TARGETING REGULATORY PATHWAYS OF *STIM1* IN NASOPHARYNGEAL CANCER

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TARGETING REGULATORY PATHWAYS OF *STIM1* IN NASOPHARYNGEAL CANCER

by

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LIST OF SYMBOLS

a	Alpha
ß	Beta
G	Gram
°C	Degree Celsius
kDa	Kilodalton
µg	microgram
µg/mL	Microgram/milliter
µL	Microliter
µML	Micromolar
mg	milligram
Ml	microliters
mM	Millimolar
mL	Milliliter
ng	nanogram
Nm	Nanometer
%	Percentage
Rpm	Revolutions per minute
RT	Room temperature
V	Volt
w/v	Weight/volume

LIST OF ABBREVIATIONS

Ago2	Argonaute 2
AKT 1	RAC-alpha serine/threonine-protein kinase
ATCC	American Type Culture Collection
ATP2A2	ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 2
β-actin	beta actin
BCL 2	B-cell lymphoma 2
BSA	Bovine serum albumin
carboxy-H2DCFDA	carboxy 2',7'-dichlorodihydrofluorescein diacetate
CDKN1B	Cyclin-dependent kinase inhibitor 1B
cDNA	complementary DNA
CO_2	carbon dioxide
CRC	Colorectal cancer
diH2O	deionized H2O
DMSO	Dimethyl sulfoxide
DsiRNA	Dicer-Substrate Short Interfering RNAs
dsRNA	Double-stranded RNA
dsRNA	double stranded RNA
DPBS	Dulbecco phosphate buffered-saline
EBV	Epstein-Barr virus.
CDH1	E-cadherin
EDTA	Ethylenediaminetetraacetic acid

EMT	Epithelial mesenchymal transition
ER	endoplasmic reticulum
ESCC	Esophageal squamous cell carcinoma
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanin cytosine
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HPRT-1	Hypoxanthine-guanine phosphoribosyl transferase
HRP	horseradish peroxidase
IHC	Immunohistochemistry
IP3	inositol triphosphate
KEGG	KEGG: Kyoto Encyclopedia of Genes and Genomes
LTBR	Lymphotoxin beta receptor
miRNA	MicroRNA
mRNA	messenger RNA
mPTPs	mitochondrial-permeability-transition pores
NCBI	National Centre for Biotechnology Information
NPC	Nasopharyngeal cancer
ORAI1	Calcium release-activated calcium channel protein 1
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PI3K/Akt	Phosphatidylinositol 3-kinase/protein kinase-B
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PVDF	poly-vinylidene difluoride
Rac1	Ras-related C3 botulinum toxin substrate 1

RIPA	Radio-immunoprecipitation assay
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleotide protein complex
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
RYR2	Ryanodine receptor 2
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SKP2	S-phase kinase-associated protein 2
STIM1	Stromal interaction molecule1
TAR	Trans-activation response
TBS	Tris buffered saline
TBS	Tris-buffered saline
TBST	Tris buffered saline tween
TEMED	Tetra methyl ethylene diamine
TGF-β1	Transforming growth factor beta 1
UTRs	Untranslated regions
VOCs	voltage-operated channels

LIST OF APPENDICES

MENSASARKAN PENGAWALATURAN *STIM1* DAN LALUANNYA DALAM KANSER NASOFARINKS

ABSTRAK

Molekul interaksi stromal 1 (*STIM1)* terlibat dalam pelbagai proses karsinogenik. Walau bagaimanapun, penglibatan dan implikasi molekul *STIM1* dalam kanser nasofarinks (NPC) tidak difahami sepenuhnya. Kajian ini bertujuan untuk menyelidiki peranan pengawalseliaan *STIM1* dan laluan isyarat yang berkaitan di Kanser nasofarinks, menggunakan model penyah fungsian gen yang dimediasi DsiRNA. Titisan-titisan sel NPC yang digunakan adalah sel-sel yang berkaitan dengan HK1/NPC-Non EBV dan C666-1/NPC EBV. Gen-gen dan laluan-laluan fungsian yang berkaitan dinilai melalui analisis bioinformatik. Ekspresi *STIM1* serta kesan transkripsi dan pasca-transkripsi penyahfungsian *STIM1* di titisan-titisan sel NPC ditentukan melalui RT-qPCR. Peranan penyahfungsian *STIM1* dikaji dari segi percambahan sel, penghijrahan sel, kemampuan pembentukan koloni, profil kalsium dan aktiviti spesies oksigen reaktif. Kemudian, kesan pasca-terjemahan *STIM1* dalam NPC dikaji melalui Western blot dan pewarnaan imunohistokimia. Ekspresi *STIM1* yang rendah dalam HK1/NPC serta ekspresi *STIM1* yang tinggi dalam C666-1/NPC dapat dilihat berdasarkan perbezaan dalam patogenesis tumor NPC. Penyahfungsian *STIM1* yang optimum dicapai pada 12 jam dan berterusan selama 72 jam di HK1/NPC, sementara penyahfungsian *STIM1* yang optimum bagi C666-1/NPC dicapai pada 48 jam dan berterusan selama 72 jam. Penyahfungsian *STIM1* mengawal selia ekspresi *PIK3CA, AKT1, Orai1, BCL2* dan *SKP2*, yang dikaitkan dengan NPC. Penyahfungsian *STIM1* juga mengawal selia *BAX*, *P27*, *PTEN*, *RYR2*, *ATP2A2* dan *CDH1*, yang dikaitkan dengan perencatan NPC. Kajian kami menunjukkan penyenyapan *STIM1*

mampu mengawal selia ekspresi miRNA 205-3p, miRNA 185-5P, mir-34a-5p, let-7b-5p, miRNA 200a-3p dan miRNA375 setelah transkripsi, menunjukkan prognosis yang baik. Penyenyapan *STIM1* mengakibatkan penindasan protein-protein STIM1, RAC1 dan ORAI tetapi meningkatkan tahap protein-protein FOXO3a dan CDKN1B(P27). Analisis lanjutan terhadap pewarnaan imunohistokimia menunjukkan ekspresi sederhana *STIM1* dalam NPC bukan keratinising dibezakan, manakala NPC bukan keratinising tidak dibezakan menunjukkan intensiti pewarnaan yang kuat. Kesimpulannya, kajian ini mencadangkan *STIM1* mempunyai hubung-kait dengan kesan kawal-selia banyak laluan serta *STIM1* adalah sasaran yang berpotensi untuk NPC. Oleh itu, menilai hubungan antara parameter patologi klinikal terhadap tahap protein NPC dan STIM1 in vivo adalah sangat penting.

TARGETING REGULATORY PATHWAYS OF STIM1 IN NASOPHARYNGEAL CANCER

ABSTRACT

Stromal interaction molecule 1 (*STIM1)* is involved in various carcinogenic processes. However, the involvement and molecular implication of *STIM1* in nasopharyngeal cancer (NPC) is not fully understood. This study aims to investigate the regulatory roles of *STIM1* and associated signaling pathways in NPC, using a DsiRNA-mediated gene knockdown model. The NPC cell lines used were HK1/NPC-Non EBV- and C666-1/NPC EBV-related cells. Genes and related functional pathways were assessed through bioinformatics analysis. *STIM1* expression and the transcriptional- and post-transcriptional effects of *STIM1* knockdown in NPC cell lines were determined through RT-qPCR. The functional roles of *STIM1* knockdown were studied in terms of cell proliferation, cell migration, colony formation ability, calcium profiles and reactive oxygen species activities. Then, the post-translational effect of STIM1 in NPC were studied through Western blot and immunohistochemical staining. Low *STIM1* expression in HK1/NPC and high *STIM1* expression in C666-1/NPC were observed because of differences in NPC tumour pathogenesis. Optimum *STIM1* knockdown was achieved at 12 h and sustained for 72 h in HK1/NPC, whereas optimum *STIM1* knockdown for C666-1/NPC was achieved at 48 h and sustained for 72 h. *STIM1* knockdown regulated the expression of *PIK3CA, AKT1, Orai1, BCL2* and *SKP2*, which were associated with NPC. *STIM1* knockdown regulated the expression of *BAX*, *P27*, *PTEN*, *RYR2*, *ATP2A2* and *CDH1*, which were associated with the inhibition of NPC. Our findings showed that *STIM1* silencing can regulate the expression of miRNA 205-3p, miRNA 185-5P, mir-34a-5p, let-7b-5p, miRNA 200a- 3p and miRNA375 after transcription, indicating good prognosis. *STIM1* silencing resulted in the suppression of STIM1, RAC1 and ORAI

proteins but increased FOXO3a and CDKN1B(P27) protein levels. Further analysis on immunohistochemical staining showed moderate STIM1 expression in non-keratinising differentiated NPC, and non-keratinising undifferentiated NPC showed strong staining intensity. In conclusion, this study suggests that STIM1 has an interlinking association with regulatory effect of many pathways and *STIM1* is a potential target for NPC. Thus, evaluating the association between the clinicopathological parameters of NPC and STIM1 protein levels in vivo is crucial.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nasopharyngeal cancer (NPC) is commonly diagnosed at the advanced stage with poor prognosis. NPC treatment is complex because of NPC's multiple risk factors. The current treatment option for NPC is concomitant chemotherapy and radiation therapy. However, treatment of NPC at advanced stage is challenging and associated with high fatality rate. The risk factors associated with NPC are Epstein–Barr virus (EBV) infection, lifestyle, and dietary factors. In Malaysia, NPC ranked as the fourth most common cancer in 2020 with 2.222 new cases, 1.450 deaths, an age-standardised rate of 6.3 per 100,000 and an age-standardised mortality rate of 3.7 people per 100,000 (Lao and Le, 2020). NPC prognosis is poor because of late lesions, which are attributed to a poor understanding of its molecular pathogenesis. The pathophysiology of cancers and unsuccessful treatment are associated with oxidative stress responses (Biswas, 2016).

The association between the molecular mechanisms of NPC and oxidative stress is still not clear; therefore, understanding the relationship might provide a suitable therapeutic target for NPC. The deregulation of the oxidant equilibrium generates oxidative stress, which alters and damage intracellular molecules. High oxidative conditions trigger a deregulated endoplasmic reticulum (ER) Ca^{2+} release, which induces oxidation of proteins, DNA and lipids leading to cellular damage and impaired repair mechanism (Dai *et al.,* 2017). Therefore, understanding the molecular basis of ER stress in NPC and its interactions with Ca^{2+} pathways is necessary. Stromal interaction molecule 1 (STIM1) protein is a Ca^{2+} sensor that regulates calcium homeostasis and is consistently deregulated in various malignancies (Wang *et al.,* 2017).

STIM1 has two EF hand motifs of which one of the EF hand motifs binds Ca^{2+} while the second EF hand motif does not bind Ca^{2+} (Soboloff *et al*., 2012). and associated with the regulation of mitochondrial bioenergetics and oxidative stress. Oxidative stresses are linked to increased reactive oxygen species (ROS) formation, the oncogenic signaling in cancer cells sustains ROS levels via the involvement of antioxidant activities. (Poprac *et al.,* 2017). Furthermore, the oncogenic activities of STIM1 in cancers occurs via aberrant expression, whereas its suppression inhibits cancer development (Xu et al., 2015a; Zhang et al., 2015). Previous studies reported that *STIM1* deregulation associated with induce hyperactivities in cancers, such as cell proliferation, migration, cell cycle alteration and inhibition in apoptosis (Yang *et al.*, 2013; Jardin *et al.*, 2018). Till date, the functions of STIM1 in NPC is not clear. Present work studied *STIM1* regulatory behavior using DsiRNA-mediated gene knockdown on NPC cell lines model; HK1/NPC-Non EBV- and C666-1/NPC EBV-related cells. Targeting STIM1 will provide an insight into the molecular effects especially oxidative stress, NPC pathophysiology and potential molecular target development that could prevention or reduce NPC cases.

1.2 **Problem statement**

1. STIM1 expression is altered in cancers. Previous evidence has revealed that deregulated *STIM1* expression contributes to tumorigenesis.
2. Deregulated expression of STIM1 in cancers results in elevated calcium influx which promotes carcinogenesis by increasing migration, colony formation. proliferation, cell cycle alteration, apoptosis inhibition and has been implicated in the promotion of cancer development.
3. The functions of STIM1 in NPC is largely unclear. Therefore, this study aims to examine the functional effects of *STIM1* expression in NPC.

1.3 Objectives

General objective:

To study the regulatory roles of STIM1 and its associated pathways in NPC.

Specific objectives

1. To evaluate the expression of *STIM1* in cell lines and determine the best target point, percentage GC content and time point for Dicer substrate small interfering RNA (DsiRNA)-mediated *STIM1* knockdown in NPC cell lines
2. To elucidate STIM1 regulatory pathways in NPC using bioinformatic platform.
3. To evaluate the effects of *STIM1* knockdown on the transcriptomic expression of genes in apoptosis, cell cycle, PI3K/AKT and calcium signalling pathways and the post-transcriptional effects of *STIM1* knockdown.
4. To assess the functional effects of *STIM1* on the cell proliferation, migration, colony formation ability, calcium deposition, intracellular calcium levels and reactive oxygen species (ROS) in NPC cell lines after *STIM1* knockdown
5. To determine the post-translational expression of STIM1, ORAI1, FOXO3a, RAC1 and CDKN1B proteins in NPC after *STIM1* knockdown
6. To evaluate the expression of STIM1 protein in NPC tissues.

CHAPTER 2

LITERATURE REVIEW

2.1 Nasopharyngeal cancer

NPC is a highly metastatic cancer that arises at the nasopharynx, behind the nasal cavity and above the mouth (Tsao *et al.*, 2014; Tu *et al.*, 2016; Okekpa *et al.*, 2019) (Figure 2.1). NPC has dissimilar geographical distribution, and Asia has the highest NPC occurrence rate (Kontos *et al.*, 2013; Tu *et al.*, 2016; Mahdavifar *et al.*, 2016). The current treatment option for NPC is the combination of chemotherapy and radiotherapy (Colevas *et al.*, 2018). However, the success of NPC treatment depends on the NPC stage (tumour, node and metastasis [TNM] stages) (Tulalamba and Janvilisri, 2012; Colevas *et al.*, 2018). NPC treatment is mostly initiated at the advanced stages because of late diagnosis and partly because of insufficient knowledge about NPC's molecular pathogenesis (Hsu *et al.*, 2014).

NPC incidence is three folds more in males than female (Bray *et al.,* 2018; Hamid, 2021). The dominance of NPC incidence in males can be associated with gender and biological differences or dissimilarity in lifestyles and environmental factors, such as alcohol consumption, smoking and exposure to hazardous occupations (Jia and Qin, 2019). NPC incidence in low-risk groups increases with age because older people have higher susceptibility and are associated with more exposure to carcinogens early in life (Wang *et al.,* 2004; Schottenfeld, and Fraumeni, 2006). NPC takes years to metastasize after the appearance of the signs; thus, persistent exposure to carcinogens contributes to NPC development (Jia and Qin, 2019).

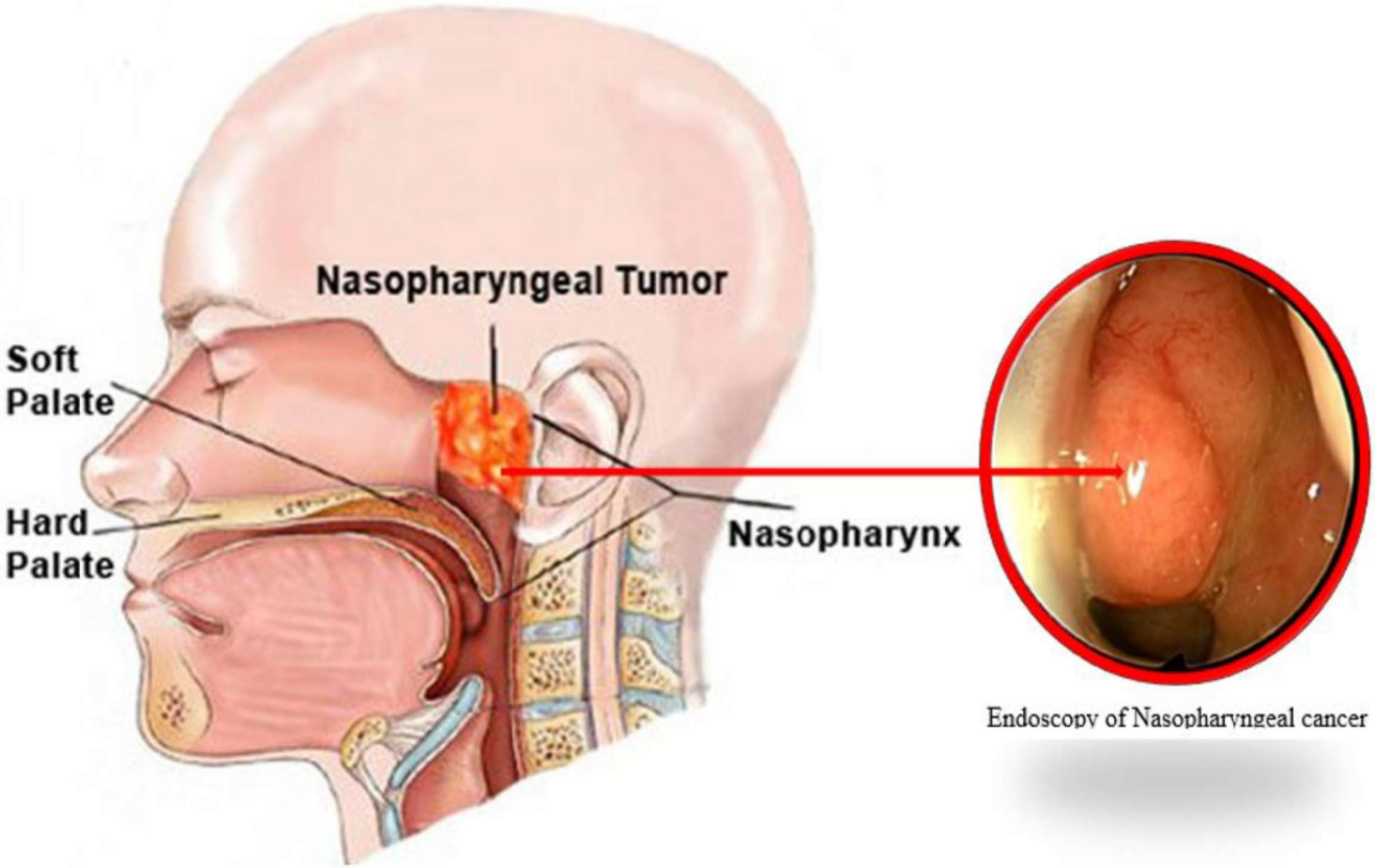

Figure 2.1 **NPC location observed using endoscopy approach.** NPC is located at the nasopharynx, behind the nasal cavity and above the mouth (https://imunews.imu.edu.my/wp-content/uploads/2015/03/Cancer-2.jpg 10 September 2020).

2.1.1 Global prevalence and mortality of NPC

Globally, the prevalence of new NPC cases in 2018 was 129,079, amongst which 93,416 (72.37 %) are males and 35,663 (27.63 %) are females. In the same year, 72,987 individuals, including 54,280 (74.37 %) males and 18,707 (25.63 %) females, died from NPC. In Southeast Asia, 34,681 new NPC cases, which include 25,895 (74.67 %) men and 8,786 (25.33 %) women, were recorded in 2018; amongst these cases, 22,231 individuals, including 15,057 males and 5,174 females, died from NPC (Lao and Le, 2020). In Malaysia, NPC was rated the fifth most common cancer in 2020 with 2.222 new cases and 1.450 deaths (Lao and Le, 2020). NPC is not prevalent in Japan, Latin America, and most Western countries, wherein the occurrence rate of NPC is less than 1 per 100,000 people (Bray *et al.,* 2018).

Global NPC incidence is fewer than 1 for every 100,000 person in many years, but, Taiwan, has the incidence of about 2.8–6.6 for every 100,000 persons (Fan et al., 2018). The five Asian countries with the highest standardised incidence of NPC are Brunei, Singapore, Indonesia, Malaysia and Vietnam, which have standardised NPC incidence rates of 9.9, 6.7, 6.6, 6.3 and 5.7 for every 100,000 people, respectively (Lao and Le, 2020). The five Asian countries with the highest NPC-related mortality are Brunei, Indonesia, Timor-Leste, Viet Nam and Malaysia, which have standardised rates of 5.8, 4.3, 4.0, 3.9 and 3.7 per 100,000 people, respectively (Lao and Le, 2020). In high NPC endemic areas, non-keratinizing-carcinoma (NKC) histotype of NPC is more prevalent whereas, keratinizing squamous-cell carcinoma (KSCC) histotype is more prevalent at low endemic risk regions (Carioli *et al.*, 2017).

2.1.2 NPC Situation in Malaysia

NPC has been shown as one of the top five cancers in Malaysia Since 1966 (Linton *et al*., 2021). In early 80s (1981 and 1982), NPC was recorded as the second most prevalent cancer in Sarawak males (AK, 1985; Linton *et al*., 2021). Precisely, the incidence rate of NPC between January 1981 and December 1982, in Bidayuh, (formerly known as Land Dayak in Sarawak) was 9.0 per 100,000 for each year, while Sarawak Chinese had 5.8 per 100,000 (AK, 1985; Linton *et al*., 2021). NPC was the most common cancer in the Sarawak males of Iban or Bidayuh ethnicity between 2007 to 2011 (Sarawak Cancer Registry, Malaysia, 2017). NPC became the fifth commonest cancer amongst Malaysian residents between 2012 to 2016 and predominantly found in male. It is the fourth most prevalent cancer in Chinese Malaysian males and has top incident rate in Sarawak males (Azizah *et al.,* 2019).

The prevalence rate of NPC in Sarawak doesn't reflect the incidence rate in other Malaysia states (Linton *et al*., 2021). Other race in Malaysia such as Melanau, Malay and Chinese also have NPC risk. Age standardised rate (ASR) of NPC in Sarawak between 2007 to 2011 was 11.6 for every 100,000 population in males and 4.8 for every 100,000 in females, with ASR-ratio of 2.4:1 in male to female (Sarawak Cancer Registry, Malaysia, 2017). Precisely, in Bidayuh males the has 24.6 for every 100,000 and 9.3 per 100,000 for females (Sarawak Cancer Registry, Malaysia, 2017). The ASR at Iban was 12.0 for each 100,000 males and 4.7 for each 100,000 females. For the Chinese, there were 11.2 for every 100,000 males and 4.1 for every 100,000 of females. Malay males have 8.1 per 100,000 ASR and 4.5 per 100,000 ASR in females (Sarawak Cancer Registry, Malaysia, 2017).

Additionally, Melanau has ASR of 4.7 per 100,000 for males and 1.9 per 100,000 for females (Sarawak Cancer Registry, Malaysia, 2017). The incidence ratio of NPC in males is 2-3 folds higher than in females. In 2020, GLOBOCAN estimated that the world ASR was 2.2 per 100,000 and 0.8 per 100,000 for male and females respectively (Ferlay *et al.,* 2020). The higher prevalent rate of NPC in male than in females might be associated with susceptibility of the X-chromosome or oestrogen (Xie *et al*., 2013; Zuo *et al.,* 2019), as well as lifestyle and environment factors (Tang *et al.,* 2016).

Previous study in NPC at Pahang referral hospitals Malaysia from 2012 to 2017 revealed that 143 new NPC cases were reported with ratio of 2.9 male to 1 female (Azizah et al., 2017). Pahang has mean ASR of 2.4 per 100,000 for males and 0.9 per 100,000 for females (Ahmad *et al.,* 2021). Malaysian National Cancer Registry 2012-2011 reported that an average of 900–1,000 cases was obtained each year from 2007–2011 with ASR of 6.4 per 100,000 for males and 2.2 per 100,000 for females. The Malaysian ethnic nationality with higher ASR was Chinese males with AR of11.0 per 100,000, Malay males with 3.3 per 100,000 and Indian males with 1.1 per 100,000(Ahmad *et al.,* 2021).

2.1.3 Risk factors of NPC

NPC has a multifactorial risk factor, which includes race, EBV infection, genetic susceptibility, diet consumption, lifestyle, and environmental factors. Tobacco smoking, salted fish consumption, preserved food and alcohol consumption are remarkably associated with NPC risk (Jia *et al.*, 2010; Polesel *et al.*, 2013; He *et al.*, 2015; Lourembam *et al.*, 2015; Ren *et al.*, 2017; Yong *et al.*, 2017; Okekpa *et al.*, 2019; Okekpa *et al.*, 2020). A study reported that the prevalence rate of NPC can be increased by the consumption of nitrosamines in preserved foods, such as salted fish (Lo *et al.*, 2004; Chattopadhyay *et al.*, 2017). An increase in NPC prevalence is considerably associated with salted fish consumption (Zheng *et al.*, 1994; Kataki *et al.*, 2011; Yong *et al.*, 2017). The processes involved in the preservation of salted fish generates carcinogenic nitrosamines (Ekburanawat *et al.*, 2010; Chattopadhyay *et al.*, 2017; Yong *et al.*, 2017).

Nitrosamine performs oxidising activities in cells, which reduces the levels of intracellular glutathione (GSH), inhibit antioxidant activities, enhance lipid peroxidation and nitric oxide release, and create oxidative stress (Waly *et al.,* 2018). Meta-analysis revealed that salted fish consumption has remarkable association with NPC development (Yong *et al*., 2017; Mydin and Okekpa, 2019). Multivariate analysis revealed that salted fish consumption substantially raises NPC risk (Yong *et al*., 2017). Chinese style salted-preserved fish causes NPC, and its mechanism for carcinogenesis is mediated by exposure to N-nitrosamines, EBV reactivating constituents, direct geno-toxins, and mutagens ((IARC 2012; Chang *et al.,* 2021). Early childhood exposure to salted fish consumption and other *N*-nitrosamines containing food was linked with carcinogenesis of NPC because N-nitrosamines can activate latent EBV or act as an independent carcinogen (Mimi *et al.,* 1986; Mimi and Yuan, 2002).

NPC incidences increase with age in non-endemic regions while the in high-risk countries such as China, the highest NPC incidence occur at ages between 45 to 54 years. However, early exposure to EBV and carcinogens may cause younger NPC onset (Parkin *et al.,* 2002; Argirion et al., 2020). In non-NPC endemic countries, frequent alcohol consumption and cigarette smoking was associated with the risk of keratinizing squamous-cell carcinoma (Vaughan *et al.,* 1996; Barrett *et al.,* 2019; Argirion et al., 2020). Previous studies suggested that alcohol consumption increases NPC risk, and the frequency of alcohol consumption is positively associated with NPC risk (Du *et al.,* 2019; Mydin and Okekpa, 2019). Alcohol consumption is linked with NPC risk because the metabolite of alcohol called acetaldehyde binds to DNAs to form carcinogenic adducts (Seitz and Mueller, 2015; Mydin and Okekpa, 2019). Smoking is associated with squamous cell NPC. Tobacco smoke has also been linked to NPC risk because it contains high levels of nitrosamines (Xue *et al.*, 2013; Xie *et al.*, 2015; Ren *et al.,* 2017).

The carcinogenic mechanism of tobacco smoke occurs through the direct interaction between nasopharynx epithelium and chemicals in smoke. Long-term cigarette smoke inhalation is also associated with the high risk of developing keratinising NPC (Vaughan *et al.*, 1996; Chang *et al.*, 2017; Long *et al.*, 2017). The cell components of the NPC microenvironment enhance EBV infection (Huang *et al.*, 1999; Lin *et al.*, 2018). EBV infection was first associated with NPC when the serum of patients with NPC was found to show high titre against EBV antigens (Old *et al.*, 1966; Tsao *et al.,* 2017; Yang *et al.,* 2019). EBV infection is associated with non-keratinising and histologically undifferentiated NPC (Lo *et al.*, 2004; Tsang and Tsao, 2015; Tsao *et al.,* 2017; Lee *et al.*, 2021).

The molecular mechanisms of EBV in NPC pathogenesis results from the establishment of aberrant latent infection in the nasopharynx epithelium, which causes pre-malignant changes, such as the deletion of *p16* and the up-regulated expression of cyclin D1(Tsang *et al.*, 2012; Cho *et al.*, 2020). Latent EBV genes regulate NPC growth to evade the immune system and suppress apoptosis (Tsao *et al.*, 2017; Lin *et al.*, 2018). Lytic EBV antigens, which are associated with NPC risk amongst Asian population, are stimulated through the medicinal use of traditional herbs (Hildesheim *et al.*, 1992; Yang *et al.,* 2019). Other factors associated with NPC risk are aerosolised toxic pollutants, such as formaldehyde, and wood dust, which cause nasopharyngeal irritation and consequent inflammation (Armstrong *et al.*, 1983; Chang and Adami, 2006; Li *et al.*, 2006; Bussu *et al.,* 2021). Many other studies have equally observed that wood dust exposure can cause nasal tumours (Hardell *et al.,* 1982; Armstrong *et al., 1*983; Fang *et al.*, 2001; Hildesheim *et al.*, 2001; Ekpanyaskul *et al.*, 2015).

The link between wood and NPC tumourigenesis is attributed to the toxic chemicals used in wood preservation (Hardell *et al.,* 1982; Ekpanyaskul *et al.*, 2015). The regular consumption of salted vegetable can also increase NPC risk (Armstrong, Yu and Henderson, 1983; Turkoz *et al.*, 2011; Polesel *et al.*, 2013; Yong *et al.*, 2017; Okekpa *et al*., 2019). Genetic predisposition was suggested as one of the main factors that contribute to the high NPC occurrence rate in Chinese population (Lu *et al.*, 2003; Lung *et al*.,2019). The association between human leukocyte antigen (*HLA*) genes and NPC was studied by many researchers with controversial outcomes (Simons *et al.*, 1974; Chan *et al.*, 1985; Ren *et al*., 1995). The first researcher to report the association between NPC risk and *HLA-A2* phenotype in Singaporean–Chinese population was (Simons *et al.*, 1974).

Further studies showed that people with *HLA* genes *BW46, AW19-B17, A2*, *A2-BW46*, *A2-B16*, $B17^{+}$, $B18^{+}$ and $B35^{+}$ have increased NPC risk (Chan *et al.*, 1985; Ren, *et al*., 1995; Lung *et al.,* 2019). The association of these HLA genes with NPC incidence led to the proposition that the HLA region may have a disease susceptibility gene that increases NPC risk (Chan *et al.*, 1985; Ren *et al.,* 1995; Ning *et al.*, 2020). The findings of genome-wide association studies showed a strong association of HLA-A, HLA-B and HLA-C with increased NPC susceptibility (Lu *et al.*, 1998; Salehiniya *et al.,* 2018). Individuals with HLA-B*55:02, HLA-A*31:01, HLA-A*11:01 and HLA-B*13:01 alleles have decreased NPC risk. The other genes that are also associated with high NPC risk are *MDS1-EVI1, MICA, ITGA9, HCG9*, *CDKN2B, GABBR, TNFRSF19 and HCP5* (Tse *et al.*, 2009, 2011; Bei *et al.*, 2010; Jia *et al.*, 2010). Genes that metabolise nitrosamine, such as *CYP2E1* and *CYP2A6*, are also susceptible to increased NPC risk (Cui *et al.*, 2016). Previous discoveries revealed that X-ray repair cross-complementing 1 (XRCC1) with genotype Trp194Trp is associated with decreased NPC risk in Cantonese tobacco smokers and males (Cao *et al.*, 2006).

2.1.4 Clinical features, diagnosis, and treatment of NPC

NPC manifests in multiple forms, but many patients present NPC at advanced stage with poor prognosis. Patients seek medical advice at a late stage, that is, patients first consult doctors at approximately 6 months after symptom appearance, because NPC has misleading and confusing symptoms and check-up of the nasopharynx is difficult to perform (Abdullah *et al.,* 2009; Waliyanti *et al*., 2018). NPC has unspecific early symptoms, such as auditory complaints, neck masses, epistaxis, tinnitus, headache, nasal obstruction, facial numbness and hoarseness, which lead to late presentation and diagnosis (Abdullah *et al,* 2009; Razak *et al.*, 2010; Tabuchi *et al.*, 2011; Waliyanti *et al*., 2018).

NPC is metastatic; about 75 % of patients with NPC have neck lumps, and distant metastasis is the major cause of patient death (Chua *et al.*, 2016). NPC diagnosis requires postnasal mirror and naso-endoscopy to reveal exophytic tumour, but many NPCs are submucosal and complex to diagnose (Abdullah *et al.,* 2009). Current NPC diagnosis is via the pathological examination of biopsy specimens (Jeyakumar *et al.*, 2006). The erythrocyte sedimentation rate (ESR) and full blood count (FBC) of patients with NPC must be investigated to rule out repeated epistaxis and high ESR, which are symptoms of anemia and lymphoma, respectively (Abdullah *et al.,* 2009). Radiological examination is used to classify NPC based on TNM staging (Liu *et al.*, 2014; Lee *et al.*, 2019). NPC stage and tumour extension are assessed using magnetic resonance imaging (MRI) and computed tomography (CT) scans (Topkan *et al.*, 2020). Advancement in technology has brought 18F-2-fluoro-2-deoxy-d-glucose positron emission tomography–CT for better NPC diagnosis, therapeutic assessment and prognostic assessment (Agarwal *et al.*, 2013; Topkan *et al.*, 2020).

Early-stage NPC is treated by radiotherapy. Intensity-modulated radiotherapy (IMRT) is preferred over conventional standard 2D radiotherapy as NPC treatment. IMRT is tumouricidal with minimum effect on adjacent healthy tissues (Luo *et al.*, 2016). The standard treatment for advanced NPC, particularly locoregional NPC, is concurrent chemoradiotherapy (Chua *et al.*, 2016; Handayani *et al.,* 2020; Topkan *et al.*, 2020). Fresh fruit, herbal tea and herbal soup consumption are associated with the reduction of NPC risk depending on the frequency of consumption (Jia *et al.*, 2010). Habitual tea consumption is linked to NPC prevention because of its polyphenolic content (Okekpa , *et al.*, 2020). A previous report established that polyphenols and antioxidant in tea prevents cancer development through enzyme inhibition (Martínez *et al.,* 2018).

2.1.5 Limitations of current NPC treatment

The current NPC treatment is radiation-therapy combine with or without chemotherapy and Nasopharyngectomy. Nasopharyngectomy causes transient complications such as submandibular necrosis and palatal fistula (Bian *et al*., 2012). Therapeutic challenge of radiation treatment is the determination of the radiation dose and normal organs tolerance. Mortality may also result from radiation-therapy due to excessive bleeding resulting from injury to the carotid artery (Hao *et al.,* 2008). Radiotherapy can cause adverse effects such as cranial nerve palsy, trismus, xerostomia, mucositis, necrosis of the nasopharynx, necrosis of the temporal lobe and hearing deficit (Bian *et al*., 2012; Qiu *et al*., 2014).

2.1.6 Histopathological classification of NPC

NPC is classified into three subtypes depending on microscopic histological appearance (Table 2.1) and differentiation level (Handayani *et al., 2*020). The classifications according to WHO are: keratinising squamous cell carcinoma, also known as type 1 and characterised by keratin protein production; differentiated nonkeratinizing carcinoma, also known as type 2 and undifferentiated carcinoma, also called type 3 (Barnes *et al*., 2005; Chen *et al.*, 2019; Gondhowiardjo *et al.*, 2020). Histologically, type 1 NPC has keratinisation features with squamous and intercellular bridges (Sharif *et al.*,2020).This type of NPC is graded into poorly or moderately differentiated. Types 2 and 3 are more common and have strong association with EBV such as Asia, whereas type 1 is not common in NPC endemic regions (Marcus and Tishler, 2010; Sharif *et al.*, 2020). Nonkeratinising differentiated NPC has similar histopathological appearances as undifferentiated NPCs, except that the cell borders of the former are stratified and pavemented (Chen *et al.*, 2019).

Undifferentiated NPC is microscopically characterised by spindle or oval shaped vesicular hyperchromatic nuclei, which possess protuberant nucleoli and mitotic activity (Wei *et al*., 2010). However, the survival rate of patients with NPC depends on cancer stage (Table 2.1). For example, people with stage I or II NPC have 72 %–90 % 5-year survival rate, whereas those with stage III NPC have a 5-year survival rate of ~55 %. People with stage IV NPC have 30% survival rate because of frequent locoregional metastasis or recurrence (Tulalamba and Janvilisri, 2012; Chattopadhyay *et al*., 2017).

Table 2.1 Classification and stages of NPC. The table shows WHO classification of NPC for 1978, 1991 and 2005. It also showed stages of NPC and the treatment in the lower part of the table

Classification of NPC (Barnes *et al*., 2005)		
WHO Classification 1978	**WHO Classification 1991**	**WHO Classification 2005**
WHO type I (well-differentiated keratinized SCC)	Squamous cell carcinoma	Keratinizing squamous cell carcinoma (SCC)
WHO type II (differentiated keratinized non-SCC)	Non-keratinizing carcinoma (a). Differentiated (b). Undifferentiated	Non-keratinizing carcinoma (a). Differentiated (b). Undifferentiated
WHO type III (undifferentiated carcinoma)		Basaloid squamous cell carcinoma
Stages of NPC and treatment (Lee *et al.*, 2015)		
Stage I	**Stage II, III, IVA and IVB**	**Stage IVC (distant metastasis**
Definitive treatment Radiotherapy to nasopharynx & Radiotherapy to neck	Concurrent chemotherapy and radiotherapy	Palliative chemotherapy Radiotherapy to alleviate symptoms
IMRT to minimise dose to critical structure	IMRT to minimise dose to critical structure	Referral to palliative care/ palliative home care

2.1.7 Molecular dysregulation in NPC

Early event in NPC progression involves heterozygosity loss on chromosomes 9p and 3p (Chan *et al.*, 2000, 2002; Chattopadhyay *et al.*, 2017). Another early events in NPC tumourigenesis are the deletion and promoter hypermethylation of chromosomes 3p (*RASSF1)* and 9p (*p16* [*CDKN2A*]) (Kwong *et al.*, 2002; Young and Rickinson, 2004). Gains on chromosomes 1q and 3q have also been reported in NPC. Oncogenes, such as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha *(PIK3CA).* ras (rat sarcoma) B cell CLL/lymphoma-2 B cell CLL/lymphoma-2*,(**Bcl-2)***, *CCDN1* (cyclin D1), B lymphoma Mo-MLV insertion region 1 homolog,(*Bmi-1)* and Lymphotoxin beta receptor (*LTBR)* are overexpressed or mutated in NPC (Lu *et al.*, 1993; Hui *et al.*, 2005; Li *et al.*, 2011; Lo *et al,* 2012; Bononi *et al., 2013;* Cho *et al.,* 2020 Tsang *et al.,* 2020*)*. The up-regulated expression of *PIK3CA* and *LTBR* contributes to NPC pathogenesis via the activation of multiple signalling pathways, such as PI3K/AKT (Lo *et al,* 2012; Tsang *et al.,* 2020). Super-enhancer-associated oncogenic transcripts, including F3, BCAR1, TBC1D2, LDLR and RNA TP53TG1, are overexpressed in NPC and function to promote NPC (Jiang *et al.,* 2017).

Previous exome sequencing analysis revealed that NPC development can be influenced by the deletions and mutation of autophagic genes (*ATG2A*, *ATG7* and *ATG13*), chromatin modification genes (*ARID1A* and *BAP1*) and genes in the ERBB–PI3K pathway (*PIK3CA*, *ERBB2* and *ERBB3*) (Lin *et al.*, 2014a; Dai *et al.*, 2016). The activation of the ERBB–PI3K pathway due to genetic alterations aggravates NPC. Oncogenic *PIK3CA* is dysregulated in NPC because of mutations at p.Glu545Lys and p.His1047Arg (Lin *et al.*, 2014). *KRAS* and Akt are amplified*,* Gly12 is mutated and *PTEN* is deleted in NPC (Lin *et al.*, 2014). *TP53* and *CDKN2* alterations in NPC affect the G1/S transition in the cell cycle.

Mutations in the *FAT* gene family, including *FAT1, FAT2* and *FAT3*, has been reported in NPC (Lin *et al.*, 2014). Previous report stated that *TP53* is mutated in NPC (Yee-Lin *et al,* 2018; Rampias,2020). *BAP1* is deleted and/or mutated in NPC; thus, *BAP1* encodes tumour suppressors that are lost during NPC pathogenesis (Lin *et al.*, 2014). ARID1A inhibits NPC cell proliferation by regulating the expression of $p21^{WAF1}$ (Guan *et al.,* 2011; Guan *et al.*, 2012). ARID1A depletion causes MYC overexpression; hence, ARID1A is the target of MYC (Lin *et al.*, 2014). In NPC, *SYNE1* mutation deregulates epithelial cell differentiation. *NOTCH* family genes are also deleted or mutated in NPC. SYNE1 protein enhances the NOTCH1-dependent differentiation of the epidermis (Williams *et al.*, 2011).

Chronic inflammation accounts for about 25 % of the factors that cause cancers (Perwez Hussain and Harris, 2007; Murata, 2018). Chronic inflammation induces inflammatory and epithelial cells to produce ROS and reactive nitrogen species (RNS), which cause oxidative stress (Pinlaor *et al.,* 2004; Murata, 2018). Chronic inflammation damages lipids, proteins and nucleic acids through oxidative stress and leads to tissue damage. Tissue injury can activate stem or progenitor cells to regenerate tissues, but ROS and RNS from the inflamed area, damage the stem or progenitor cells and lead to the accumulation of mutations and the generation of more cancer stem cells (Ohnishi, *et al.,* 2013; Wang *et al.,* 2016; Murata, 2018). Stemness markers, such as ALDH1A1 and CD44v6, have been detected in NPC tissues (Wang *et al*., 2016). ALDH1A1- or CD44v6-positive NPC cells form 8-nitroguanine (inflammation-specific carcinogenic marker) through the reaction between nitric oxide and superoxide anions, which causes mutagenesis via oxidative stress (Kawanishi et al., 2006; Wang et al., 2016).

2.1.8 NPC pathogenesis model.

Persistent contact of the nasopharyngeal mucosa with lifestyle and environmental carcinogens induces DNA damage, leading to genetic alterations in the nasopharyngeal epithelial cells which stimulate the establishment of EBV infection (Yang et al., 2019). The process of transformation induces *p16* gene (*CDKN2A*) inactivation on chromosome 9 and *RASSF1* inactivation on chromosome 3 resulting in cyclin D1 over-expression or amplification chromosome 11 regions which allows for latent EBV infection as well as epigenetic and genetic changes (Figure 2.2). Abnormal methylation of CDKN*2A* and *RASSF1* are considered as early critical events of NPC tumorigenesis (Tsao et al 2014; Dai et al., 2016) Genetic/epigenetic changes facilitate and encourage EBV infection and cause inflammation and molecular alteration, which result in the malignant transformation of the nasopharynx epithelial cells (Figure 2.2).

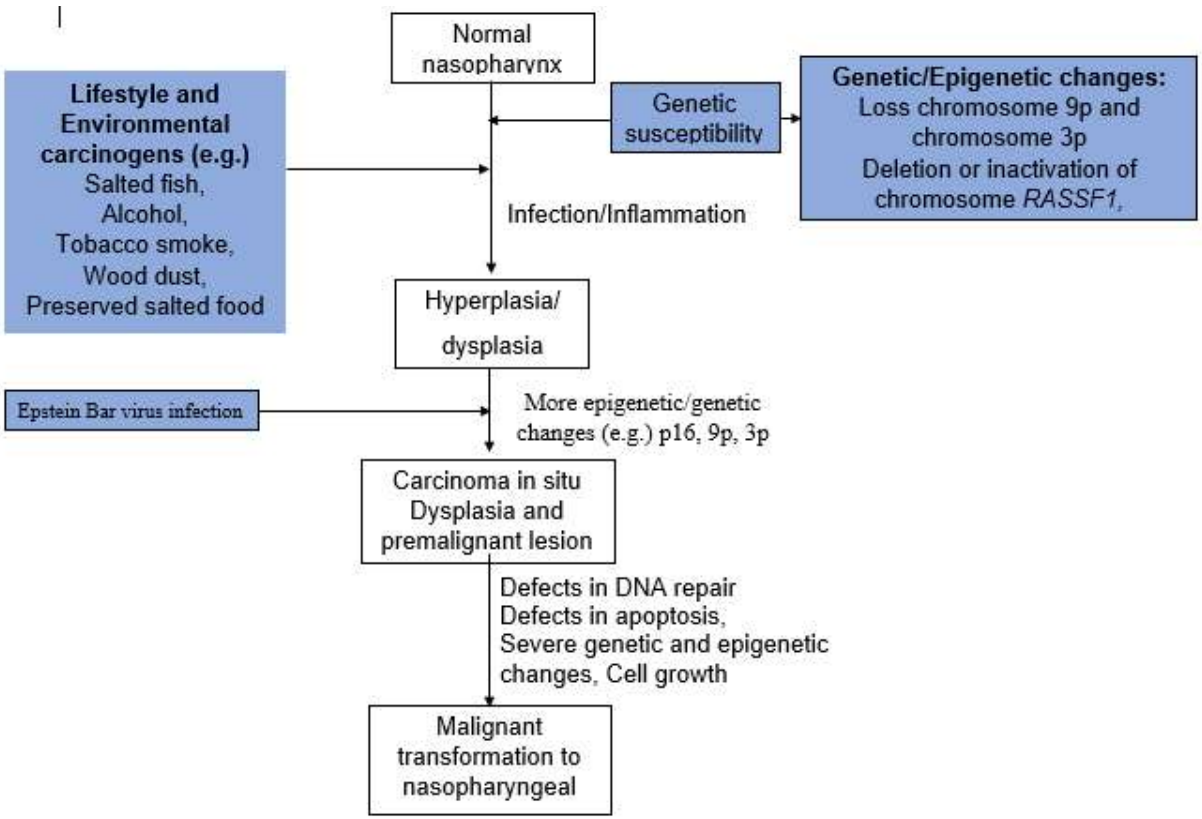

Figure 2.2 **NPC pathogenesis model.** Loss of chromosomes 3p and 9p as well as inactivation of RASSF1A and CDKN2A are the early pathogenic events in NPC that promote hyperplasia or low-grade dysplasia. Genetic/epigenetic changes facilitate and encourage EBV infection and cause inflammation and molecular alteration, which result in malignant transformation and NPC carcinogenesis (Adapted from Tsao *et al* 2014).

2.1.9 Oxidative stress and cellular homeostasis

Oxidative stress is an abnormal cellular status that arises from the lack of antioxidant reserve against reactive oxygen species (ROS) Excessive ROS and elevated levels of active oxygen regulate ageing and the induction of diseases, such as cancer (Xu *et al.,* 2014). ROS includes hydroxyl radicals ($^{\bullet}OH$), free radicals (such as superoxide [$O_2^{\bullet-}$]) and non-polar hydrogen peroxide (H_2O_2) molecules, which contribute to oxidative stress. High ROS levels mediate the oxidation of macromolecules, including proteins, DNA, and lipids, and ultimately cause cellular damage by altering intracellular redox homeostasis, inhibiting repair mechanisms, and inducing apoptotic signalling (Schieber *et al.,* 2014; Chen *et al.,* 2017). Evidence have shown that oxidative stress contributes to the induction of cell ageing and disease progression (pollina & Brunet, 2011; Chen *et al.,* 2017; Liguori *et al*., 2018). Oxidative stress-mediated damage occurs in the form of mitochondrial dysfunction, lipid peroxidation, protein denaturation and DNA modifications (Liguori *et al*., 2018).

At the transcription level, ROS oxidises the cysteine (Cys) residues of activator protein-1, hypoxia-inducible factor-1 alpha, p53 and NF-κB to alter their DNA-binding sites (Georgieva *et al.*, 2017). ROS oxidises methionine and Cys to generate sulfoxide and sulfonic acids, respectively, which change the tertiary structures of proteins by degradation, activation or inhibition (Grimsrud *et al.,* 2008; Morry *et al.,* 2017). Dysregulated cellular ROS generation can induce DNA damage or inactivate DNA repair mechanisms (Morry *et al.,* 2017). In cancer, the accumulation of DNA damage due to insufficient DNA repair mechanisms leads to gene mutation. This instability in the genome will activate oncogenes, which cause unregulated metabolic activities and reduce antioxidant production to increase intracellular ROS generation (Morry *et al.,* 2017).

2.2 Structure of STIM1

STIM1 IS a transmembrane protein type 1 which is made up of 685 amino acids. It comprises of EF hands with two Ca^{2+} binding sites, one disulfide linkage, numerous glycosylation sites, cytosolic localized coiled-coil domains, one sterile-alpha motif (SAM) domain in the luminal-domain, as well as lysine, proline, and serine rich regions (Soboloff *et al*., 2012). One previous report revealed that STIM1 has two EF hand motifs in which one binds Ca^{2+} while the second one is 'hidden' and cannot bind Ca^{2+}. The motifs of EF-hand interact closely with SAM known as EFSAM to form a compact-domain (Figure 2.3). STIM1 senses a decreased Ca^{2+} levels in the ER via EF hand Ca^{2+} binding-motif, which increases the oligomerization of STIM1. The STIM1 EF hand domain has equilibrium dissociation-constant (K_d) that ranges from 0.2–0.6 mM which is consistent with fluctuations in the ER Ca^{2+} levels (Gudlur *et al*., 2018). Ca^{2+} signaling activates dynamic changes in the ER causing movement of the STIM1 transmembrane protein, which involves sensing of the Ca^{2+} concentration in the lumen, and direct contact with Orai1 Ca^{2+} pump near the plasma membrane

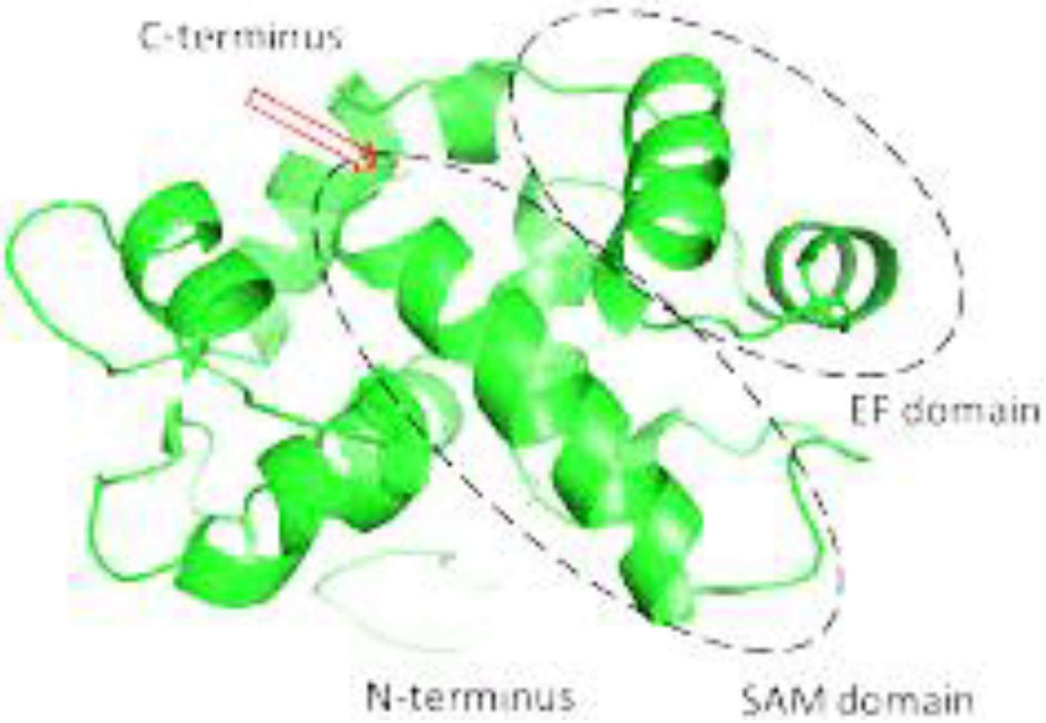

Figure 2.3 **Structure of STIM1 and its luminal domain**. ER luminal domain of STIM1 showing the location of EF-hand domain (EF) and SAM domain (Adapted from Stathopulos *et al.*, 2008).

2.2.1 STIM1 calcium signaling and cancer

STIM1 is a transmembrane protein encoded by the *STIM1* gene that is positioned in the ER and has a transmembrane domain (Jardin *et al.*, 2018). *STIM1* is a novel human gene mapped to the chromosome 11p15.5 region (Wang *et al.*, 2017; Ge *et al.*, 2019). Its protein regulates calcium homeostasis and intracellular calcium concentration (Wang *et al.,* 2017). STIM1 has N-termini that extend into the lumen of the ER and C-termini that extend into the cytosol. *STIM1*'s N-terminal senses Ca^{2+} concentration changes (Figure 2.4). Reduction in Ca^{2+} concentration leads to the activation of *STIM1*, which causes STIM1 to bind to ORAI1. The ORAI1 channels are localised to the puncta to induce Ca^{2+} influx into the cell and triggered by receptor-associated phospholipase C(PLC) activation to generate inositol triphosphate (IP3). IP3 induces the opening of IP3-responsive Ca^{2+} channels in the ER membrane to release ER-stored Ca^{2+} into the cytoplasm (Jardin *et al.*, 2018).

Persistent Ca^{2+} signals can come as Ca^{2+} spikes or oscillations that lead to recurring morphological changes due to proteolysis. STIM1 overexpression can cause epithelial-to-mesenchymal transition (EMT). EMT is a morphogenic process that induces the loss of epithelial features in cells to transform into mesenchymal features, which results in the loss of cell-to-cell contact, the loss of cell polarity and increased motility. EMT creates invasive status in tumour cells and prevents apoptosis and senescence in the cell. EMT is a morphological indication of metastasis. A scientific report associated STIM1-triggered Ca^{2+} influx with EMT (Chen *et al.*, 2011). A recent report showed that STIM1 is implicated in the tumour metastasis of colorectal cancer (CRC) (Zang *et al.*, 2016). STIM1 regulates cell proliferation, cell cycle, apoptosis and cell migration during human cancer development (Jardin *et al.*, 2018).

Previous work by Sun *et al*. on hypopharyngeal carcinoma indicates that silencing the *STIM1* gene inhibits tumour growth and promotes apoptosis and cell cycle arrest (Sun *et al.*, 2014, 2015). *STIM1* repression remarkably inhibits cell migration in prostate cancer and inactivates the PI3K/Akt pathway (Zhou *et al.*, 2017). *STIM1* regulates various cellular activities, such as gene transcription, hormone production and neurotransmitter release (Lao and Le, 2020). Numerous studies have reported that *STIM1* silencing can suppress cell proliferation and migration in many cancer types (Chen *et al.*, 2011; Yang *et al.*, 2013; Wang *et al.*, 2015; Tu *et al.*, 2016; Zhou *et al.*, 2017). *STIM1* helps in the developmental processes and functional activities of numerous cell types, such as myoblasts, lymphocytes, skeletal muscle cells, smooth muscle cells, neurons, and adipocytes (Wang *et al* 2017).

Additionally, countless studies have shown that the down-regulated expression of *STIM1* can suppress cancer growth and progression in CRC, gastric cancer, breast cancer, prostate cancer, lung cancer and hepato-cellular carcinoma (Umemura *et al.*, 2014; Pei *et al.*, 2016; Wang *et al.*, 2017; Zhou *et al.*, 2017; Karacicek *et al.,* 2019). The mechanism of *STIM1* is shown in Figure 2.4. *STIM1* up-regulation increases Ca^{2+} influx and accelerates cancer progression (Chen *et al.,* 2013). Calcium (Ca^{2+}) functions as an intracellular messenger that is crucial in cellular processes, such as proliferation and apoptosis (Abdul et al., 2008). *STIM1* senses Ca^{2+} release from the ER and triggers Ca^{2+} loss from STIM1 EF-hand, which induces STIM1 oligomer formation. The oligomer interacts and activates ORAI1 (Kawasaki *et al.,* 2009).