# ELUCIDATING THE FUNCTION OF REPRESSOR ELEMENT SILENCING TRANSCRIPTION FACTOR IN HUMAN BREAST CANCER AND ITS RELATION WITH VOLTAGE-GATED SODIUM CHANNELS-MEDIATED METASTASIS

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# ELUCIDATING THE FUNCTION OF REPRESSOR ELEMENT SILENCING TRANSCRIPTION FACTOR IN HUMAN BREAST CANCER AND ITS RELATION WITH VOLTAGE-GATED SODIUM CHANNELS-MEDIATED METASTASIS

**by**

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## LIST OF SYMBOLS AND ABBREVIATION

| | |
|---|---|
| ® | Registered |
| °C | Degree Celsius |
| × g | Times graviti |
| α | Alpha |
| β | Beta |
| β-actin | Beta actin |
| μ | Micro |
| μg | Microgram |
| μl | Microliter |
| μM | Micro molar |
| AP | Ammonium persulfate |
| ATCC | American Type Cell Culture |
| ATP | Adenosine 5'-Triphosphate |
| bp | Base pair |
| BDNF | Brain-derived neurotrophic factor |
| 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 |

| | |
|---|---|
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| CTCL | Cutaneous T-cell lymphoma |
| CTCs | Circulating tumour cells |
| CTLA4 | Cytotoxic T-lymphocyte associated antigen 4 |
| CypB | Cyclophilin B |
| D3 | Domain 3 |
| D4 | Domain 4 |
| $dH_2O$ | 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 |
| EDTA | Ethylene diamine tetra acetic acid |
| EGF | Epidermal Growth Factor |
| EMSA | Electrophoretic mobility shift assays |
| EMT | Epithelial-mesenchymal transition |
| ER | Estrogen receptor |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| GATA3 | GATA binding protein 3 |
| gDNA | Genomic DNA |
| 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 |
| HIF | Hypoxia-inducible factor |
| HMTs | Histone methyltranferases |
| hr | Hour |
| HRP | Horseradish peroxidase |
| IARC | International Agency for Research on Cancer |
| $IC_{50}$ | Half maximal inhibitory concentration |
| IDT | Integrated DNA Technologies, Inc. |
| kb | Kilo base |
| kDa | Kilo Dalton |
| L | Litre |
| mA | Milliampere |
| 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 |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PNS | Peripheral nervous system |
| 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 |
| TNBC | Triple-negative breast cancer |
| TRANSFAC | Transcription Factor database |
| TTX | Tetrodotoxin |
| ™ | Trademark |
| TSA | Trichostatin A |
| UV | Ultraviolet |
| V | Voltage |
| VEGF | Vascular endothelial growth factor |

| | |
|---|---|
| VGSC | Voltage gated sodium channel |
| VHL | Von Hippel-Lindau |
| ZEB1 | Zinc finger E-box-binding homeobox 1 |

**MENJELASKAN PERANAN *REPRESSOR ELEMENT SILENCING TRANSCRIPTION FACTOR* DALAM KANSER PAYUDARA MANUSIA DAN HUBUNGANNYA DENGAN *VOLTAGE-GATED SODIUM CHANNELS*-METASTASIS PENGANTARA**

## ABSTRAK

Pengekspresian *voltage-gated sodium channels* (VGSCs), terutamanya jenis Nav1.5 dan varian sambatan neonat didapati sangat tinggi di dalam kalangan pesakit kanser payudara dan berkait rapat dengan keupayaan metastatik yang tinggi. *Repressor element silencing transcription* (REST) pula merupakan faktor transkripsi yang bertindak sebagai penindas tumor yang mana pengurangan pengekspresiannya telah dikaitkan dengan sel karsinoma fenotip yang agresif. Matlamat keseluruhan kajian ini adalah untuk mengkaji peranan REST dalam mengawal ekspresi Nav1.5 dan nNav1.5 di dalam sel kanser payudara manusia. Tindak balas rantai polimerase (*PCR*) dan *Western blot* telah digunakan untuk menganalisa tahap pengekspresian mRNA dan protin sasaran (Nav1.5, nNav1.5, REST, HDAC1, HDAC2, HDAC3, MMP2, N-cadherin) di antara sel epitelium payudara bukan kanser (MCF-10A), sel kanser payudara kurang agresif (MCF-7) dan sel kanser payudara sangat agresif (MDA-MB-231). Tapak pengikatan REST pada promoter Nav1.5 pula telah dikenal pasti menggunakan perisian atas talian JASPAR[2018]. Penyahfungsian REST menggunakan siRNA, perencat HDAC, dan trichostatin A (TSA) hanya dilakukan terhadap sel MCF-7 kerana pengekspresian REST telah dilaporkan lebih tinggi dalam sel tersebut. Pertumbuhan dan kelakuan metastatik sel dinilai menggunakan kaedah *MTT*, motiliti sisi dan migrasi. Pengekspresian mRNA dan protin REST telah dikesan dalam semua sel tetapi ekspresi tertinggi dilihat di dalam sel MCF-7 (mRNA $p<0.05$ dan protin $p<0.05$). Pengekspresian mRNA Nav1.5 ($p<0.01$) dan

nNav1.5 ($p<0.05$) didapati lebih tinggi dalam sel MDA-MB-231 berbanding sel MCF-7, manakala tidak dikesan dalam sel MCF-10A. Pengekspresian protin Nav1.5 juga didapati lebih tinggi di dalam sel MDA-MB-231 berbanding dengan sel MCF-7 ($p<0.05$). Sebanyak dua belas tapak pengikatan REST di dalam kawasan promoter Nav1.5 telah diramal oleh JASPAR$^{2018}$. Walaupun penyahfungsian REST terhadap ekspresi mRNA dan protin REST telah berjaya dilakukan ($p<0.05$), namun tiada perubahan ketara ke atas paras pengeksperisian mRNA Nav1.5 dan nNav1.5. Tambahan pula, walaupun pengekspresian mRNA HDAC2 lebih tinggi dengan ketara dalam sel MCF-7 ($p<0.05$) berbanding sel MDA-MB-231 (daripada analisa perbandingan ekspresi asas), pengekspresian mRNA HDAC1 menurun dengan ketara ($p<0.05$) dalam sel MCF-7 yang mengalami penyahfungsian REST. Rawatan dengan TSA ke atas sel MCF-7 mendapati pengekspresian mRNA HDAC2 menurun dengan ketara pada 1000 dan 10 000 ng/ml ($p<0.001$) dan juga menurunkan dengan ketara pengekspresian mRNA REST pada 100 ($p<0.05$), 1000 ($p<0.001$) dan 10 000 ($p <0.0001$) ng/ml. Menariknya, TSA meningkatkan pengekspresian mRNA Nav1.5 dengan ketara (pada 1000 ($p<0.05$) dan 10 000 ($p<0.01$) ng/ml) dan nNav1.5 (pada 10 000 ng/ml, $p<0.01$). Ini diikuti dengan peningkatan migrasi sel (pada 1000 dan 10 000 ng/ml, $p<0.05$) yang disahkan oleh peningkatan ketara dua gen yang berkait dengan metastasis iaitu MMP2 ($p<0.01$) dan N-cadherin ($p<0.05$). Kesimpulannya, REST mengawal pengekspresian Nav1.5/nNav1.5 dalam sel kanser payudara yang kurang agresif melalui perencatan oleh HDAC2 (yang mana tidak berlaku di dalam sel kanser payudara agresif kerana kekurangan REST), seterusnya membuktikan bahawa pengekspresian Nav1.5/nNav1.5 dalam kanser payudara boleh dikawal oleh epigenetik.

**ELUCIDATING THE FUNCTION OF REPRESSOR ELEMENT SILENCING TRANSCRIPTION FACTOR IN HUMAN BREAST CANCER AND ITS RELATION WITH VOLTAGE-GATED SODIUM CHANNELS-MEDIATED METASTASIS**

## ABSTRACT

Voltage-gated sodium channels (VGSCs), particularly isoform Nav1.5 and its neonatal splice variant, nNav1.5, is found to be highly upregulated in human breast cancer and its expression/activity associates with strong metastatic potential. Whilst repressor element silencing transcription factor (REST) was discovered as tumour suppressor in various type of carcinomas in which loss/lack of its expression has been linked to aggressive phenotype. The overall aim of this study was to investigate the role of REST in regulating Nav1.5 and nNav1.5 expression in human breast cancer cells. Real-time PCR and Western blotting were conducted to compare the mRNA and protein expression levels of target molecules (Nav1.5, nNav1.5, REST, HDAC1, HDAC2, HDAC3, MMP2, N-cadherin), respectively, 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). The possible REST binding sites on Nav1.5 promoter sequence was predicted using online software JASPAR$^{2018}$. Since MCF-7 cells has been reported to expressed higher REST expression, siRNA-mediated REST knockdown and treatment using histone deacetylase (HDAC) inhibitor, trichostatin A (TSA) was performed only on MCF-7 cells. Cell growth and metastatic behaviours of the cells were also assessed by functional assays (MTT, lateral motility and migration assays). REST mRNA was detected in all three cell lines with the highest expression in MCF-7 cells ($p<0.05$). Similarly, REST protein expression

was also detected in all three cell lines, with the highest expression in MCF-7 cells ($p<0.05$). The mRNA expression of Nav1.5 ($p<0.01$) and nNav1.5 ($p<0.05$) was higher in MDA-MB-231 cells compared to MCF-7 cells and not detected in MCF-10A cells. Correspondingly, Nav1.5 protein expression was also higher in MDA-MB-231 cells compared to MCF-7 cells ($p<0.05$). Twelve REST binding sites in Nav1.5 promoter were predicted by JASPAR[2018]. Although mRNA and protein expression of REST were successfully knockdowned ($p<0.05$), however, no significant change was observed on Nav1.5 and nNav1.5 mRNA level. Additionally, although only HDAC2 mRNA expression was significantly higher in MCF-7 cells compared to MDA-MB-231 cells ($p<0.05$) (from the basal comparison analysis), instead, in the MCF-7-REST knockdown cells, HDAC1 mRNA expression was significantly reduced ($p<0.05$). In the TSA treated MCF-7 cells, HDAC2 mRNA level was significantly reduced (at 1000 and 10 000 ng/ml, $p<0.001$), similarly, REST mRNA expression was significantly reduced (at 100 ($p<0.05$), 1000 ($p<0.001$) and 10 000 ($p<0.0001$) ng/ml)). Interestingly, TSA significantly enhanced mRNA expression of Nav1.5 (at 1000 ($p<0.05$) and 10 000 ($p<0.01$) ng/ml) and nNav1.5 (at 10 000 ng/ml, $p<0.01$). This was followed by enhanced cell migration (at 1000 and 10 000 ng/ml, $p<0.05$), which was confirmed by significant upregulation of two metastasis-related genes, MMP2 ($p<0.01$) and N-cadherin ($p<0.05$). In conclusion, REST regulates Nav1.5/nNav1.5 expression via inhibition by HDAC2 in the less aggressive breast cancer cells (which did not occur in aggressive breast cancer due to lack of REST), providing a revelation that Nav1.5/nNav1.5 expression in breast cancer could be regulated by epigenetics.

# CHAPTER 1

# INTRODUCTION

## 1.1 Overview of breast cancer

Breast cancer has become the most common diagnosed cancer and the leading cause of cancer mortality among women worldwide. Based on the International Agency for Research in Cancer (GLOBOCAN) 2018 database, an estimated 2 million new cases of breast cancer in females with 4.2 million of breast cancer death were reported globally in 2018 (Bray *et al.*, 2018). In south east Asia countries, breast cancer is the leading cause of cancer mortality which accounted 18% of all other cancer cases (American Cancer Society, 2015). According to the latest data from Malaysian National Cancer Registry Report 2007-2011, breast cancer is the most common cancer among Malaysian females, which comprised of 32.1% of all cancers, which is followed by colorectal, cervical and ovarian cancer cases reported from 2007-2011 (Ab Manan *et al.*, 2016). These scenarios have set an alarming extent as the incidence rate of breast cancer will continue to rise in Asia Pacific countries including Malaysia (Youlden *et al.*, 2014).

### 1.1.1 Subtypes of breast cancer

Breast cancer, a heterogeneous disease, originates in tissue of breast which comprised of lobules (milk production glands) and ducts, which function as connector from lobule to the nipple **(Figure 1.1)** (Weigelt *et al.*, 2005; Carol, 2012). Breast cancer is classified into several molecular subtypes; luminal A, luminal B, HER2/neu positive and basal-like. Both of luminal A and B are estrogen receptor (ER) positive breast cancer (Nielsen *et al.*, 2004). This ER positive subtypes show a good prognosis and response well towards hormone therapy but luminal A has a

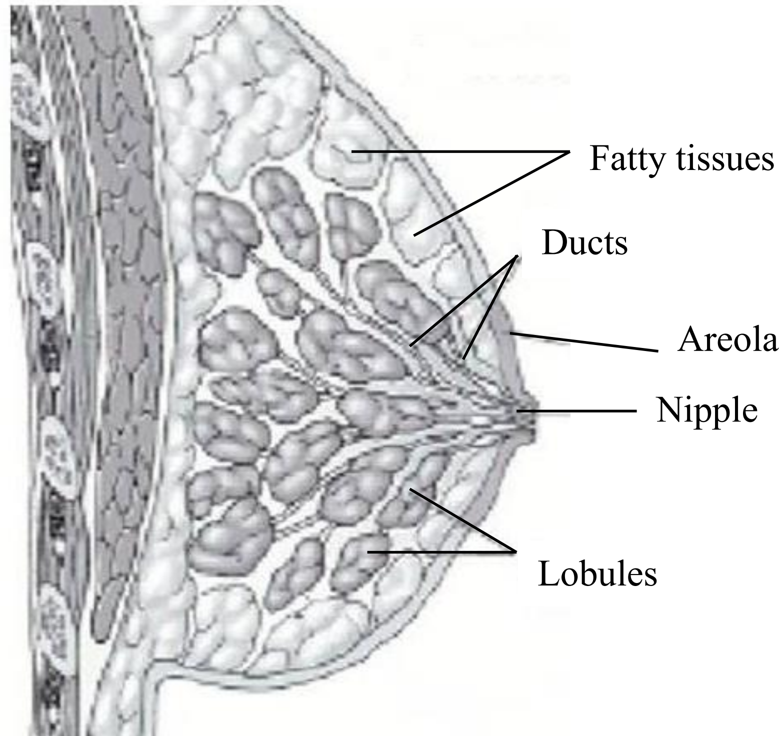


Figure 1.1 Adult female breast anatomy

Breast cancer begins in breast tissue that consist of lobules (glands for milk production) and the ducts which function as connector from lobule to the nipple. Image adapted from Shareef *et al.*, 2016.

better prognosis compared to luminal B due to higher proliferation rate and express lower progesterone receptor (PR) (Perou *et al.*, 2000; Masood, 2016). Subtype of HER2/neu positive represents breast cancer with either ER positive or ER negative but this subtype is controlled by receptor tyrosine kinase erythroblastic oncogene B (ERBB2, also known as HER2) and it comprises 10–15% of all cases of breast cancer (Jin and Mu, 2015; Masood, 2016). Additionally, HER2/neu subtype is linked to poor prognosis and is commonly found in ductal carcinoma in situ (Perou *et al.*, 2000). The basal-like group is recognised for lacking of ER, PR and HER-2/neu oncogene and it has high proliferation and mitotic rate, which makes it known as triple-negative breast cancer (TNBC) (Masood, 2016). This basal-like is the most studied breast subtypes which consists of separate characterisation of immunocytochemical, genetic expression from other subtypes and represents the worst prognosis (Masood, 2016). To date, there is no targeted therapy available to treat this type of breast cancer (Masood, 2016).

### 1.1.2 Treatment of breast cancer

Due to the heterogeneity of breast cancer, it is a major challenge to treat this disease as breast cancer is genetically and molecularly different in each patient. Several approaches are available in treating breast cancer based on tumour size, stage and hormonal status. Commonly, primary tumour is surgically removed (lumpectomy or mastectomy) and the patients will follow up several adjuvant therapies such as chemotheraphy, radiation, hormonal therapy and targeted therapy, in order to eliminate remaining tumour (Jin and Mu, 2015). Despite of advances of therapies available, unfortunately, about 25-40% breast cancer patients developed metastatic disease (Guarneri and Conte, 2009). Since breast cancer comprises of

several subtypes, it is crucial to identify the correct analysis for each patient as each subtype requires different therapeutic approaches. For instance, hormonal therapy and targeted therapy using tamoxifen and trastuzumab are applied to HER2 positive and HER2 negative patients, respectively (Guarneri and Conte, 2009). The complexities of breast cancer itself provide challenges for effective treatment for breast cancer patients in controlling their disease progression.

### 1.1.3 Breast cancer metastasis

Metastasis is the main cause of breast cancer mortality. Metastasis is defined as the spread of cancer cells from primary tumour sites to distant organs and tissues, which accounts for over 90% of mortality in cancer patients (Weigelt *et al.*, 2005; Li *et al.*, 2007). Consequently, many studies have been conducted to understand the mechanism of metastasis particularly in breast cancer.

Cancerous cells or tumour can spread out from the primary site and invade the surrounding breast tissues. In most cases, development of distant metastasis to various organs has become the major cause of death from breast cancer (Lacroix, 2006; Carol, 2012). Within three years after the first detection of the primary tumour, it is reported about 10-15% breast cancer patients will develop distant metastasis (Weigelt *et al.*, 2005). In breast cancer, the ER positive subtype predominantly invades the bone and less detected in brain and lung (Soni *et al.*, 2015). The HER2 positive subtype usually detected in the liver whilst the TNBC subtype commonly affected the brain, lungs and less often in liver (Soni *et al.*, 2015; Jin and Mu, 2015).

One of the important factors that influence the organ preference by cancer cells for distant metastasis has been long hypothesised by the 'seed and soil' theory in which the cancer cells (the seed) tend to metastasize to any organ (the soil) that provides a compatible environment for the growth of cancer cells (Paget, 1889). Additionally, each organ is composed of specific vessels structure and circulation system which greatly impact the spread of metastasis (Obenauf and Massagué, 2015). It is reported that bone is one of the main target sites for breast cancer metastasis. About 60% of breast cancer patients were diagnosed with initial distant metastasis at the bone and the patients had a higher survival rate (5 years) in comparison to those who had initial distant metastasis at other sites (Xiong *et al.*, 2018). Available evidence suggest that the vasculature of blood flow from breast to bone through the vertebral-venous plexus contribute to a high risk for the breast cancer-bone metastasis (Coleman, 2006). It is noted that estrogen hormone is important in maintaining bone remodeling and homeostasis but the cancer cells particularly the ER positive type accommodating in the bone marrow after metastasis may utilise the hormone for growth and proliferation (Nakamura *et al.*, 2007; Jin and Mu, 2015). In addition, it has been shown that matrix cells of the bone release various type of growth factors for example, transforming growth factor β (TGF-β) and insulin-like growth factor (IGF) which further promote the proliferation of cancer cells (Yu and Rohan, 2000; Xu *et al.*, 2015).

## 1.2 Mechanism of metastasis

Many studies have been conducted to elucidate the mechanism and elements that contribute towards the progression of metastasis in cancer research. The process of metastasis is undoubtedly complex and consists of several steps including: 1)

local invasion of surrounding tissues and detachment from the primary tumour site, 2) entrance into circulation and lymphatic system, 3) survival in circulation, 4) extravasating and residing into target organ, 5) survival in foreign microenvironment of the host and 6) initiation of tumour growth in the target organ (**Figure 1.2**) (Jin and Mu, 2015).

Tumour cells acquire certain aggressive traits and surpass multiple barriers in order to propagate uncontrollably in primary tumour site. Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are critical processes that involve in the transition of cell phenotype between epithelial and mesenchymal state (Yao *et al.*, 2011). The most common example of EMT occurs during embryonic development in which epithelial cells lose their adhesive characteristic, therefore making them increase in motility which required for gastrulation and organogenesis (Thiery *et al.*, 2009; Polyak and Weinberg, 2009). This EMT model was used to explain early progression of metastasis process together with reversal of EMT, MET occurs upon extravasation process when tumour cells re-attach and colonise in a new site of distant tissue or organ (May *et al.*, 2011).

In epithelial cancers, EMT regulates the first step for local invasion of tumour cells in primary tumours by losing cell to cell junctions, secreting enzymes such as matrix metalloproteinaises (MMPs) that destruct extracellular matrix (ECM), increase in mesenchymal phenotype and becoming migratory (Samatov *et al.*, 2013; Jin and Mu, 2015). This transition process is controlled by various transcription factors such as zinc finger E-box-binding homeobox 1 and 2 (ZEB1 and ZEB2), Snail and Slug which resulting in downregulation of epithelial markers such as E-cadherin and activation of mesenchymal marker (for example N-cadherin

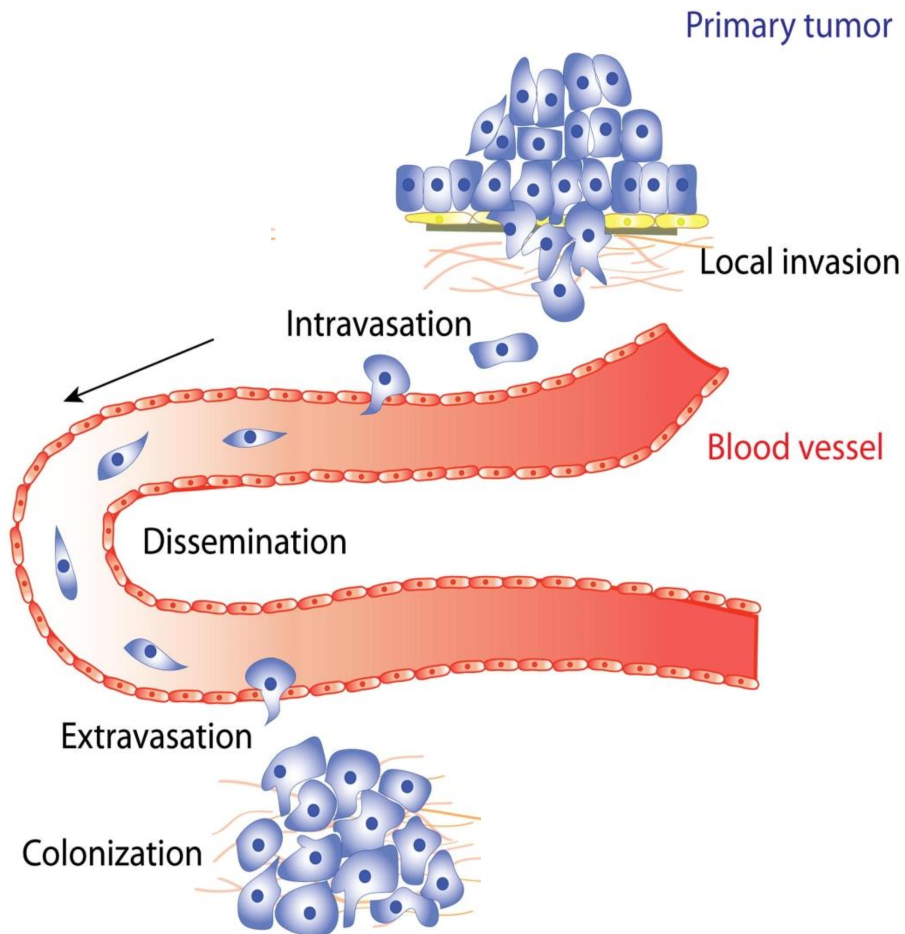


Figure 1.2 A schematic illustration of multisteps process of metastasis.

Metastasis starts with local invasion of tumour cells at the primary tumour site to surrounding tissues. The tumour cells undergo the EMT transformation in order to reach the blood vessel and overcome the immune barrier to survive in the circulation system. Once the tumour cells arrest at the target organ, the cells undergo MET to extravasate for organ colonisation. Image adapted and modified from Saxena and Christofori, 2013.

and vimentin) (Garg, 2013). Once tumour cells gained all these characteristics, invasion of surrounding tissues occurs and the cells start to intravasate into the blood vessel in order to reach circulation system.

In the circulatory system, the tumour cells are now termed as circulating tumour cells (CTCs). In order to survive in circulation, these CTCs need to defy shear forces, immunological barriers and anoikis. Anoikis is defined as induction of cell death after the detachment from primary tumour site or ECM (Guo and Giancotti, 2004). CTCs have been reported to overexpress tyrosine receptor kinase B and Wnt Family Member 2 (Wnt2) to survive anoikis (Douma *et al.*, 2004; Yu *et al.*, 2012). Consequently, blood platelets also facilitate CTCs survival by coagulating the cells and then forming emboli, which protecting the CTCs from immunological attack, for instance, by natural killer cells (Gay and Felding-Habermann, 2011).

After the dissemination of CTCs in circulatory system, extravasation takes place when cancer cells escape the vascular vessels into parenchyma of distant tissues (Hanahan and Weinberg, 2011). Disseminated tumour cells (DTCs) are now in need to overcome new microenvironments such as differences in tissue configuration and stromal composition in distant organ. As in the case of breast cancer metastasised to bone marrow, several soluble factors such as C-X-C motif chemokine 12 and insulin-like growth factor, which are secreted by the bone marrow cells further promote the DTCs survival and growth in new host microenvironment (Müller *et al.*, 2001; Mundy, 2002). This unique ability of cancer cells is critical for adaptation in order to survive in distinct environment (Yilmaz and Christofori, 2010). Therefore, many factors including various gene programming, transition of

epithelial- mesenchymal phenotype and adaptation in different microenvironments are interrelated in these multisteps processes of metastasis cascade.

### 1.2.1 Motility and migration of cancer cells

One of important aspects of metastasis is the ability of cancer cells to move. Movement of cancer cells is crucial in the steps of metastasis, particularly during detachment of the cells from primary tumour site. In order to migrate to distant organ, detachment of cancer cells begins when the cells loss their adhesiveness property followed by disintegration of cell to cell tight junctions, which initiated by downregulation of E-cadherin, an important molecule of adherens junctions in epithelial cells (Yilmaz and Christofori, 2010). Loss of functional E-cadherin is then followed by increase of a mesenchymal marker, N-cadherin, further fuel-up metastasis progression due to established interaction between N-cadherin and numerous growth factors (such as platelet-derived growth factor and fibroblast growth factor) (Suyama *et al.*, 2002; Kong *et al.*, 2008).

Early step for cancer cells to migrate is extension of cell configuration which termed as 'protrusion'. Cell protrusions such as invadopodia were detected in highly invasive carcinoma in which their formations are initiated by localisation of polymerised actin, growth factors and chemotactic signals (Pollard and Borisy, 2003; Yamaguchi *et al.*, 2005). The formation of invadopodia is further assisted by activation of actin regulatory proteins such as cofilin and cortactin, in forming the barbed-end structure and generating branched-actin network of cell protrusions, respectively (Bailly *et al.*, 2001; Goley and Welch, 2006). These extended cell protrusions facilitate cancer cells to overcome physical barriers present in ECM and

degrade the basement membrane of blood vessels which crucial in migration and invasion.

Cancer cells are identified to migrate as a single cell and collectively attached cell streams *in vivo* (Friedl and Alexander, 2012). *In vitro* studies have suggested that the individually migrating cells are controlled by Rho pathway and prefer amoeboid-like migration (Wolf *et al.*, 2003; Sahai and Marshall, 2003). Moreover, Rho GTPases family involves in cell migration and invasion by promoting actin stress fiber and controlling cytockeletal formations, which then affecting cell adhesions (Yilmaz and Christofori, 2010). Other than actin reformation, Rho GTPases also has been shown to be able to activate MMP, which contribute to enhance cancer cells invasion and metastasis (Lozano *et al.*, 2003).

### 1.3 Overview of ion channels

Cell membranes are embedded with variety of proteins including ion channels. Ion channels can be categorised into several type namely, voltage-gated such as sodium, calcium and potassium channel and ligand-gated channel. These ion channels act as barriers that selectively control the movement of ions from/between intracellular and extracellular environment of the cells. Over the past few decades, studies on the ion channels undergo a vast improvement after the development of voltage clamp by Hodgkin and Huxley in which they use it to study electrical changes in a nerve fibre of a giant squid axon responsible in the generation of action potential (Hodgkin and Huxley, 1939). Almost 40 years later, the patch clamp which is an improved version of the voltage clamp was invented by Neher and Sakmann, which then has become one of the most important contributions in the cell physiology field (Neher and Sakmann, 1976). Patch clamp method allowed

measurement of ion flow from a single ion channel by limiting a small area of cell membrane with a miniscule glass pipette tip onto the muscle cells of a denervated frog (Neher and Sakmann, 1976). Later on, the technique was improved by their group and a lot of other electrophysiologists, which then serve as a fundamental tool to study the functional activity of ion channels.

### 1.3.1 Voltage-gated Sodium Channels (VGSCs)

A basic concept of sodium ion ($Na^+$) influx (ion flow into the cells) was highly connected to the rise of membrane potentials was first proved by Hodgkin and Katz, and later on, series of experiments by Hodgkin and Huxley found that the important role of $Na^+$ conductance involved in membrane potential (Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952a; Hodgkin and Huxley, 1952b; Hodgkin and Huxley, 1952c). The gating property of sodium channel was then discovered by Armstrong and Bezanilla, when they successfully measured a small amount of current generated by 'gating particle' by using tetrodotoxin (TTX), a sodium channel blocker derived from pufferfish, which inhibited the movement of ion via sodium channel (Armstrong and Bezanilla, 1973). From there on, unique features and functions/regulations of voltage-gated sodium channels (VGSCs) in excitable and non-excitable cells, as well as in various diseases are slowly elucidated up until now.

#### 1.3.1(a) Structure of VGSCs

In recent years, VGSCs have emerged to be a subject of interest in the study for their functional expression particularly in excitable as well as non-excitable cells. In order to understand the mechanism of VGSCs, it is important to study the

structure first. The discovery of primary structure of subunits of VGSCs was first made by Beneski and Catterall by utilising scorpion toxin together with photoaffinity labelling method, conveyed α and β-subunits of VGSC which sized ~260 kDA and ~30-40 kDA, respectively (Beneski and Catterall, 1980; Catterall, 2000).

VGSCs are transmembrane proteins that composed of a central pore-forming α-subunit with one or more auxiliary β (β1–4)-subunits (Catterall, 2000). Each α-subunit consist of four homologous domains (D1-D4) and every individual domain resides six transmembrane segments (S1-S6) where segment 5 and 6 form the pore, whilst segment 4 is the voltage sensor which is an important component contribute to its major function (Catterall, 2000) (**Figure 1.3**). As illustrated in figure 1.3, whilst α-subunit VGSCs is the central pore-forming of the ion channels, both of the β- subunits are immunoglobulin-like fold shaped and composed of an extracellular N-terminal domain and a C-terminal region in cytoplasmic of the cells (Isom, 2002). Typically, VGSCs form heteromers when coupled with one or several auxiliary β-subunits (Catterall, 2000). Other than functioning as generator for action potential, sodium channel which is not voltage-gated also occurs in cellular physiology for instance, epithelial sodium channel of the EnaC/Degenerin gene family. In term of structure, this type of sodium channel has no relation at all with VGSCs and it carries function of sodium transporter in epithelial and other types of cells (Yu and Catterall, 2003). In this study, we only focus on the one with voltage-gated as VGSCs have many unique features that contribute to its function and mechanism in biology.

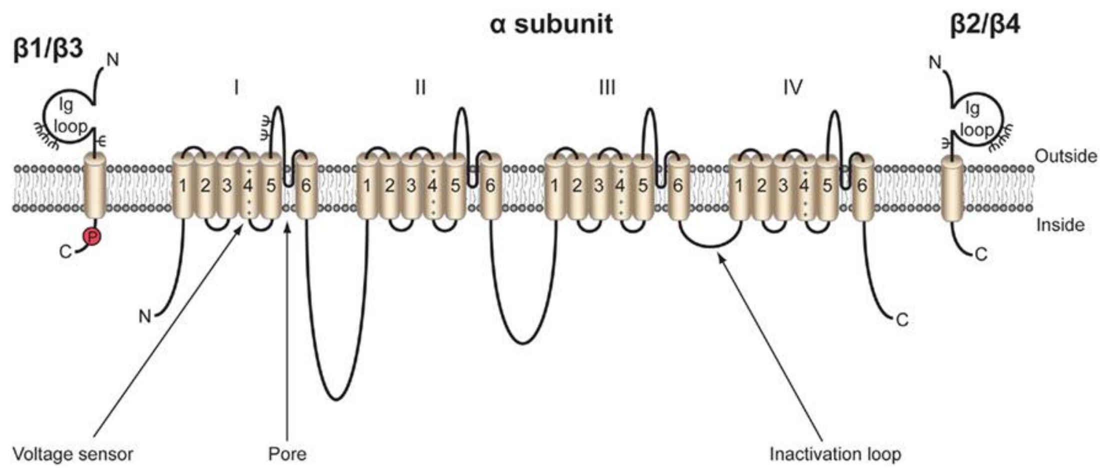


Figure 1.3 Structure of VGSC.

α-helical segments represented by four repetitive domain in cylindrical shaped. Each domain consists of six segments; segment 4 is the voltage sensor and pore-forming lies between segment 5 and 6. The extracellular domains of the β1 and β2 subunits are shown as immunoglobulin-like folds. Ψ, sites of probable N-linked glycosylation; P, sites of demonstrated protein phosphorylation by protein kinase A (red circle). Image adapted from Brackenbury and Isom, 2008.

#### 1.3.1(b) VGSC α-subunits (VGSCα) isoforms

Serve as the core part of VGSC with pore-forming lies within its structure, the α-subunits play a crucial role in generating action potential in membrane of excitable cells since the mRNA encoding α-subunits has been shown to be adequate for the functional expression of VGSCs, thus, in this study, the abbreviation 'VGSCs' represents the α-subunit (Goldin *et al.*, 1986). In mammals, there are ten VGSCα isoforms (Nav1.1-Nav1.9 and Nax) which encoded by genes *SCN1A-SCN11A* (Goldin, 2001) (**Table 1.1**). Each of the isoforms are distributed differently in excitable cells inside peripheral nervous system (PNS), central nervous system (CNS), skeletal muscle and cardiac muscle (Goldin, 2001). In the CNS, the highly expressed isoforms are Nav1.1, Nav1.2 and Nav1.3. Additionally, Nav1.7, Nav1.8 and Nav1.9 are abundantly expressed in the PNS. Nav1.6 is expressed in both of PNS and CNS. VGSC isoforms of Nav1.4 and Nav1.5 are expressed primarily in skeletal muscle and in cardiac muscle, respectively. The tenth isoform of VGSCα, Nax, which is expressed in cardiac muscle and skeletal muscle, has been found to have no functional expression of VGSCs (inactivation gate) but serve as sodium sensor when Nax-knockout mice exhibited deficiency in detecting extracellular salt level in the brain (Hiyama *et al.*, 2002). Although all of the isoforms have similar molecular structure, they composed of different length of amino acids sequence and demonstrated different electrophysiological and pharmacological characteristics when expressed (Plummer and Meisler, 1999; Diss *et al.*, 2004)

Table 1.1 List of isoforms, genes name and tissue distribution of VGSCα (adapted from Goldin, 1999).

| **Protein isoforms** | **Genes symbol** | **Tissue distribution** |
|---|---|---|
| Nav1.1 | *SCN1A* | CNS |
| Nav1.2 | *SCN2A* | CNS |
| Nav1.3 | *SCN3A* | CNS |
| Nav1.4 | *SCN4A* | Skeletal muscle |
| Nav1.5 | *SCN5A* | Cardiac muscle |
| Nav1.6 | *SCN8A* | CNS, PNS |
| Nav1.7 | *SCN9A* | PNS |
| Nav1.8 | *SCN10A* | PNS |
| Nav1.9 | *SCN11A* | PNS |
| Nax | *SCN6A, SCN7A* | Cardiac muscle, skeletal muscle |

Abbreviations: CNS, central nervous system; PNS, peripheral nervous system

#### 1.3.1(c) Basis function of VGSCα

Activation of VGSCs are driven by modified electrical field from charged residues that move from outer environment which then creates voltage-dependence activation (Hodgkin and Huxley, 1952). The core part of VGSCs, the segment 4 (S4) which functions as voltage sensor, is composed of repeated motifs of positively charged residue (generally arginine) and two other hydrophobic residues (Catterall, 2012). The arrangement of these residues possibly create a helical conformation of positive charge through this channel (Yu and Catterall, 2003). Modification of electrical field of these charged residues lead to its movement to the outer environment which then creates voltage-dependent activation (Hodgkin and Huxley, 1952). Interestingly, several studies have shown that during the transition from resting (inactivated) to activated state, the S4 of VGSCs move outward in order to exchange/transport the ion/charges during membrane depolarisation (Yang and Horn, 1995; Yang *et al.*, 1996; Chanda and Bezanilla, 2002)

All of the four VGSCα domain are connected by two large interdomain (ID) loops for instance ID1-2 and ID2-3 and a short loop between ID3-4 **(Figure 1.4)** (Plummer and Meisler, 1999). VGSCs exist in three basic states: 1) Activated (open), 2) Deactivated (closed) and 3) Inactivated (closed) (Chichili *et al.*, 2013). During action potential, the channel is activated by external electrical signal and then closed rapidly (inactivated state) within milliseconds to stop another signal coming in (Chichili *et al.*, 2013). Previously, Vassilev and his group have proven that short loop between ID3 and ID4 is important for fast inactivation of the channels using side-directed anti peptide antibodies (Vassilev *et al.*, 1988). Additionally, they found that this loop undergoes conformational change by folding into the channel to prevent accessibility to antibodies during transition from

Figure 1.4 Detailed structure of VGSCα.

VGSCα composed of 4 interconnected domains by loops. The N and C- terminus, ID1-2 and ID2-3 carry function of protein interaction modulation whilst ID3-4 is responsible in fast inactivation of VGSC. Image adapted from Diss *et al*., 2004.

activated to inactivated state of VGSCs (Vassilev *et al.*, 1988). Other study conducted by Stuhmer and his group support the involvement of this ID3 and ID4 loop in fast inactivation when the cleavage of the loop resulted in slow inactivation rate (Stühmer *et al.*, 1989). These findings suggest that the loop between ID3 and ID4 are important in fast inactivation since this process is crucial for repetitive firing during action potential in nerve system and excitable cells (Catterall, 2000). The channels are in deactivated state when there is no external signals present (Chichili *et al.*, 2013).

Other important element during fast inactivation is amino acid residues in intracellular pore region. Three amino acid residues, isoleucine, phenylalanine and methionine (IFM), serve as pore blocker by binding to its receptor in the pore segment (West *et al.*, 1992). The hinged-lid mechanism of inactivation gate was proposed by Kellenberger and colleague when they found that glycine and proline residue flanking the IFM motif had allowed the inactivation gate to move during inactivation process (Kellenberger *et al.*, 1997) (**Figure 1.5**). This hinged-lid model and the intracellular loop connecting D3 and D4 are interconnected in a way that interaction with receptor sites of hydrophobic IFM motif resulted in pore-blocking when the loop is folded into the channel during inactivation of VGSCs (Kellenberger *et al.*, 1997).

#### 1.3.1(d) VGSC β-subunits (VGSCβ) family

Previously, the expression of VGSCα alone is found to be sufficient for functional expression of VGSCs (Goldin *et al.*, 1986). However, VGSCβ also

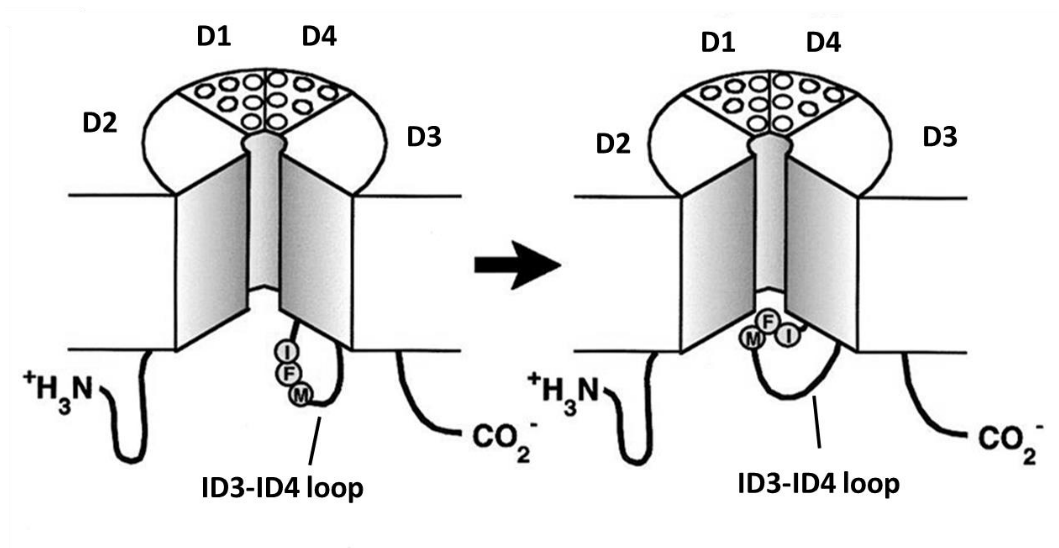


Figure 1.5 Mechanism of inactivation of VGSCs.

The intracellular loop connecting domains III and IV of the VGSCs is depicted as forming a hinged lid. The critical residue phenylalanine (F) is shown occluding (blocking) the intracellular mouth of the pore during the inactivation process. Image adapted and modified from Catterall, 2000.

contributes a vital role in kinetics of VGSCs and VGSCs expression on cell surface (de Lera Ruiz and Kraus, 2015). The VGSCβ belong to the immunoglobulin (Ig) superfamily of cell adhesion molecule (CAM) which majorly involved in cell adhesion related activities (Isom *et al.*, 1995; Yu *et al.*, 2003). Each of individual VGSCβ consists of extracellular N-terminal domain and a C-terminal region in cytoplasmic of the cells (Isom, 2002). Similar to the VGSCα, each member of the VGSCβ is identical in term of structures but the subunits consist of different length of amino acid sequence (Catterall, 2000).

In mammalian, there are four members of VGSCβ, β1-β4, which encoded by *SCN1B-SCN4B* genes, respectively (Brackenbury and Isom, 2008). In nervous system, VGSCβ are expressed in excitable and non-excitable cells for instance in PNS, inside glia in CNS and adrenal gland (**Table 1.2**). The β1 subunit shares 43% homology with β3, whilst the β2 and β4 subunits share 35% homology identity (Diss *et al.*, 2004). In α-subunit, β1and β3 are non-covalently attached to the region whilst β2 and β4 subunits bound to α region by covalently disulphide-linked (Isom *et al.*, 1995).

#### 1.3.1(e) Basis function of VGSCβ

The VGSCβ are essential in refining normal kinetics and voltage dependence of gating of VGSCs even though the expression of VGSCα is enough for channel functional expression (Isom *et al.*, 1992; Isom *et al.*, 1995). Basis function and role of VGSCβ are slowly deciphered after Isom and his group were the first to successfully clone the complementary DNA encoded β1 subunit from the rat brain by using polymerase chain reaction and library screening methods (Isom *et al.*, 1992). They discovered the co-expression of β1-subunit with α-subunit increase the

Table 1.2 List of proteins, genes name and tissue distribution of VGSCβ (adapted from Brackenbury and Isom, 2008).

| **Protein** | **Genes symbol** | **Tissue distribution** |
|---|---|---|
| β1 | *SCN1B* | CNS, PNS, cardiac, skeletal muscle |
| β2 | *SCN2B* | CNS, PNS, cardiac |
| β3 | *SCN3B* | CNS, PNS, adrenal gland, kidney |
| β4 | *SCN4B* | Skeletal muscle, cardiac, CNS, PNS |

Abbreviations: CNS, central nervous system; PNS, peripheral nervous system

peak of sodium current, increase inactivation rate and shifting voltage-dependence inactivation of VGSCs in rat brain (Isom *et al.*, 1992). Consequently, other evidences also showed the VGSCβ also enhanced the channel gating property of VGSCs (Patton *et al.*, 1994; Makita *et al.*, 1996; Morgan *et al.*, 2000).

Other than conducting function of membrane excitability with VGSCα, the VGSCβ also involve in a numerous cell adhesion related activities (Isom *et al.*, 1995; Yu *et al.*, 2003). VGSCβ has been shown to interact with other cell adhesion molecules (CAMs) and proteins of extracellular matrix (ECM), for instance, β1 interacts with neurofascin, nodal CAM, ankyrin, β2 and contactin (Ratcliffe *et al.*, 2001; McEwen and Isom, 2004; Kazarinova-Noyes *et al.*, 2001). Interestingly, interaction of VGSCβ with contactin, β2 subunit and neurofascin have contributed to sodium current enhancement (Kazarinova-Noyes *et al.*, 2001; Ratcliffe *et al.*, 2001). Therefore, those evidences have proven VGSCβ dependent adhesion involves in the regulation of VGSCα excitability.

### 1.3.2 Alternative splicing of VGSCs

Alternative splicing is an event of generating more transcripts encoding protein with/without modification of novel function, which contribute to increase of protein diversity (Graveley, 2001). As for VGSCs, this splicing event in specific subtypes generates multiple gene isoforms and increases the functional diversity of this channel (Diss *et al.*, 2004).

In mammals, the involvement of two alternative exons (5' and 3' genomic) encoding part of domain 1, segment 3 (D1:S3) and domain 1, segment 3 to segment 4 (D1:S3-S4) extracellular linker are example of alternative splicings occur in the

VGSCα genes (Diss *et al.*, 2004; Onkal *et al.*, 2008). The 5' genomic and 3'genomic alternatives in exon 6 are generally differentiated by the absence or presence of an aspartate residue in 5'-exon and 3' exon, respectively, at the S3-S4 linker (Onkal *et al.*, 2008). Previously, two groups of researcher, Gustafon et al and Sarao et al, were the first to show this splicing event in two VGSCs isoforms, Nav1.2 and Nav1.3 in rat brain (Sarao *et al.*, 1991; Gustafson *et al.*, 1993). They found that the splicing was considered to be developmentally regulated because the transcripts consisting of upstream 5'exon were plentiful at birth but then were immediately replaced by transcripts consisting of downstream 3'exon after post-natal day ten. With regards to this discovery, the term 'neonatal' was named for channels with 5'-exon variant and channels with 3'-exon variant was termed as 'adult' (Sarao *et al.*, 1991; Gustafson *et al.*, 1993). In term of functional aspect, the neonatal splice variant was found to have different electrophysiological modulation of VGSCs, for instance, when compared to the adult Nav1.2, the neonatal Nav1.2 exhibited a small hyperpolarised activation and steady-state inactivation of the channel (Auld *et al.*, 1990). This alternative splicing of D1:S3 also occur in other VSGC isoforms of Nav1.1 (Copley, 2004), Nav1.5 (Diss *et al.*, 2004), Nav1.6 (Plummer *et al.*, 1998) and Nav1.7 (Raymond *et al.*, 2004).

Other splicing event also has been found in the longest interdomain region ID1-2 of VGSCα isoforms including Nav1.1, Nav1.3, Nav1.6 and Nav1.7, which resulted in two Nav1.1 isoform (1 and 1A)(Schaller *et al.*, 1992), three isoforms of Nav1.3 (3, 3A and 3B) (Schaller *et al.*, 1992), two isoforms of Nav1.6 (8 and 8A) (Dietrich *et al.*, 1998; Plummer *et al.*, 1998) and two Nav1.7 isoforms (9 and 9A) (Raymond *et al.*, 2004). Splicing in ID1-2 also could have more functional effect

because a possible phosphorylation sites also resides within this region (Diss *et al.*, 2001).

Alternative splicing can also occur in VGSCβ. One of the VGSCβ, β1 subunit (encoded gene SCN1B) has been reported to have species-specific alternative splicing. The splicing of this subunit via retention of intron 3 encoding C-terminus and a stop codon produced two splice variant, β1 and β1B (also called β1A in rat) (Kazen-Gillespie *et al.*, 2000; Qin *et al.*, 2003). In rats, the β1A splice variant is found to be developmentally regulated in brain. Although the β1A in rat and β1B in human share similar splicing pattern and regulatory properties, they are totally different in terms of expression pattern and sequence, for instance, the C-terminal region of both β1A and β1B share less than 33% of sequence identity (Qin et al., 2003).

### 1.3.3 Post-translational modification of VGSCs

Other than conducting permeation of $Na^+$ influx for activation and inactivation of channel, extracellular and cytoplasmic region of VGSCα also subjected to post-translational modification for such glycosylation and phosphorylation, respectively (Diss *et al.*, 2004). Extracellular pore-lining regions of D1 and D3 consist of multiple glysosylation sites and carbohydrate (mostly sialic acid) has been shown to be involved in glycosylation, which is important in maintaining cell surface localisation and expression in VGSCs (Marban *et al.*, 1998; Bennett, 2002). In addition, glysosylation modulation are subtypes specific; Nav1.1, Nav1.2, Nav1.3 and Nav1.4 are highly glycosylated with 15-30% of carbohydrate, whereas only 5% carbohydrate in Nav1.5 and Nav1.9 (Tyrrell *et al.*, 2001; Diss *et al.*, 2004). Other than that, glycosylation also have been reported to modify the