise to seven amino acid changes in the extracellular loop of D1:S3/S4 region of the channel in nNav1.5 as shown in Figure 2.3(b) (Chioni *et al*., 2005). The 7^{th} positioned amino acid in the sequence is a positively-charged lysine instead of a neutrally charged residue. The remaining 6 amino acids in the sequence are located in the extracellular D1:S3/S4 linker region (Onkal *et al*., 2008) as seen in Figure 2.3(c). From these findings, Chioni *et al*., (2005) generated an anti-peptide polyclonal antibody named NESOpAb which selectively identify nNav1.5 often from the alternative splicing event in EBNA-293

cells. NESOpAb is tested to evaluate the developmental expression of nNav1.5 in mouse tissues by immunohistochemistry (Chioni *et al*., 2005; Onkal, 2011). Lastly, considering the alternative splicing of D1:S3 Nav1.5, operative expression of nNav1.5 is proved to be present in the human late stage of breast cancer (Fraser *et al*., 2005; Brackenbury *et al*., 2007; Gillet *et al*., 2009).

nNav1.5 can further elevate the invasiveness of breast cancer by changing the pH regulation system. Not only that, nNav1.5 overexpression by oncofoetal gene can alter the cell signalling pathway as well (Koltai, 2017; Rhana *et al*., 2017). These statements conclude that oncofoetal gene can upregulate nNav1.5 in breast cancer despite that nNav1.5 would usually be expressed only during foetal growth (Ben-Porath *et al*., 2008). Ultimately, cancer cells are able to enter the vascular system through the metastasis cascade, travel and proliferate at the other body parts (Chambers *et al*., 2002). Therefore, this current study is executed by referring to a concept of nNav1.5 as a new biomarker for metastatic breast cancer (Yamaci *et al*., 2017).