GLUTAMATE REGULATION OF VOLTAGE-GATED SODIUM CHANNELS (VGSCS) IN BREAST CANCER CELL LINES OF DIFFERENT METASTATIC POTENTIAL

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by

IRFAN IRSYAD BIN AZAHAR

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LIST OF ABBREVIATIONS AND SYMBOLS

[ER+|PR+]	tumours with either ER or PR positivity
[ER+|PR+] HER2-	tumours with either ER or PR positivity, and HER2 negativity
[ER+|PR+] HER2+	tumours with either ER or PR positivity, and HER2 positivity
× g	Times gravity
®	Registered
°C	Degree Celsius
AP	Ammonium persulfate
APM	Airborne particulate matter
AR	androgen receptor
ATCC	American Type Cell Culture
ATP	Adenosine 5'-Triphosphate
BBB	blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMU	Best Matching Unit
bp	Base pair
BRCA	Breast cancer gene
BSA	Bovine Serum Albumin
C	Cytidine
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CHGA	Chromogranin A
ChIP	Chromatin immunoprecipitation assay
CK	cytokeratins
CMB	Chemical Mass Balance
CMF	cyclophosphamide, methotrexate, 5-fluorouracil
CNS	Central nervous system
CO2	Carbon dioxide
CPCA	Consensus PCA
CSI	Class Separation Indices
CSM	Class Sample Matrix
CSV	Class Sample Vector
CTCL	Cutaneous T-cell lymphoma
CTCs	Circulating tumour cells
CTLA4	Cytotoxic T-lymphocyte associated antigen 4
CWM	Class Weight Matrix
CWV	Class Weight Vector
CypB	Cyclophilin B
D3	Domain 3
D4	Domain 4

dH2O	Distilled water
DMEM	Dulbecco‟s modified Eagle‟s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTCs	Disseminated tumour cells
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDC	Euclidean Distance to Centriods
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal Growth Factor
EGFR	epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assays
EMT	epithelial to mesenchymal transition
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GATA3	GATA binding protein 3
gDNA	Genomic DNA
GEP	gene expression profiling
GTPase	Guanosine triphosphates
HATs	Histone acetyltransferases
HCl	Hydrochloric acid
HDACs	Histone deacetylases
HEK293	Human embryonic kidney cells 293
HER2	human epidermal growth factor receptor 2
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HMTs	Histone methyltranferases
HR	hormonal receptors
hr	Hour
HRP	Horseradish peroxidase
IARC	International Agency for Research on Cancer
IC50	Half maximal inhibitory concentration
IDT	Integrated DNA Technologies, Inc.
IHC	immunohistochemistry
IPS	Institut Pengajian Siswazah
kb	Kilo base
kDa	Kilo Dalton
L	Litre
mA	Milliampere
MAC	ER-PR-AR+, also named molecular apocrine
MBC	metaplastic breast cancers
MEF2	Myocyte enhancer factor-2
MET	Mesenchymal-epithelial transition
mg	Milligram

miRNA	MicroRNA
ml	Milliliter
MMP2	Matrix metalloproteinase 2
MNCR	Malaysian National Cancer Registry
MoI	Motility index
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
mV	Millivolt
MW	Molecular weight
Na+	Sodium ion
Nav	Voltage-gated sodium channel
NCBI	National Center for Biotechnology Information
NCR	National Cancer Registry
NLS	Nuclear localisation signal
nm	Nanometer
nNav1.5	neonatal Nav1.5
NRSF	Neuron-Restrictive Silencer Factor
PBM	Protein binding microarray
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween-20
PCR	Polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
PR	progesterone receptor
PR	Progesterone receptor
qPCR	Quantitative Real-time Polymerase Chain Reaction
Rcf	Relative centrifugal force
RE1	Repressor element 1
REST	RE1-silencing transcription factor
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT buffer	Reverse Transcription buffer
SCN5A	Sodium channel, Voltage-gated, Type V, Alpha subunit
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELEX	Systematic evolution of ligands by exponential enrichment
SEM	Standard Error of the Mean
siRNA	Small interfering RNA
SNAP25	Synaptosome Associated Protein 25
TAE buffer	Tris-acetate-EDTA buffer
TEMED	N, N, N‟, N‟- tetramethylethylenediamme

TFBS	Transcription factor binding site
TIC	tumor initiating cell
TNBC	Triple-negative breast cancer
TOP2A	Topoisomerase II alpha
TRANSFAC	Transcription Factor database
TSA	Trichostatin A
TTX	Tetrodotoxin
™	Trademark
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Voltage
VEGF	Vascular endothelial growth factor
VGSC	Voltage gated sodium channel
VHL	Von Hippel-Lindau
VIM	vimentin
VRD	vitamin D receptor
ZEB1	Zinc finger E-box-binding homeobox 1
α	Alpha
β	Beta
β-actin	Beta actin
µ	Micro
µg	Microgram
µl	Microliter
µM	Micro molar

LIST OF APPENDICES

KAWALATUR GLUTAMAT OLEH *VOLTAGE-GATED SODIUM CHANNELS* (VGSCS) DI DALAM SEL BARAH PAYUDARA DENGAN KEUPAYAAN METASTASIS BERBEZA

ABSTRAK

Peningkatan ekspresi dan aktiviti *voltage-gated sodium channels* (VGSC), khususnya varian neonatal, nNav1.5, di dalam sel barah payudara manusia umumnya dikaitkan dengan keupayaan untuk bermetastasis. Pada masa yang sama, peningkatan kandungan sejenis neurotransmiter umum, glutamat pula didapati di dalam pelbagai jenis barah manusia, termasuk barah payudara. Ini menghasilkan satu kemungkinan wujudnya fungsi kawalatur oleh nNav1.5 terhadap kandungan glutamat dalam sel barah payudara yang mana kawalatur ini (VGSCs-glutamat) sememangnya berlaku dalam fisiologi normal sel neuron. Oleh itu, tujuan keseluruhan kajian ini adalah untuk mengkaji kawalatur nNav1.5 terhadap glutamat yang terdapat di dalam sel barah payudara serta pengaruhnya terhadap potensi metastasis. Asai glutamat dilakukan untuk membandingkan kandungan dasar glutamat di dalam dan luar sel barah, manakala pewarnaan glutamat untuk pengimejan mikroskopi sel dan *real-time* PCR dilakukan untuk membandingkan tahap mRNA nNav1.5 di antara sel epitelium payudara bukan barah (MCF-10A), sel barah payudara manusia yang kurang agresif (MCF-7) dan sel barah payudara manusia yang sangat agresif (MDA-MB-231). Oleh kerana MDA-MB-231 mempunyai kandungan glutamat dan tahap ekspresi mRNA nNav1.5 tertinggi, maka sel ini menjadi subjek untuk ujian perencatan nNav1.5 menggunakan agen seperti siRNA, perencat khusus VGSCs, iaitu tetrodotoxin (TTX) dan perencat penghasilan glutamat, iaitu riluzole (pada kepekatan IC_{50} yang diperolehi dari ujian pertumbuhan, MTT). Pertumbuhan sel dan keupayaan

bermetastasis sel pula diukur melalui ujian pertumbuhan MTT dan ujian invasi. Kandungan dasar glutamat dikesan di dalam ketiga-tiga sel dengan kandungan tertinggi dikesan di dalam sel MDA-MB-231 ($p < 0,05$). Ini disahkan juga oleh imej *flourescence* mikroskopi yang mana pewarnaan merah glutamat didapati paling menonjol di dalam sel MDA-MB-231. Ekspresi mRNA nNav1.5 dikesan di dalam ketiga-tiga jenis sel, dengan ekspresi tertinggi juga di dalam sel MDA-MB231 ($p < 0.01$). Seterusnya, mRNA nNav1.5 berjaya direncatkan menggunakan agen perencat siRNA di dalam sel MDA-MB-231, ($p < 0.01$), TTX ($p < 0.01$) dan riluzole ($p < 0.05$). Rawatan agen ini juga turut mengurangkan kepekatan glutamat di dalam dan luar sel; TTX ($p < 0.05$), siRNA ($p < 0.05$) dan riluzole ($p < 0.05$). Pengimejan mikroskopi glutamat menunjukkan penurunan pewarnaan (merah) glutamat bagi ketiga-tiga rawatan ($p < 0,05$). Menariknya, perencatan glutamat melalui riluzole, siRNA dan TTX membawa kepada penurunan yang ketara terhadap ekspresi mRNA biopenunjuk metastatik dari kumpulan matrix metalloproteinase, MMP1 ($p < 0.01$), MMP8 ($p < 0.01$), dan MMP11 ($p < 0.01$) menyokong pengurangan invasi sferoid 3D. Di dalam ujian invasi sferoid 3D, kadar invasi MDA-MB-231 yang tidak dirawat meningkat sehingga hari ke-3 dan kemudiannya mendatar sehingga hari ke-7. Bagaimanapun, bagi sferoid 3D MDA-MB-231 yang dirawat dengan agen perencat, diameter perimeter sepanjang 7 hari eksperimen mendatar, menunjukkan rawatan yang diberikan berjaya mengekang diameter perimeter ataupun berlaku pengurangan invasi. Kesimpulannya, tahap ekspresi nNav1.5 berhubung kait dengan kandungan glutamat dan potensi metastasis sel barah payudara. Paling utama, kajian ini adalah yang pertama menunjukkan wujudnya fungsi kawalatur nNav1.5 terhadap kandungan glutamat dan seterusnya potensi metastatik di dalam sel barah payudara.

GLUTAMATE REGULATION OF VOLTAGE-GATED SODIUM CHANNELS (VGSCS) IN BREAST CANCER CELL LINES OF DIFFERENT METASTATIC POTENTIAL

ABSTRACT

The upregulation of voltage-gated sodium channels (VGSC) expression and activity, specifically its neonatal splice variant, nNav1.5, in human breast cancer is commonly related to metastatic potential. Concentration of a common neurotransmitter, glutamate is also found to be high in multiple types of human cancers, including breast cancer. This raises the potential of regulatory control by nNav1.5 on glutamate in breast cancer cells as in normal neuronal physiology (VGSC-glutamate). Thus, the overall aim of the study is to investigate the regulatory control of nNav1.5 on the glutamate concentration in breast cancer cells and its metastatic potential. Glutamate assay was conducted to compare basal concentration of endogenous and exogenous glutamate, glutamate staining for microscopy imaging of cellular glutamate and real-time PCR was conducted to compare the mRNA of nNav1.5 between the non-cancerous breast epithelial cell line (MCF-10A), the less aggressive human breast cancer cell line (MCF-7) and the highly aggressive human breast cancer cell line (MDA-MB-231). Since MDA-MB-231 was found to have significantly highest glutamate concentration along with upregulation of nNav1.5 mRNA expression, the cells were subjected for nNav1.5 inhibition using agents; siRNA, VGSCs specific blocker, tetrodotoxin (TTX) and glutamate released inhibitor, riluzole (at IC_{50} concentration produced from MTT assay). Cell growth and metastatic potential of the cells were also assessed by MTT and invasion assay, respectively. Glutamate concentration was detected in all the three cell lines, where

the highest concentration detected in MDA-MB-231 cells ($p<0.05$). This was confirmed with fluorescence microscopy images showing prominent red staining in MDA-MB-231. nNav1.5 mRNA expression was also detected in all the three cell lines, again, with the highest expression in MDA-MB231 cells ($p<0.01$). The nNav1.5 mRNA expression was successfully knocked down using siRNA ($p<0.01$) in MDA-MB-231 cells, similarly, for MDA-MB-231 cells treated with TTX ($p<0.01$) and riluzole ($p<0.05$). Followed by significant reduction of endogenous and exogenous glutamate by TTX ($p<0.05$), siRNA ($p<0.05$) and riluzole ($p<0.05$) as confirmed by reduction of the red staining of glutamate by all the three treatments ($p<0.05$) through fluorescence microscopic imaging. Inhibition of glutamate by riluzole led to significant downregulation of mRNA expression of the metastatic biomarkers of the matrix metalloproteinase family, MMP1 ($p < 0.01$), MMP8 ($p < 0.01$), and MMP11 ($p < 0.01$) respectively, followed by suppression of 3D spheroid invasion by these agents. Accordingly, the untreated MDA-MB-231 3D spheroid invasion rate increased until day 3 and started to plateau. However, for the treated MDA-MB-231 3D spheroids, the perimeter diameter of the 3D spheroid plateaued throughout the 7 days experiments, indicating no observable invasion. In conclusion, nNav1.5 expression correlate with glutamate concentration and metastatic potential of breast cancer cells. Importantly here, this study showed for the first time a possible regulatory control of nNav1.5 on glutamate concentration and so does the metastatic potential of the cancer cell.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

It has been proven that voltage gated sodium channel (VGSC) expression in tumour correlates with poor prognosis in several types of cancers such as cancer of prostate, colon, cervical, ovarian, breast and melanoma (Patel and Brackenbury 2015). While VGSCs are actively involved in excitable cells, studies have recently shown that VGSCs play significant role in cancer aggressiveness - increased expression of VGSCs in human carcinomas (cancer of the epithelial origin) such as cancer of the breast, prostate and cervical cancer, among those that have been associated with aggressive phenotype *in vitro* and *in vivo* (Djamgoz et al. 2019; Litan and Langhans 2015; Patel and Brackenbury 2015).

There are more than nine isoforms of VGSCs have been reported and each isoform could have several other variants (Patel and Brackenbury 2015). Different isoform or variant has been shown to be associated with different type of cancer, but research now is showing that in carcinomas the most common isoforms to appear are the Nav1.5 and Nav1.7 (and their variants) as shown in **Table 1.1.**

The major VGSCs isoform expressed in breast cancer is Nav1.5 but predominantly in the form of its splice variant, ‘neonatal’ Nav1.5, nNav1.5 (Brackenbury et al. 2007; Dutta et al. 2018; Yamaci et al. 2017). In the earlier work, most investigation on confirming the role of Nav1.5/nNav1.5 in breast cancer involved TTX (at μM concentration, because Nav1.5/nNav1.5 are TTX resistant isoform) (Brackenbury et al. 2007) as well as using other VGSC modulators drugs

such as brevotoxins, batrachotoxin, α and β scorpion toxins (ScTx) and also local anaesthetic and anticonvulsants (Clare et al. 2000).

Table 1.1 Tissue and cancer expression of VGSCs (Patel and Brackenbury 2015)

Subtype	Gene	Tissue location	Cancer type
Nav1.1	SCN1A	CNS, PNS, heart	Ovarian
Nav1.2	SCN2A	CNS, PNS	Cervical, mesothelioma, ovarian, prostate
Nav1.3	SCN3A	CNS, PNS	Ovarian, prostate, small cell lung cancer
Nav1.4	SCN4A	Skeletal muscle	Cervical, ovarian, prostate
Nav1.5	SCN5A	Heart, brain, Uninervated skeletal muscle	Breast, colon, lymphoma, neuroblastoma, non-small cell lung cancer, ovarian, small cell lung cancer
Nav1.6	SCN8A	CNS, PNS, heart	Breast, colon, lymphoma, neuroblastoma, non-small cell lung cancer, ovarian, small cell lung cancer
Nav1.7	SCN9A	PNS, neuroendocrine cells, sensory neurons	Breast, cervical, lymphoma, mesothelioma, non-small cell lung cancer, ovarian, prostate
Nav1.8	SCN10A	sensory neurons	Prostate
Nav1.9	SCN11A	sensory neurons	Lymphoma, small-cell lung cancer

But later, when specific antibody against nNav1.5, NESOpAb was developed, the role of nNav1.5 in breast cancer was further defined (A.-M. Chioni et al. 2005b). Furthermore, using all the agents mentioned, not only downstream mechanisms regulated by nNav1.5 was revealed but also, the upstream mechanisms that regulate nNav1.5 was also elucidated (S. P. Fraser et al. 2014). Accordingly, exploitation of TTX in understanding the upstream regulation of nNav1.5 has been revealed to involve through mechanism that involved transcription initiation sites - VGSCs contains upstream promoter within a distinct cytosine-guanine (CpG) island for luciferase that expressed in tandem (Diss et al. 2008) e.g. the binding of estrogen to

nuclear transcription factor estrogen receptor through β-estradiol (E2) could amplify the current of VGSCs in breast cancer cells, MDA-MB-231 but was blocked by TTX treatment (S. P. Fraser et al. 2010).

However, this study was designed to investigate the downstream regulation of nNav1.5 in potentiating invasion of breast cancer. Previously, several mechanisms have been discovered such as pH regulation via sodium-hydrogen exchanger 1 (NHE1) – increased NHE activity reduced pH at the pericellular space which then induces proteolysis and eventually promote invasiveness (Brisson et al. 2011). Furthermore, reduced pH and the shifting of H+ efflux was also able to activate cysteine cathepsin expression and promote epithelial-to-mesenchymal transition (EMT), respectively, all of which led to potentiation of metastasis and invasiveness of breast cancer cells (Luo et al. 2020).

Due to the mentioned research, this study hypothesized that increase expression of nNav1.5 in breast cancer has a role in regulating glutamate concentration thus, promoting invasion in cancer cells. In lieu of the hypothesis, it came to light that other than through reduced pH or increase proteolytic enzymes activity to promote invasion, increased glutamate concentration has also been associated with cell invasion (Sontheimer 2008; Yi et al. 2019). This ability is especially true for neuronal cancers where increased glutamate led to excitotoxicity (like during brain damage) that promotes cell death/damages of the surrounding cells to allow tumour expansion (Noch and Khalili 2009; Ye and Sontheimer 1999). Increased expression and activity of VGSCs during excitotoxicity in brain damage have already been well-known, thus we expect the study to show the toxic effect of

glutamate in promoting agrresiveness *in vitro*. In testing the hypothesis, objective, and specific objectives below were conceived.

1.2 General objective

To investigate the role of VGSCs in the regulation of glutamate released by breast cancer cells and its effect on breast cancer aggressiveness. Overall study framework is shown in **Figure 1.1**. Specific objectives are as follows:

1. To measure and compare glutamate concentration of the weakly and aggressive breast cancer, MCF-7, MDA-MB-231 and the non–cancerous human breast epithelial, MCF-10A cell lines using glutamate assay and immunofluorescence staining
2. To assess the effects of TTX, riluzole and siRNA of nNav1.5 on MDA-MB-231 cells using glutamate assay and immunofluorescence staining
3. To assess the effects of TTX, riluzole and siRNA of nNav1.5 on MDA-MB-231 cells using glutamate assay and immunofluorescence staining

Design of the experiments involved for the study is detailed in the following schematic workflow:

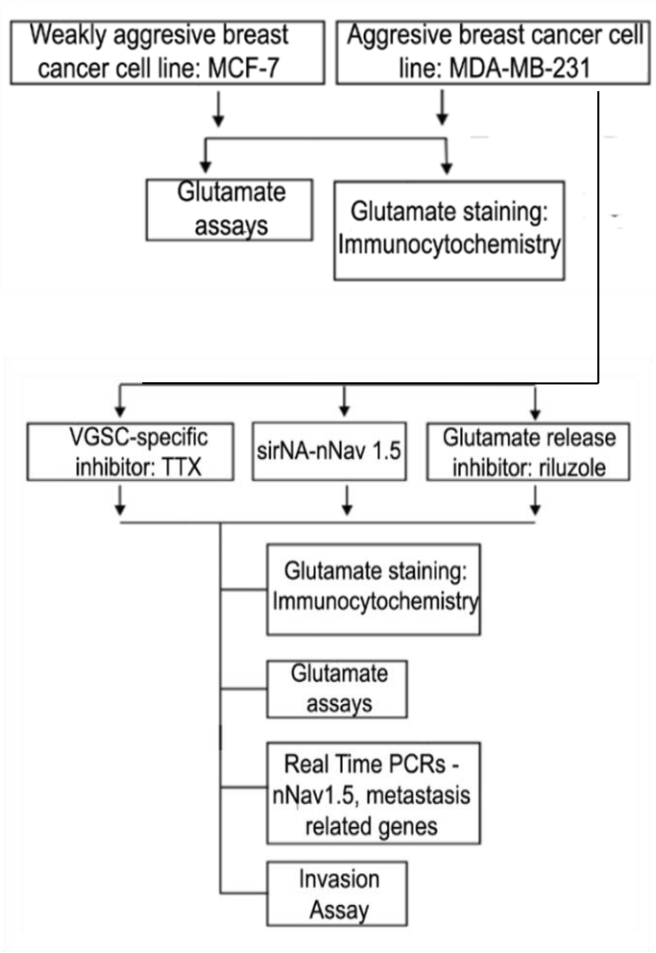


Figure 1.1 The flowchart of the experiments for this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer

The female breast comprises of mammary glands (lobules) and ducts, acts mainly as milk producing organ (**Figure 2.1**), is the most prone organ to cancer. Breast cancer is the abnormal growth of cells that develops from the breast tissue and falls under the category of epithelial-derived cancer – carcinoma (National Cancer Institute 2014).

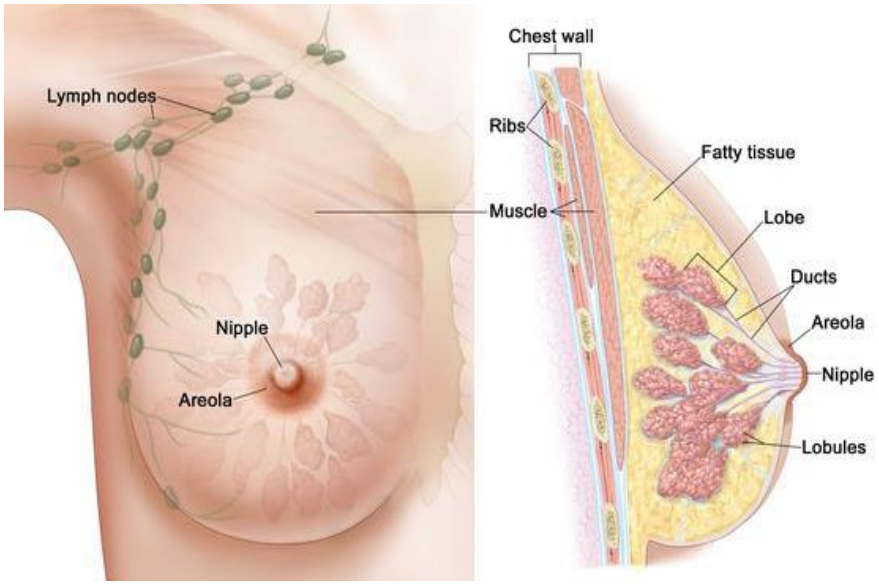
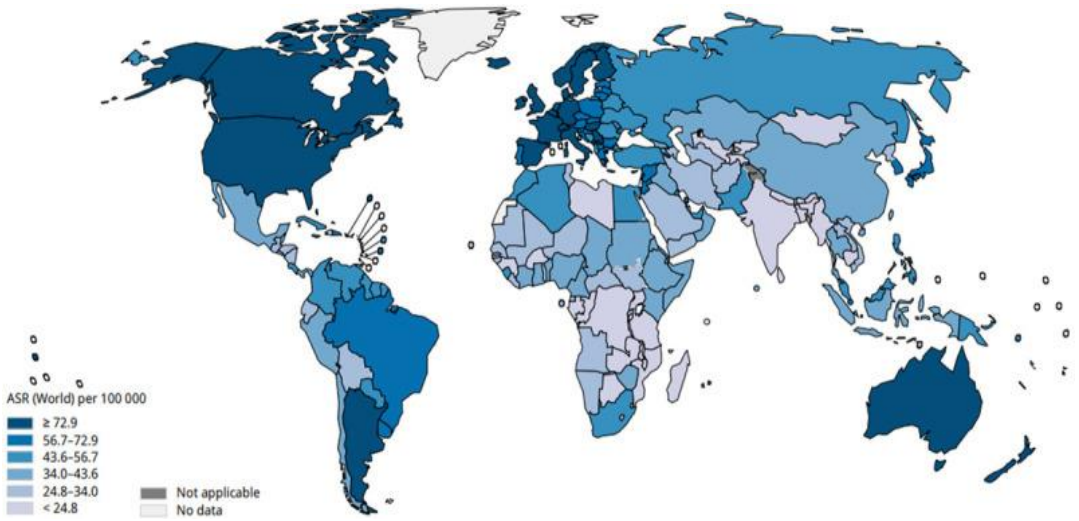


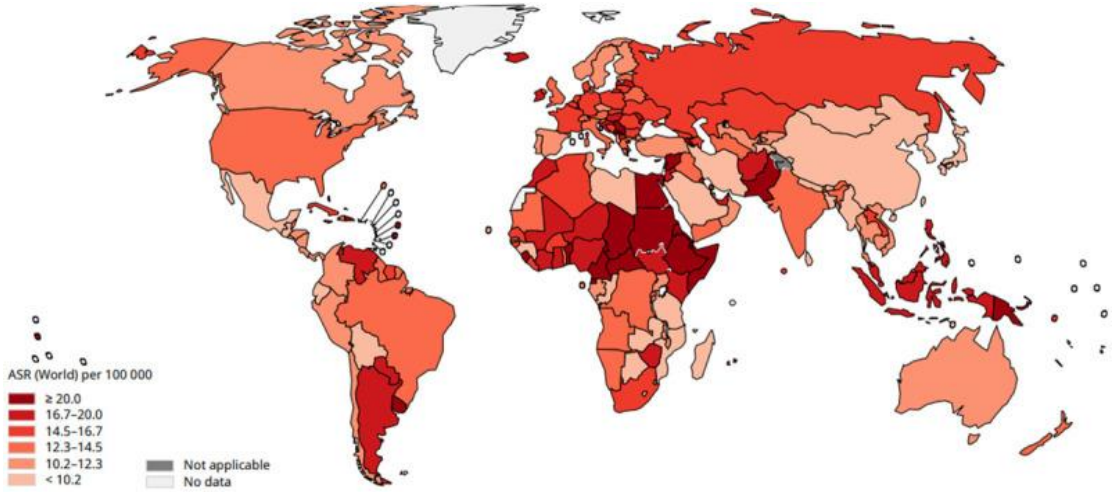
Figure **2.1** The adult breast anatomy, whereby cancer can originate from either the lobules or ducts tissues (National Cancer Institute 2014).

Post 2010, breast cancer is the most diagnosed type of cancer and among the leading cause of mortality among women worldwide. Based on the International Agency for Research in Cancer (GLOBOCAN, 2018) **(Figure 2.2),** an estimated of 2 million new cases of breast cancer in females with 4.2 million of breast cancer death were reported globally which is worrying since 99% of breast cancer affects females.

A


ASR (World) per 100 000
≥ 72.9
56.7–72.9
43.6–56.7
34.0–43.6
24.8–34.0
< 24.8
Not applicable
No data

B


ASR (World) per 100 000
≥ 20.0
16.7–20.0
14.5–16.7
12.3–14.5
10.2–12.3
< 10.2
Not applicable
No data

C

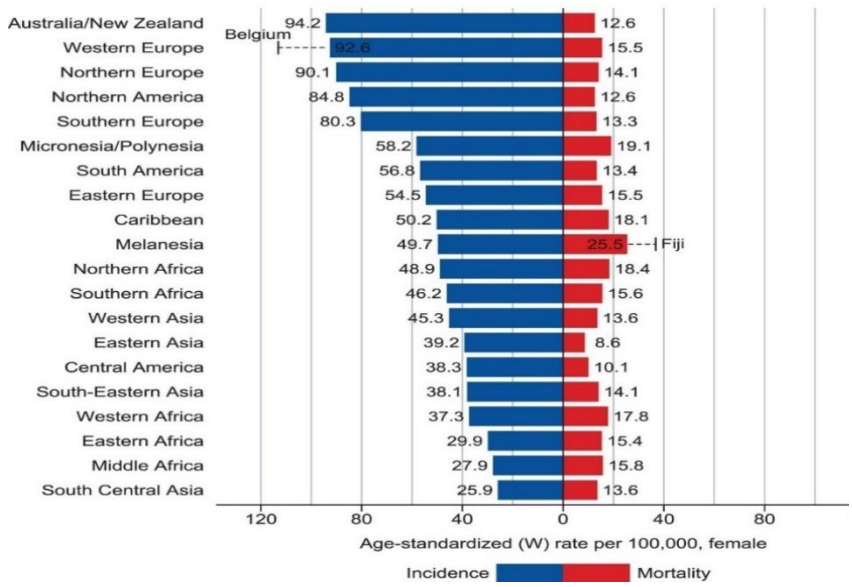


Figure 2.2 Worldwide breast cancer rate. **A**. Breast cancer incidence global map. **B.** Breast cancer death global map. **C**. Regional breast cancer incidence versus mortality graph (Bray et al. 2018).

Breast cancer like most type of cancers is a well-managed disease if detected earlier. In most of developed countries such as the US, UK, Japan etc. patient's overall survival rate is >80%. But, inversely, the developing country with low-income tends to have higher mortality rate even when they have a lower incidence rate (Bray et al. 2018).

Among the Association of Southeast Asian (ASEAN) countries, breast cancer is the leading cause of cancer mortality which accounted for 18% of all other cancer cases (American Cancer Society, 2015). These scenarios have set a distressing situation as the occurrence rate of breast cancer will continue to grow in Asia Pacific countries including Malaysia (Youlden et al. 2014). According to the latest data from the Malaysia National Cancer Registry (2007-2011), 32.1% of all cancers in female is breast cancer followed by colorectal, cervical and ovarian cancer (Azizah et al. 2016). But this is made worst when Malaysia is ranked among the worst breast cancer survival in the region (Abdullah et al. 2013) (**Table 2.1** & **2.2**).

Table 2.1 Malaysian breast cancer patient ethnicity breakdown (Abdullah et al. 2013).

Ethnic	Percentage (%)
Malay	53.8
Chinese	27.1
Indians	9.6
Other Malaysians	7.7
Non-Malaysians	1.9

Table 2.2 Malaysian breast cancer patient survival rates ethnicity breakdown (Abdullah et al. 2013).

Ethnicity	1 year	3 years	5 years
Malay	58.9	49.7	45.1
Chinese	61.2	52.8	49.1
Indian	68.3	58.3	54.2

Note: Breast cancer survival rate in developed countries such the US, UK and Japan is >80% (R. Sharma 2019).

2.2 Classification of breast cancer

The classification of breast cancer can be divided into histological and molecular classification. According to the histological diagnosis, breast tumour can be categorized as restricted to the epithelial component of the breast or has invaded the surrounding locality, and whether this tumour appeared in the mammary ducts or lobules (**Figure 2.1**) (Nascimento and Otoni 2020), whilst based on the microanatomy of the tumour and tissue structure, breast cancer can be sorted as in situ (non-invasive) such as ductal or lobular, or into invasive carcinoma category such as infiltrating ductal, mucinous or invasive lobular (Malhotra et al. 2010). The other classification is the molecular classification, emerged during the era of molecular biology after

realization that sole reliance on physical or histology of tumours is inadequate in predicting its malignancy and correct treatment to be administered. This classification is based on gene expression studies where four distinct molecular characteristics or subtypes are used to define breast cancer subtypes (Vuong et al. 2014) (**Table 2.3**), Molecular targets specifically found in cancers such as hormone receptors, estrogen receptor (ER) and progesterone receptor (PR) and the Human Epidermal Growth Receptor 2 (HER2) and the absence of which would the title triple negative breast cancer (TNBC) (Schiff et al. 2003). A more distinct and consistent classification is achieved with molecular classification which results in the better prognosis prediction and type of therapy to be administered based on presented biomarkers (Kanwar et al. 2020).

Table 2.3 Molecular Classification of Breast Cancer (Vuong et al. 2014)

Molecular Subtypes	Biomarkers detected	Frequency of Cases (%)	Prognosis
Luminal A	ER+ PR+ HER2- low Ki67	40–50	Good
Luminal B	ER+ PR- HER2-/+ high Ki67	20–30	Intermediate
HER2 Enriched	ER- PR- HER2+ High Ki67	15–20	Poor
TNBC	ER- PR- HER2- High Ki67	10–20	Poor

Among those from the molecular classification, a lot of interest has been put on the triple negative breast cancer (TNBC) generally due to the poor prognosis associated with the subtype. In terms of frequency, 1 in 10, and can as high as 1 in 5

cases constitutes of this subtype (De Laurentiis et al. 2010; Nascimento and Otoni 2020). Worse still, it rakes up a death toll highly disproportionate to the incidence (De Laurentiis et al. 2010). The lack of common molecular targets has rendered the usual targeted treatments ineffective causing physicians to rely on systemic therapy using cytotoxic agents (Lee and Djamgoz 2018). Stated poor prognosis also stems from tumour's features of having a more antagonistic phenotype and a high tendency for metastatic development (Yao et al. 2017). There is also significant heterogeneity in TNBCs which decelerates the progress for a definitive targeted therapy (Caswell-Jin et al. 2020; Yao et al. 2017), differing in vital biological pathways and prognosis, of which 6 subtypes were deduced; the basal-like 1 and 2 (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) (Lehmann et al. 2011).

2.3 Treatment of breast cancer

Treatment of this disease revolves around an ultimate goal that is the complete removal of cancer without suffering damage to other parts of the body. However, heterogeneity of cancers presents a major obstacle in achieving this goal, as the diverging molecular trait in individual patient will leads to varying response in treatment. Thus, multiple approaches are required to overcome the conditions presented that could involve surgery, radiotherapy, chemotherapy and, targeted therapy and currently, the new addition, immunotherapy (Caswell-Jin et al. 2020).

Treatment by surgery usually involves tumour removal (lumpectomy) or breast removal (mastectomy), a direct and theoretically simple approach, but surgeries are considered invasive and impossible for advanced stages at multiple sites. Neoadjuvant drug therapies refer to treatments delivered before surgery with the goal

of shrinking a tumour or stopping the spread of cancer to make surgery less invasive and more effective (Bonadonna et al. 1990; Glynne-Jones et al. 2006). Meanwhile, adjuvant drug therapies are administered after surgery to kill any remaining cancer cells with the goal of reducing the chances of recurrence.

Radiotherapy utilises radiation to kill cancer cells, a method that could damage surrounding cells, but safe dosage is carefully precalculated. This can be administered externally using machinery to deliver radiation from outside the body or internally after surgery temporarily via radiation-delivery device near the tumour site (Ben et al. 2020).

Chemotherapy utilises cytotoxicity targeting high mitotic targets (**Table 2.4**). Therefore, affecting normal highly dividing cells. While progress was made in the last decade, it has remained an unfavourable method of therapy (Crown et al. 2012; Glynne-Jones et al. 2006; Hassan et al. 2010). In metastatic cases, TNBCs though being 'invincible' to most targeted therapy, its high mitotic rate causes it being somewhat sensitive towards this type of treatment, and being more effective in an adjuvant or a neoadjuvant setting (Hassan et al. 2010).

Targeted therapy, as the name suggests, act on molecular targets specifically found in cancers such as hormone receptors, estrogen receptor (ER) and progesterone receptor (PR) and the Human Epidermal Growth Receptor 2 (HER2) (Schiff et al. 2003). HER2 upregulation in particular, has been revealed to be an important part in the development and progression of certain aggressive types of breast cancer and gained notoriety with 30% of breast cancer cases, significant enough to be designated as an important biomarker (Mitri et al. 2012; Subik et al. 2010). A monoclonal antibody trastuzumab, branded commercially as Herceptin is good example of a

targeted therapy for HER2 and has been known to reverse adverse conditions in patients in as little as 6 to 12 months and was approved by the United States Food and Drug Administration (FDA) as early as 1998, with EU followed suite 2 years after (Baldo 2016; Mates et al. 2009; Mitri et al. 2012) (**T able 2.4**). Another monoclonal antibody, pertuzumab was also approved in June of 2012 by the FDA (**Table 2.5**), and can be paired with trastuzumab (**Table 2.5**), and it also prevents dimerisation in HER3 as well as HER2 (Baldo 2016).

Table 2.4 Chemotherapies and targeted therapies approved for breast cancer (The American Cancer Society 2021)

Treatments	**Target**
Tamoxifen (TAM)	17β-estradiol (E2)
Doxorubicin	topoisomerase II
Lapatinib (Tykerb)	HER2
Neratinib (Nerlynx)	HER2
Tucatinib (Tukysa	HER2
Palbociclib (Ibrance),	CDK4, CDK6
ribociclib (Kisqali)	CDK4, CDK6
abemaciclib (Verzenio)	CDK4, CDK6
Everolimus (Afinitor	mTOR
Alpelisib (Piqray)	PI3K
Olaparib (Lynparza)	PARP, BRCA1, BRCA2
talazoparib (Talzenna)	PARP, BRCA1, BRCA2
Sacituzumab govitecan – AB-drug conjugate (Trodelvy)	Trop-2

The next generation of treatments include immunotherapy, which is and the latest form of targeted therapy deserving its own classification (Couzin-Frankel 2013). This method aims to amplify, stimulate, or activate immune response toward target cancer, using the own immune system to destroy the cancer, instead of using chemical agent to destroy the tumour. This method of course has higher specificity with less side effects (Lee and Djamgoz 2018). The currently recognised immunotherapy are

the use of humanized antibodies against immune checkpoints protein which responsible for tumour escape e.g. programmed death ligand, PDL-1, atezolizumab, recently approved for breast cancer (Heimes and Schmidt 2019). Among other antibodies which have been approved by the FDA of USA for breast cancer immunotherapy are in **Table 2.5**. Importantly, immunotherapy is suitable for the most difficult to treat breast cancer subtype, the TNBCs where the absence of the three key receptors, reduces the options for targeted therapy, coupled with the high malignancy and metastatic incidence.

The high specificity of targeted and immunotherapy makes them a preferred choice of therapies where induction of cell death or inhibition of growth and proliferation occurs without affecting normal cells, reducing toxicity and side effects in patients. Immunotherapy is suitable for TNBCs since the absence of the three key receptors, reduces the options for targeted therapy, coupled with the high malignancy and metastatic incidence.

Table 2.5 Approved monoclonal antibodies used for cancer therapy (Baldo 2016)

Monoclonal antibody and trade names	Target
Catumaxomab[e] (Removab®)	EpCAM/CD3[f]
Blinatumomab[g] (Blincyto®)	CD19/CD3[h] epsilon
Ibritumomab tiuxetan[i] (Zevalin®)	CD20[j]
Obinutuzumab (Gazyva®, Gazyvaro®)	CD20
Ofatumumab (Arzerra®)	CD20
Rituximab (MabThera®, Rituxan®)	CD20
Brentuximab vedotin[m] (Adcetris®)	CD30[n]
Alemtuzumab[o] (Campath®, MabCampath®)	CD52[p]
Cetuximab (Erbitux®)	EGFR
Panitumumab[r] (Vectibix®)	EGFR
Necitumumab (Portrazza®)	EGFR
Bevacizumab (Avastin®)	VEGF
Ramucirumab (Cyramza®)	VEGFR-2
Pertuzumab (Perjeta®)	HER2, HER3
Trastuzumab (Herceptin®)	HER2
Ado-trastuzumab emtansine[x] (Kadcyla®)	HER2
Denosumab (Xgeva®, Prolia®)	RANKL
Ipilimumab (Yervoy®)	CTLA-4[y]
Siltuximab (Sylvant®)	IL-6
Nivolumab (Opdivo®)	PD-1
Pembrolizumab (Keytruda®)	PD-1
Dinutuximab (Unituxin®)	GD2
Daratumumab (Darzalex®)	CD38
Elotuzumab (Empliciti®)	SLAMF7[af]

2.4 Hallmarks of cancer: Understanding cancer progression in search of tumour markers for cancer future management

Research in human cancer has identified several hallmarks associated with cancer to motivate/facilitate researchers in creating a proper strategy to manage the disease. These hallmarks have been expanded from their original when new notion is discovered with the advancements of technology (**Table 2.6**).

A tumour marker refers to the presence or production of key characteristics by cancer cells or other cells of the body as reaction to cancer or certain benign (noncancerous) conditions that is indicative of a cancer, such as level of aggressiveness, possibility of targeted therapy, or the level of response towards treatment (Mayeux 2004). Tumour markers could be classified into two; biomarkers of exposure, as mentioned are to predict risk levels, and biomarkers of disease, which serve diagnostic purpose as well as disease progression (Mayeux 2004). Because of that, tumour markers are vital for drug development. Estimation of a patient's response towards drugs is normally by analysing the level of expression of the molecular target or pathway in them – so called predictive marker (Mayeux 2004; van 't Veer and Bernards 2008). But as of now, the predictive factor definition has extended to include potentially markers that either located within the treatment target, the neighbouring stroma, assist as modulators or epiphenomena associated to expression and/or function of the target, or they might also be markers where research has revealed to fortuitously respond very well to existing HER2-targeted therapy. Adversely, basal-like or triple-negative subtype does not express ER, PR, and HER2 but expresses basal cytokeratin 5/6 and 17, they have a poor prognosis and a high recurrence rate (Bertozzi et al. 2018; Kanwar et al. 2020; Malhotra et al. 2010) and

does not react positively against specific drug although they can be separately classified (Munster and Norton 2001).

Table 2.6 The hallmarks of cancer including the expanded discoveries (Hanahan and Weinberg 2011).

Original hallmarks	**Revised Hallmarks**
Self-sufficiency in growth signals	Sustaining Proliferative Signalling
Insensitivity to anti-growth signals	Evading Growth Suppressors
Evading apoptosis	Resisting Cell Death
Limitless replicative potential	Enabling Replicative Immortality
Sustained angiogenesis	Inducing Angiogenesis
Tissue invasion and metastasis	Activating Invasion and Metastasis
	Genome Instability and Mutation
	Tumour-Promoting Inflammation
	Reprogramming Energy Metabolism
	Evading Immune Destruction

Many molecular markers have been studied in the recent decades to identify both a useful prognostic tool and able to determine severity of the tumour and to avoid unnecessary cost and adverse treatment effect to patients with favourable prognosis shown in **Table 2.7** and more markers is under trial (**Table 2.8**). As of recent times, genomic and molecular classification based upon mentioned markers are being grouped into prognosis description, with the main grouping as in **Figure 2.3**. For example, luminal A subtype is described with high levels of ER expression are usually benign/less aggressive, with a low distant recurrence rate while Luminal B subtype expresses fewer ER-related genes, proliferation rate increase and upregulation of

HER2, so that they usually require to be treated with both hormonal therapy and chemotherapy.

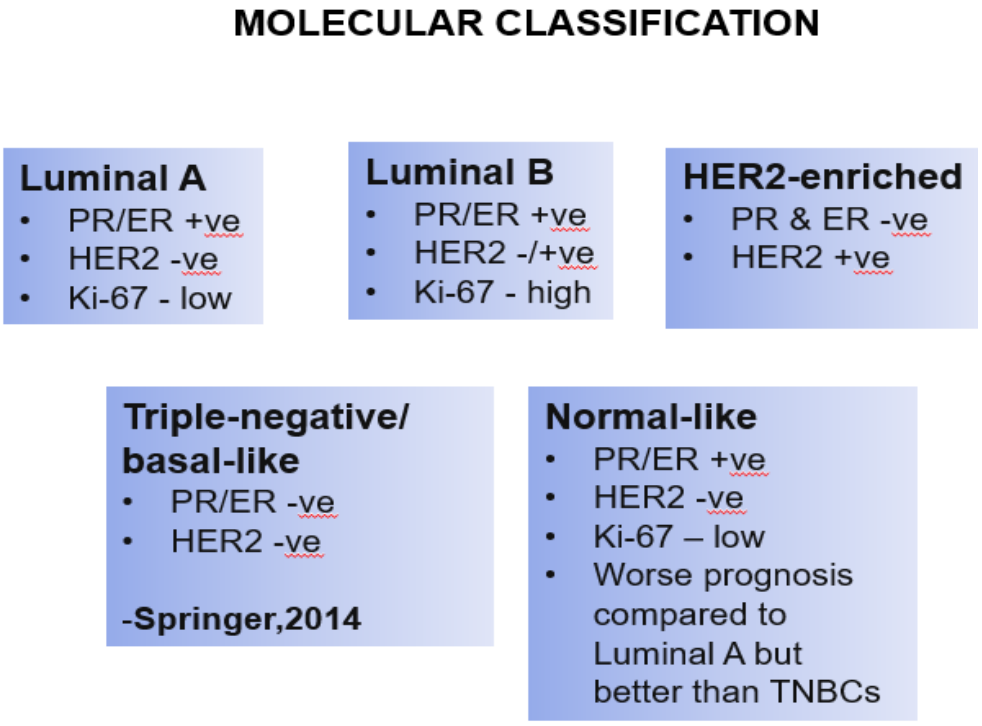


Figure 2.3 Multiple marker groupings based on prognosis (Malhotra et al. 2010)

2.4.1 Cancer hallmark: Invasion and metastasis of breast cancer

Malignancy cannot be separated from invasion and metastasis. Metastasis is a common cause of cancer death, with effective treatment unavailable which overcome a series of barriers (Wei et al. 2013). Metastasis for advanced cases. Metastasis is defined as the spread of cancer cells from primary tumour sites to distant organs and tissues, which contributes to over 90% of mortality in cancer patients (Weigelt et al. 2005). Consequently, many studies have been conducted to understand the mechanism of metastasis. Metastasis does not occur randomly but rather by accumulating enough mutations to allow metastatic cascade (Scully et al. 2012) begins with the local invasion surrounding the primary tumour, detachment of the cells continues until the

cells invade and intravasate into blood or lymphatic vessels (**Figure 2.4**) (Ha et al. 2013).

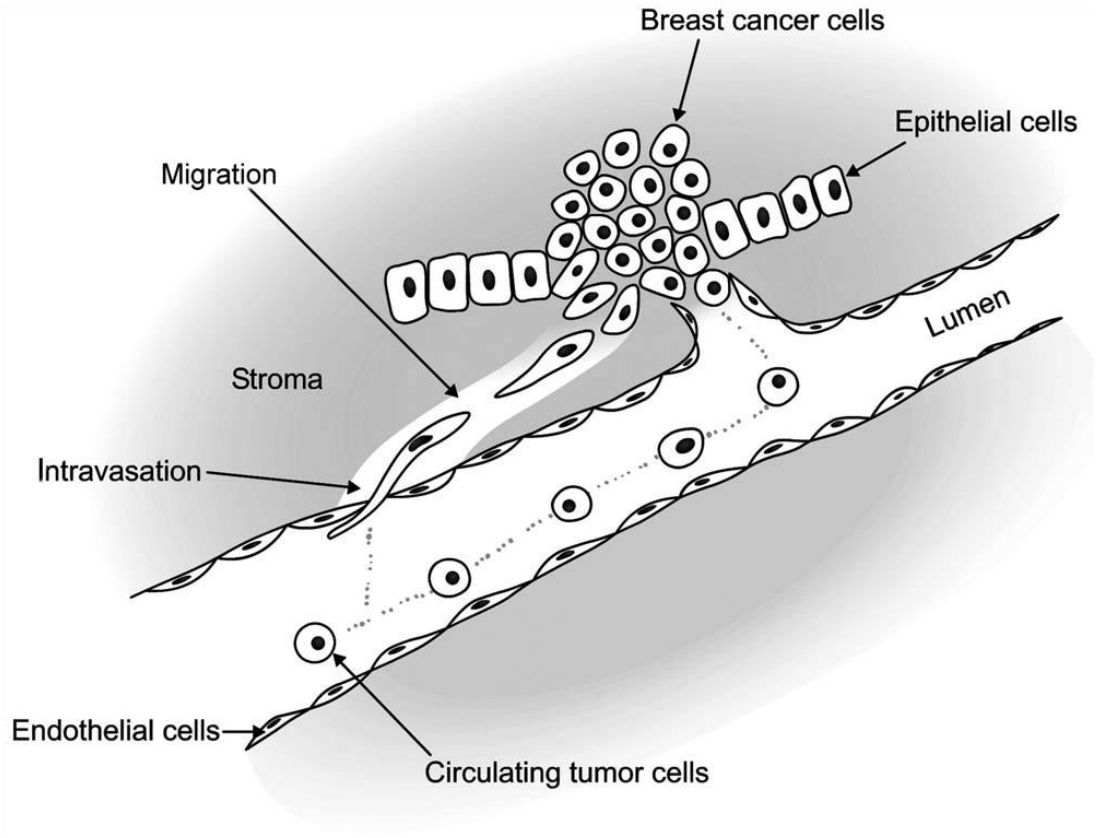


Figure 2.4 The migration of tumour in the blood circulation. Image was acquired and revised (Scully et al. 2012)

In the circulation system, the tumour cells are dispersed to distant organs. Subsequently, the tumour cells undergo cell cycle arrest and adhere to capillary beds within the target organ, before extravasating into the organ parenchyma, proliferating and promoting angiogenesis and evade the host's immune response and apoptotic signals in order to survive (S.-J. Kim et al. 2009; Talmadge and Fidler 2010). Acquiring multiple aggressive traits is imperative as it need to overcome certain barriers before successfully proliferate at the new site. One of the first barriers are from the apical structure of epithelial cells itself, which has tight junctions (TJ) which controls diffusion and avoid cohesion loss that could detached a tumour from a site of

origin and overcoming that would be a step closer to avoid immune destruction (Martin and Jiang 2009).

Table 2.7 Breast tumour markers and their prognosis (Bertozzi et al. 2018)

Marker	Prognosis
HER2	Positive expression often translates to better prognosis
ER	Positive expression often translates to better prognosis
PR	Positive expression often translates to better prognosis
BRCA1, 2	Positive expression indicates inherited susceptibility but dependant on penetrance of the gene
Ki-67	Positive expression often translates to worse prognosis, higher proliferation rate.

Table 2.8 Tumour markers used or under trial in clinical setting for breast cancer/cancers for each cancer hallmarks (Dai et al. 2016; Dewadas et al. 2019; Zeng et al. 2011)

Hallmarks	Biomarkers
Sustaining proliferative signalling	ER, PR, HER2, Ki67
Enabling replicative immortality -	Kin17
Resisting cell-death -	BCL2
Genome instability and mutation -	TP53
Activating invasion and metastasis -	CK 5/6, EGFR
Deregulating cellular energetics -	VRD, AR
Inducing angiogenesis	HIF1α, VEGFA
Avoiding immune destruction	STAT1, SP110
Evading growth suppressors	cyclin-dependent kinase CDK4/6
Tumour-Promoting Inflammation	Interleukin-1beta

This acquisition of new traits occurs via unique transformation, from an epithelial cancer to mesenchymal (EMT) and back (MET) when reaching favourable secondary site (Scimeca et al. 2020). Accordingly, in EMT, tumour cells lose their epithelial characteristics, cell-to-cell adhesion, epithelial polarity, with acquisition of mesenchymal phenotype and migratory capacity (Guarino et al. 2007). In epithelial cancers, EMT controls the initial step for local incursion of tumour cells in primary tumours by losing cell to cell junctions, secreting enzymes such as matrix metalloproteinases (MMPs) that eradicate extracellular matrix (ECM), increase in mesenchymal phenotype and become migratory (Li and Li 2015). The cadherin family has been documented to play a large role in mediating cell-to-cell adhesion and plays predominant roles in breast cancer metastasis. E-Cadherin maintains cell-cell junctions, while the downregulation of E-cadherin was shown to be a determinant in the outgrowth of metastatic breast cancer cells. The downregulation of E-cadherin has been reported to reflect progression and metastasis in breast cancer associated with poor prognosis (Berx and Van Roy 2001). Mutations in E-cadherin which lead to its functional loss were discovered in lobular breast carcinoma (Keller et al. 1999). N-Cadherin on the other hand, is closely associated with mesenchymal cells (Yan et al. 2015). (Kotb et al. 2011) showed that the expression of N-cadherin in place of E-cadherin caused the formation of fibrosis and cysts in mammary glands and eventually led to malignant breast tumour in mice. In addition, as reported by (Guarino et al. 2007), down-regulation of E-cadherin and up-regulation of N-cadherin were frequently observed in cancer cells of most epithelial cancers during stromal invasion. Down-regulation of E-cadherin is believed to result in the loss of adhesion between epithelial breast cancer cells and other epithelial cells, while increase in N-cadherin, and possibly other mesenchymal cadherins, permits the adhesion of tumour cells to

stromal cells and subsequently, the invasion of tumour cells into the stroma (Loh et al. 2019).

After the spread through circulatory system and reaching a suitable site, the MET process occurs and complete the metastasis of the tumour (Li and Li 2015). While MET was not completely understood as well as EMT, several factors have been appealed as trigger to MET, such as the prominence of 3'-5'-cyclic adenosine monophosphate (cAMP) and targeting protein kinase A (PKA) downstream, in superseding the transition of cells (Pattabiraman et al. 2016). The study also points out that regulating PKA through cholera toxin (CTx) can induce MET process in human mesenchymal, N8 cell lines as well as finding PKA's role in maintaining the epithelial state from ever transforming in the first place.

Development of distant metastasis to various organs has become the major cause of death from breast cancer (DeSantis et al. 2019; Lacroix 2006). In three years after the first detection of the primary tumour, it was reported about 10-15% breast cancer patients will develop distant metastasis (Weigelt et al. 2005). It is reported that the ER positive breast cancer predominantly invades the bone and to a lesser occurrence, detected in brain and lungs (Soni et al., 2015). The series of events explained above such as trait accumulations, EMT-MET-EMT transformations, detachment and adhesion are all part of the sequence of metastatic cascade (Scully et al. 2012). Successful metastatic cascade will lead to invasion of surrounding cells and beginning of secondary tumour site (Lacroix 2006; Scimeca et al. 2020; Yin et al. 2005). Ultimately, research on investigating hallmarks of cancer (**Figure 2.5**) has led to the birth of many new novel markers for cancer.

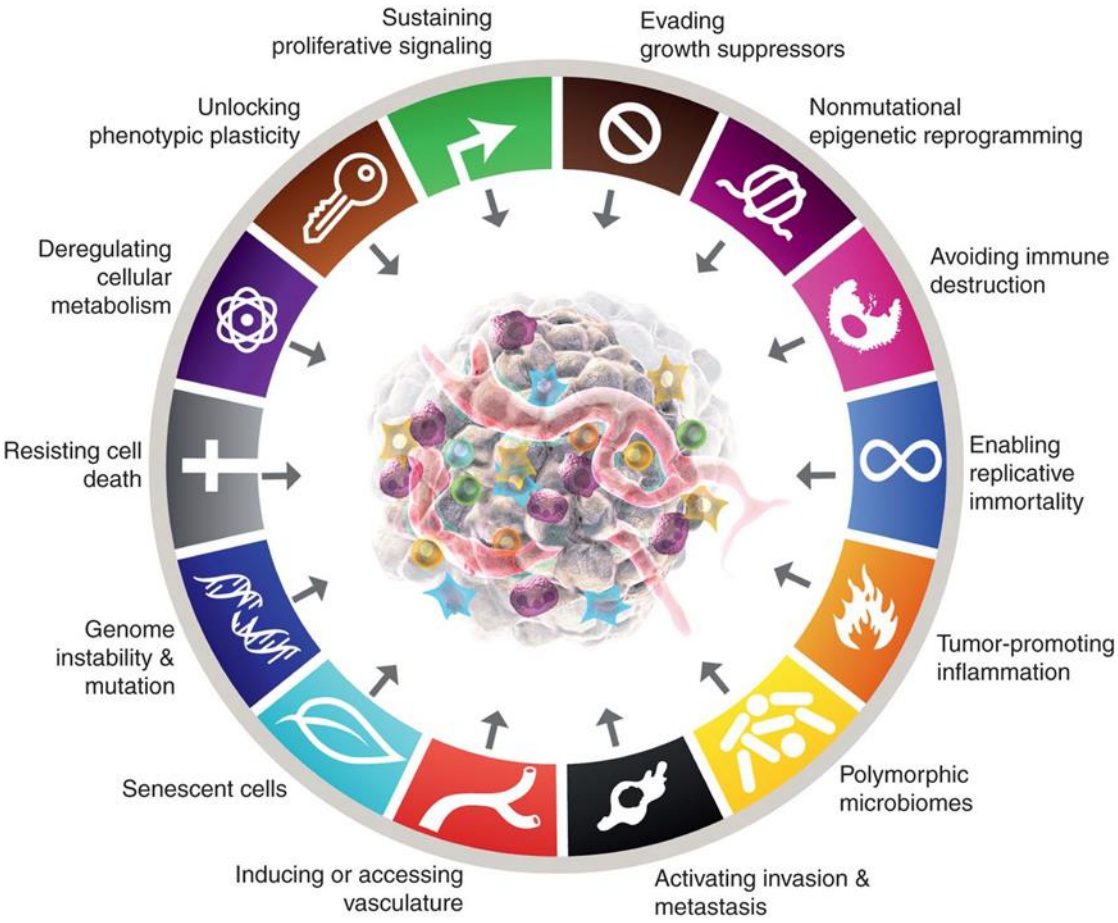


Figure 2.5 Diagram of the hallmarks of cancer with categorized functional groups (Hanahan 2022)

2.5 VGSCs: A common neuron hallmarks but now a new metastatic tumour marker

Integral and peripheral membrane proteins are important for the maintenance of many cellular functions such as signal transduction, cell integrity, intracellular and extracellular transport of molecular solutes and cell-to-cell communication (Lodish et al. 2000). The pore-forming membrane proteins allow ions to pass through the channel pore function, establishing a resting membrane potential, shaping action potentials and other electrical signals by gating the flow of ions across the cell membrane, controlling the flow of ions across secretory and epithelial cells, and regulating cell volume.

Membrane ion channels are also essential for cell proliferation and appear to have a role in the development of cancer and its progression (Bennett et al. 2004; Brackenbury 2012; Kunzelmann 2005; Pedersen and Stock 2013; Sontheimer 2008). This has initially been demonstrated for potassium channels and is meanwhile also suggested for other cation channels and Cl− channels. For some of these channels, like voltage-gated ether à go-go and Ca2+-dependent potassium channels as well as calcium and chloride channels, a cell cycle-dependent function has been demonstrated (Marble 2008)2008). Along with other membrane conductance, these channels control the membrane voltage and Ca2+ signalling in proliferating cells. Homeostatic parameters, such as the intracellular ion concentration, cytosolic pH and cell volume, are also governed by the activity of ion channels.

VGSCs are transmembrane protein expressed abundantly in excitable cells such as neurons and muscle cells. Diverse subtypes of VGSC are differentially expressed all over the body. Nav1.1, Nav1.2, Nav1.3, Nav1.6, Nav1.7, Nav1.8 and Nav1.9 are expressed in nervous tissue whereas Nav1.5 and Nav1.4 are expressed in skeletal and cardiac muscle respectively (J. B. Kim 2014). The VGSC family consists of nine different α-subunits (Nav1.1–Nav1.9). The α subunit consists of four domains (1, 2, 3 and 4) each with six transmembrane segments, The linker between domains 3 and 4 is the fast inactivation gate, shortly after opening (Angus and Ruben 2019) (**Figure 2.6**).