ANTI-APOPTOTIC PROTEINS MCL-1 AND BCL-XL ARE CRUCIAL FOR NASOPHARYNGEAL CARCINOMA CELL SURVIVAL

SITI FAIRUS BINTI ABDUL RAHMAN

UNIVERSITI SAINS MALAYSIA

2022

ANTI-APOPTOTIC PROTEINS MCL-1 AND BCL-XL ARE CRUCIAL FOR NASOPHARYNGEAL CARCINOMA CELL SURVIVAL

by

SITI FAIRUS BINTI ABDUL RAHMAN

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

November 2022

ACKNOWLEDGEMENT

In the name of Allah, The Most Gracious, The Most Merciful

Alhamdulillah, highest praise to Allah for His Mercy and Gracious, that I am able to successfully complete my study. First and foremost, I would like to express my deepest and sincere gratitude and appreciation to my supervisor, Dr. Nethia Mohana-Kumaran for her continuous advice, professional guidance and motivation throughout the completion of this study. I would not have completed this study without her endless guidance and tireless support. I also would like to thank my co-supervisor, Associate Professor Dr. Mohd Ghows Mohd Azzam for his continuous guidance and ideas for the betterment of this study.

Next, I would like to express my foremost gratitude to my beloved family especially my parents and my husband for their untiring moral support as well as their earnest understanding and encouragement which enabled me to stay confident throughout my work. I would also like to extend my thanks to all my dearest laboratory mates who have contributed in my study especially Dr. Kalaivani Muniandy and Dr. Azali Azlan for their continuous assistance and guidance. Their knowledge and research experience, tremendously impacted my outlook on research which indirectly contributed to the completion this study.

I would also like to thank laboratory staff and assistants from the School of Biological Sciences, USM and the Malaysia Institute of Pharmaceuticals and Nutraceuticals (IPHARM) for their cooperation and support throughout my research work. Last but not least, I would like convey a huge gratitude to the Program Pelajar Cemerlang (PPC) Scholarship from Jabatan Perkhidmatan Awam for funding me throughout the duration of my study.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
5-FU	5-Flurouracil
AC	Adjuvant chemotherapy
ACTB	Beta-actin
Akt	Protein kinase B
APAF-1	Apoptotic protease activating factor 1
ApoG2	Apogossypolone
ASR	Age standardized incidence rates
B2M	Beta-2-microglobulin
BAD	BCL-2 antagonist of cell death
BAK	BCL-2 antagonist killer 1
BART	*Bam*HI-A region rightward 12 transcript
BAX	BCL-2 associated X protein
BCL-2	B-cell-lymphoma-2
BCL-XL	B-cell-lymphoma-extra-large
BCL-w	B-cell-lymphoma-2 like protein
BCL-2 ASO	BCL-2 oligodeoxynucleotide antisense
BFL-1/A1	B-cell related protein A1
BH	BCL-2 homology
BID	BCL-2 interacting domain death agonist
BIK	BCL-2 interacting killer
BIM	BCL-2 interacting mediator of cell death
BMF	BCL-2 modifying factor
BOK	BCL-2 related ovarian killer

CCRT	Concurrent chemo-radiotherapy
CDH-1	Cadherin 1
CDKN2A	Cyclic dependent kinase inhibitor 2A
CRISPR	Clustered regularly interspaced palindromic repeats
DHMEQ	Dehydroxymethylepoxyquinomicin
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBER	EBV-encoded small RNA
EBNA	EBV-determined nuclear antigens
EBNA-LP	EBNA-leader protein
EBV	Epstein-Barr virus
EBV-GC	EBV associated gastric carcinoma
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMEM	Eagle's Minimum Essential Medium
FASL	Fas ligand
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDR	Homology directed repair
HHV	Human herpes virus
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase
HRK	Harakiri
IC	Induction chemotherapy
InDels	Insertion/deletions
IHC	Immunohistochemistry
IFN-γ	Interferon gamma

IL	Interleukin
IMRT	Like intensity-modulated Radiotherapy
LMP	Latent membrane protein
LOH	Loss of heterozygosity
MCL-1	Myeloid cell leukaemia 1
MEK	Mitogen-activated protein kinase
MNCR	Malaysia National Cancer Registry
MOMP	Mitochondrial outer membrane permeabilization
mTOR	Mammalian target by Rapamycin
NF-κB	Nuclear factor κ-B
NHEJ	Non-homologous end joining
NPC	Nasopharyngeal carcinoma
OMM	Outer mitochondrial membrane
PAM	Protospacer adjacent motif
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cells
PI3K	Phosphoinositol-3-kinase
PIK3CA	Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha
PUMA	p53-upregulated modulator of apoptosis
PDGFR	Platelet-derived growth factor receptor
PTLD	Post-transplant lymphoproliferative disease
qPCR	Quantitative PCR
RASSF1A	*Ras* association domain family 1A

RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute 1640
RT	Radiotherapy
sgRNA	Single guide RNA
ssODN	Single-stranded oligonucleotides
STAT	Signal Transducers and activators of transcription
tBID	Truncated BID
TM	Transmembrane
TMN	Tumor-node-metastasis
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis-inducing ligand
ULA	Ultralow-attachment
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

PROTEIN ANTI-APOPTOTIK MCL-1 DAN BCL-XL ADALAH PENTING UNTUK KELANGSUNGAN HIDUP SEL KANSER NASOFARINK

ABSTRAK

Malaysia mencatatkan kes kanser nasofarinks nasional antara yang tertinggi di Asia Tenggara. Merawat pesakit dengan kanser nasofarink metastatik sering menjadi cabaran kerana pesakit akan membina ketahanan terhadap terapi anti-kanser sistemik seperti kemoterapi dan merawat barah berulangan dengan radioterapi mempunyai banyak batasan. Oleh itu, strategi rawatan yang lebih baik diperlukan untuk mendapat hasil kesembuhan pesakit yang lebih baik. Peningkatan pengawalaturan protein anti-apoptosis BCL-2 merupakan salah satu strategi yang digunakan oleh sel kanser untuk mengelak proses kematian sel. Oleh itu, protein ini telah menjadi sasaran terapi yang menarik. Memandangkan populasi sel yang berbeza bergantung pada protein anti-apoptotik yang berbeza untuk hidup, adalah sangat penting untuk menentukan protein mana yang penting untuk kelangsungan hidup kanser nasofarink.. Kajian terdahulu melaporkan bahawan perencatan bersama BCL-2 dan BCL-XL tidak mendorong kepada kematian sel kanser nasofarink, ini menunjukkan bahawa MCL-1 ataupun BFL-1 mungkin memainkan peranan penting dalam kelangsungan hidup sel kanser nasofarink. Memandangkan peranan MCL-1 dan BFL-1 masih kurang dikaji, kajian ini akan memberi tumpuan teruatamanya untuk menentukan peranan kedua molekul tersebut dalam kelangsungan hidup sel dengan menggunakan pendekatan penyuntingan genetik dan juga keadah farmakologi. 'RT^2 Profiler PCR Array' digunakan untuk mengetahui ekspresi gen anti-apoptosis dalam sel kanser nasofarink, HK-1 dan C666-1. Sel HK-1 mengekpresi semua gen anti-apoptotis (*MCL-1, BFL-1, BCL-2, BCL-XL,* dan *BCL-w*).

Begitu juga dengan sel C666-1 yang mengekpresi semua gen anti-apoptotis kecuali *BFL-1* (tahap tidak dapat dikesan). Kedua sel kanser nasofarink mempunyai ekspresi gen *MCL-1* yang paling tinggi dan ini menunjukkan bahawa apoptotis sedia berlaku dalam kedua sel tersebut. Penghapusan *MCL-1* dan *BFL-1* menggunakan teknik 'CRISPR /Cas9' tidak menghalang percambahan sel, menunjukkan bahawa protein anti-apoptosis lain mungkin terlibat dalam kelangsungan hidup sel. Selain itu, penghapusan *MCL-1* mengakibatkan peningkatan BCL-XL sebagai mekanisme kompensasi untuk kelangsungan hidup sel. Sel yang mempunyai penghapusan *MCL-1* menunjukkan tindak balas perencatan yang baik apabila dipekakan kepada perencat selektif BCL-XL, A-1331852 menunjukkan bahawa MCL-1 dan BCL-XL mungkin penting untuk kelangsungan hidup sel kanser nasofarink. Kepekaan sel HK-1 yang mempunyai penghapusan gen *BFL-1* kepada rawatan A-1331852, menunjukkan bahawa BFL-1 dan BCL-XL mungkin penting untuk kelangsungan hidup sel NPC, walaubagaimanpun kajian lebih lanjut diperlukan. Gabungan perencat selektif yang mensasar MCL-1 dan BCL-XL, mengakibatkan kematian sel yang tinggi dalam model sel kultur dua- dan tiga-dimensi menunjukkan bahawa MCL-1 dan BCL-XL adalah kunci untuk kelangsungan hidup sel kanser nasofarink. Hasil kajian ini memberikan gambaran mengenai kepentingan MCL-1 dan BCL-XL untuk kelangsungan hidup sel kanser nasofarink dan rangka kerja bagaimana protein anti-apoptosis ini dapat disasarkan secara ideal untuk rawatan kanser nasofarink.

ANTI-APOPTOTIC PROTEINS MCL-1 AND BCL-XL ARE CRUCIAL FOR NASOPHARYNGEAL CARCINOMA CELL SURVIVAL

ABSTRACT

Malaysia has one of the highest national incidences of Nasopharyngeal carcinoma (NPC) in South-East Asia. Treating patients with metastatic NPC is often a challenge as patients develop resistance to systemic anti-cancer therapies such as chemotherapy and retreating local recurrence with radiotherapy have many limitations. Hence, improved treatment strategies are needed for better patient outcome. Up-regulation of the anti-apoptotic protein BCL-2 is one of the strategies that cancer cells adopt to evade cell death. Hence, these proteins have become attractive therapeutic targets. Given that different cell population rely on different anti-apoptotic proteins for survival, it is crucial to determine which proteins are important for NPC cell survival. Previous study reported that co-inhibition of BCL-2 and BCL-XL did not induce cell killing in NPC cells, which indicate that either MCL-1 or BFL-1 may play an important role in the survival the NPC cells. Given that the roles of MCL-1 and BFL-1 for NPC cell survival are under-explored, this study will primarily focus on delineating the functional roles of these two molecules for cell survival using gene editing and pharmacological approaches. A human apoptosis RT^2 Profiler PCR Array was first employed to profile the anti-apoptotic gene expressions in NPC cell lines HK-1 and C666-1. The HK-1 cells expressed all of the anti-apoptotic genes (*MCL-1, BFL-1, BCL-2, BCL-XL, and BCL-w*). Similarly, the C666-1 cells expressed all of the anti-apoptotic genes except *BFL-1* (undetectable level). Notably, both cell lines highly expressed *MCL-1* suggesting that the cells were "primed" for apoptosis. Deletion of *MCL-1* and

BFL-1 using the CRIS PR/Cas9 technique did not inhibit cell proliferation, suggesting that other anti-apoptotic proteins may be involved in cell survival. Moreover, deletion of *MCL-1* resulted in the up-regulation of *BCL-XL* as a compensatory mechanism for cell survival. Cells harbouring *MCL-1* deletion were sensitized to BCL-XL selective inhibitor A-1331852 suggesting that MCL-1 and BCL-XL may be important for NPC cell survival. Deletion of *BFL-1* sensitized HK-1 cells to A-1331852 treatment, indicating that BFL-1 and BCL-XL may be important for NPC cell survival, warranting further interrogation. Combinations of BH3-mimetics that selectively target MCL-1 and BCL-XL, resulted in considerable cell killing in both the two- and three-dimensional cell culture models implying that MCL-1 and BCL-XL are key for NPC cell survival. Taken together the study provides both an insight into the importance of MCL-1 and BCL-XL for NPC cell survival and a framework on how these anti-apoptotic proteins can be ideally targeted for NPC treatment.

CHAPTER 1

INTRODUCTION

Nasopharyngeal Carcinoma (NPC) is a type of head and neck cancer which begins at the nasopharynx, upper part of the nose. NPC is a rare cancer worldwide but endemic in Asia especially in South-East Asia (GLOBOCAN 2020). Malaysia is ranked as one of the top five countries in the world, with the highest age standardized incidence rates (ASR) for NPC (GLOBOCAN, 2020). The incidences of NPC were more prevalent among males of Chinese descent compared to males of other ethnic backgrounds (Malaysia National Cancer Registry 2012-2016).

Patients diagnosed with NPC early (stage I and II), respond well to concurrent chemo-radiotherapy (CCRT), the standard of care treatment for NPC (Chang *et al*., 2004). However, treating patients with metastatic NPC is often a challenge as patients tend to develop resistance to CCRT. So far, the mechanism of chemo-resistance in NPC has not been fully elucidated and there have not been a comprehensive review on this issue. Targeted therapy has been one of the strategy employed for treating metastatic NPC cases. Majority of drugs tested target either the Epidermal Growth Factor Receptor (EGFR), Vascular Endothelial Growth Factor (VEGF) or Vascular Endothelial Growth Factor Receptor (VEGFR). Testing targeted therapies alone demonstrated poor prognosis where the overall survival rate of the NPC patients were not longer than a year (Hui *et al*., 2011; Xue *et al*., 2013; Hui *et al*., 2018; Ma *et al*., 2015). Hence, improved treatment strategies are needed for better patient survival.

The BCL-2 family of proteins are critical regulators of the apoptosis pathway. The members of the family are grouped into pro- and anti-apoptotic proteins (Kurschat *et al*., 2021). The anti-apoptotic proteins (BCL-2, BCLXL, BCL-w, MCL-1 and BFL-1) are up-regulated in many cancers and hence have emerged as potential therapeutic targets. The interest to target these proteins increased with the development of BH3-mimetics (small molecule inhibitors) which specifically target these proteins (Ashkenazi *et al*., 2017). BH3-mimetic ABT-263 which inhibits BCL-2, BCL-XL and BCL-w showed impressive clinical efficacy in haematological cancers but turned out to be a disappointment in most solid cancers mainly due to the induction of MCL-1, Survivin expression and the direct (or indirect) down-regulation of which, were seen as beneficial to ABT-263 efficacy (Wilson *et al*., 2010). Cancer cells escape apoptosis via high expression of the anti-apoptotic proteins and the adaption to anti-apoptotic proteins vary from tumour to tumour. Some tumours may rely on just one anti-apoptotic proteins for survival and some may depend on more than one anti-apoptotic protein for survival (Singh *et al*., 2019). It became apparent that different cell population is addicted to different anti-apoptotic proteins for survival, which "prime" these cells for apoptosis (Leverson *et al*., 2015). Thus, it is important to identify which anti-apoptotic proteins does a cancer cell population is addicted to so that the appropriate molecules can be targeted optimally to induce maximal cell killing.

The study by Xiang *et al.*, (2020) unveiled that co-inhibition of BCL-2 and BCL-XL did not induce cell killing in NPC. This led to the re-evaluation of the utility of BCL-2 and BCL-XL as therapeutic targets in NPC. The study findings raised two questions (1) do NPC cells depend on other anti-apoptotic proteins for survival? (e.g. MCL-1 or BFL-1); OR (2) do they depend on combination of two anti-apoptotic proteins

that are not partly targeted by ABT-263, for example MCL-1 and BCL-XL? This study will primarily focus on investigating the functional relevance of MCL-1 and BFL-1, given the limited efforts in addressing their roles in NPC survival.

There are a few strategies to determine the dependencies of cancer cells to anti-apoptotic protein(s) namely the dynamic BH3 profiling technique (Montero and Letai, 2018), the CRISPR/Cas9 genome editing technique (Aubrey *et al*., 2015) and chemical parsing approach using BH3-mimetics, given their selectivity in inhibiting specific anti-apoptotic proteins (Leverson *et al*., 2015). This study will utilize combination of the CRISPR/Cas9 technique and pharmacological inhibition approaches to delineate the contributions of the anti-apoptotic proteins for NPC cell survival. Given that from the previous study, co-inhibition of BCL-2 and BCL-XL were not sufficient to induce cell death in the NPC cells, the first objective of the study will focus on investigating the functional relevance of *MCL-1* using the CRISPR/Cas9 technique. The manipulated *MCL-1* cells, once generated will be subjected to microarray analysis to study the gene regulation of other anti- and pro-apoptotic genes as a result of *MCL-1* deletion. The manipulated cells will also be subjected to treatment with BH3-mimetics which selectively target BCL-XL and BCL-2 to determine how *MCL-1* levels will influence sensitivity of the NPC cells to these BH3-mimetics. The second objective will focus on investigating the functional relevance of *BFL-1* in NPC cell survival using the CRISPR/Cas9 technique. The *BFL-1* manipulated cells will be later subjected to treatment with BH3-mimetics which selectively target BCL-XL, BCL-2 and MCL-1 to determine how *BFL-1* levels will influence sensitivity of NPC cells to these BH3-mimetics. The final aim of the study will investigate the sensitization of the NPC cells to

either ABT-199 (selective inhibitor of BCL-2) or A-1331852 (selective inhibitor of BCL-XL) by MCL-1 selective inhibitor S63845, using 2D and 3D cell culture models. This part of the study will complement the first objective of the study and at the same time provide translational relevance to the BH3-mimetics as potential therapy for NPC management. The aims of the study are presented as an illustration in Figure 1.0. Collectively, the objectives of this study are envisaged to provide insights on the relevant ant-apoptotic proteins to be targeted in NPC for optimal cell killing.

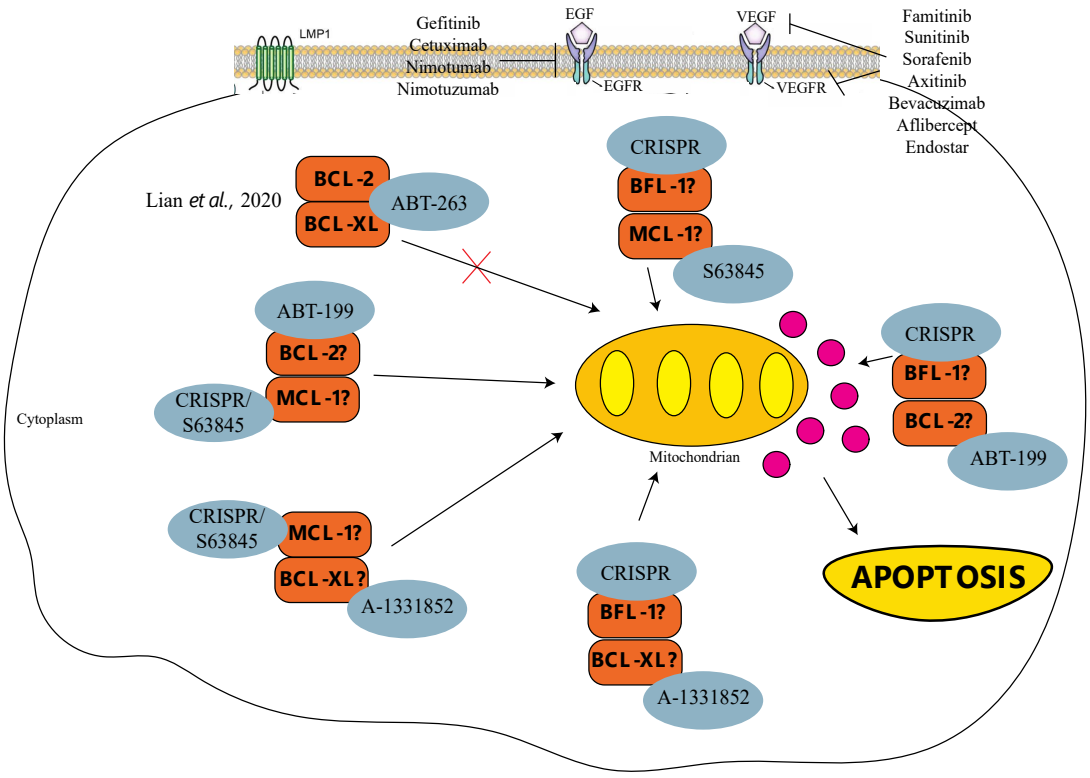

Figure 1.0: Illustration of study aims. Most of the recent targeted treatment efforts for NPC, are focused on targeting the receptor kinases such as EGFR and VEGFR and their respective ligands such as the VEGF. Targeting the apoptosis pathway is an attractive treatment avenue for NPC but it is crucial to determine which anti-apoptotic proteins do NPC cells depend for survival. Xiang *et al,* reported that co-inhibition of BCL-2 and BCL-XL does not induce apoptosis in NPC cells indicating that the NPC cells are not

dependant on these two proteins for survival (Xiang *et al.,* 2020). Given that the roles of MCL-1 and BFL-1 for NPC cell survival are under-explored, this study will primarily focus on delineating the functional roles of these two molecules for cell survival. The functional relevance of MCL-1 for NPC cell survival will be determined using the CRISPR/Cas9 technique. The *MCL-1* manipulated cell lines will be later subjected to treatment with either ABT-199 or A-1331852 to determine if additional inhibition of other anti-apoptotic proteins are necessary to induce cell killing. The second aim of the study will focus on parsing the functional relevance of BFL-1 for NPC cell survival, again using the CRISPR/Cas9 technique. The *BFL-1* manipulated cell lines will be later subjected to treatment with either ABT-199 or A-1331852 or S63845 to determine if additional inhibition of other anti-apoptotic proteins are necessary to induce cell killing. The third aim of the study will explore the therapeutic potential of the immediate inhibition of MCL-1, BCL-XL and BCL-2 using BH3-mimetics which selectively target these proteins. ***Note:*** "?" = uncertainty of which anti-apoptotic proteins will be crucial for NPC cell survival; orange boxes = anti-apoptotic proteins; blue ovals = approaches to target the anti-apoptotic molecules, either via CRISPR/Cas9 mutagenesis or selective BH3-mimetics; ABT-199 = selective BCL-2 inhibitor; A-1331852 = selective BCL-XL inhibitor; S63845 = selective MCL-1 inhibitor.

1.1 Objectives

Objective 1: To delineate the the functional importance of *MCL-1* for the survival of NPC cells via gene deletion of *MCL-1* using CRISPR/Cas9 technology and inhibition with BH3-mimetics.

Objective 2: To parse the role of *BFL-1* for the survival of NPC cells via gene deletion of *BFL-1* using CRISPR/Cas9 technology and inhibition with BH3-mimetics.

Objective 3: To assess the therapeutic potential of immediate inhibition of MCL-1, BCL-XL and BCL-2 by targeting the NPC parental cells with BH3-mimetics which selectively target these molecules, using two- and three-dimensional cell culture models.

CHAPTER 2

LITERATURE REVIEW

2.1 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is an epithelial carcinoma which manifests in the nasopharyngeal mucosal lining. More precisely, the tumour establishes in the pharyngeal recess (fossa of Rosenmüller) of the nasopharynx (Sham *et al*., 1990) (Figure 2.1). The cancer is distinctly different from other head and neck tumours despite originating from the same tissue lineages (Chen *et al.,* 2019). Patients with NPC are often associated with one or more clinical symptoms namely presence of tumour mass in the nasopharynx, painless neck lumps, epistaxis, nasal obstruction, blood discharge from nasal, ear blocking, otitis media, hearing loss, headache and diplopia (Wu *et al*., 2018).

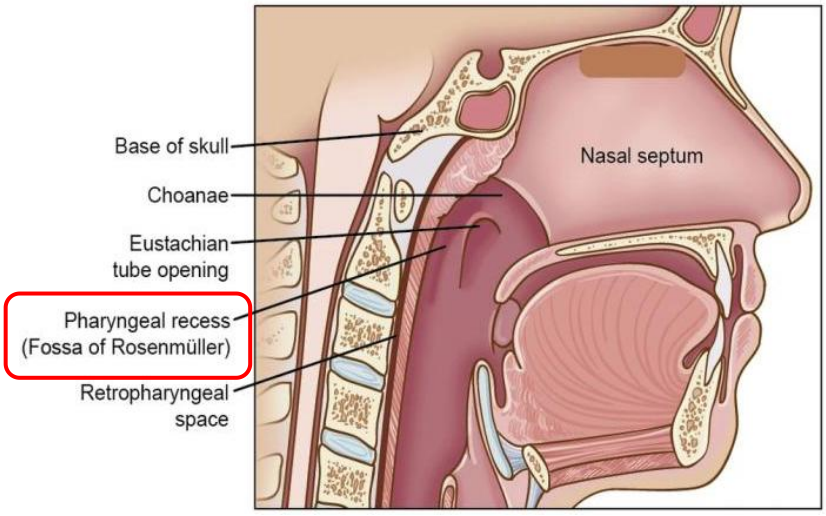

Figure 2.1: Anatomy of the human pharynx. Nasopharyngeal carcinoma manifests in the pharyngeal recess (fossa of Rosenmüller) (see red box) of the nasopharynx. Figure adapted from American Society of Clinical Oncology.

In 1978, the World Health Organization (WHO), classified NPC into three histologic subtypes namely keratinizing squamous cell carcinoma (WHO type 1), non-keratinizing carcinoma (WHO type 2), and undifferentiated carcinoma (WHO type 3) (Shanmugaratnam and Sobin, 1978). The WHO NPC classifications were amended in 1991. The revised classification in 1991 eliminated the use of numerical designations and combined WHO types 2 and 3 as non-keratinizing squamous cell carcinoma while retaining the keratinizing squamous cell carcinoma classification (formerly known as WHO type I). The non-keratinizing NPC carcinomas were further divided into differentiated and undifferentiated squamous cell carcinoma (Shanmugaratnam, 1991). The recent NPC classification maintains the revision made in 1991 with addition of another category known as basaloid squamous cell carcinoma, a rare NPC histology (Barnes, 2005).

2.2 Nasopharyngeal Carcinoma (NPC) Geographical Distribution

Nasopharyngeal carcinoma has distinct geographical and racial distributions in comparison to other head and neck cancers. NPC is a rare cancer in western countries but it is endemic in certain world regions namely South-Eastern Asia, Micronesia, Eastern Asia, Northern Africa and Polynesia (GLOBOCAN, 2020) (Figure 2.2). Among the South-Eastern Asia countries, Malaysia is ranked as one of the top five countries with the highest age standardized incidence rates (ASR) for NPC, along with Brunei, Maldives, Indonesia and Vietnam. ASR is a weighted mean of the age-specific rates where the weights are taken from the population distribution of a standard population; the ASR is expressed per 100,000 (GLOBOCAN, 2020) (Figure 2.3).

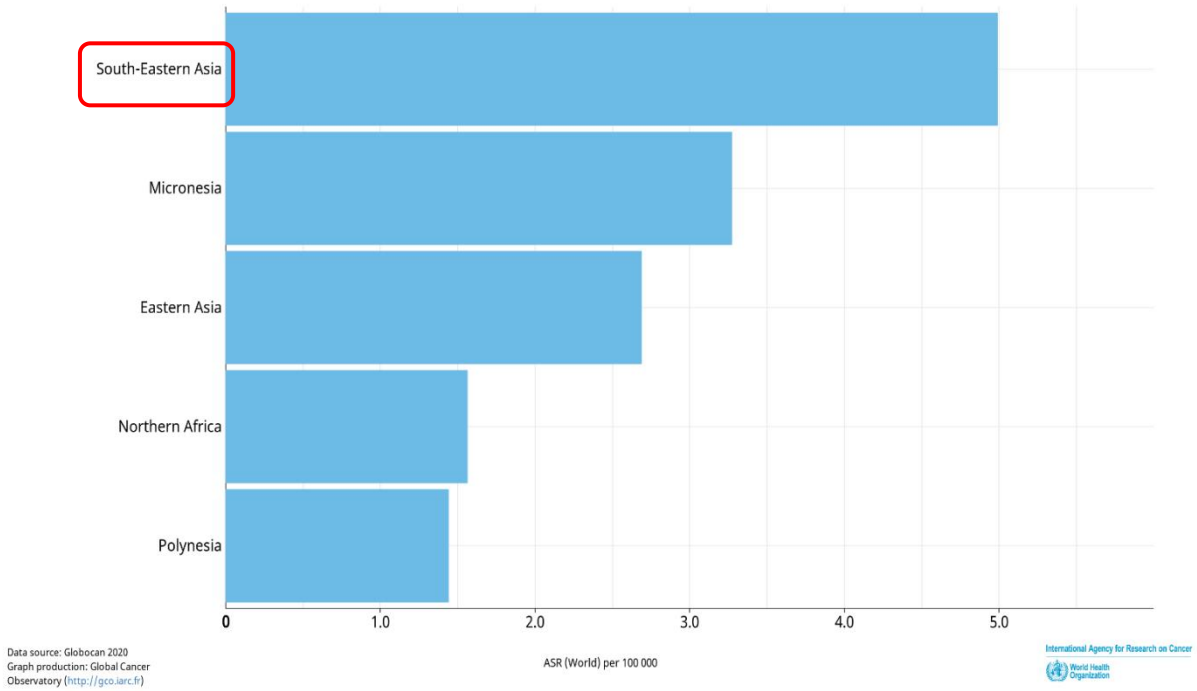

Figure 2.2: Estimated age-standardization incidence rates (ASR) for NPC by world regions. ASR for NPC is highest in the South-Eastern Asia region (red box) followed by Micronesia, Northern Africa and Eastern Africa. Figure adapted from GLOBOCAN 2020, IARC.

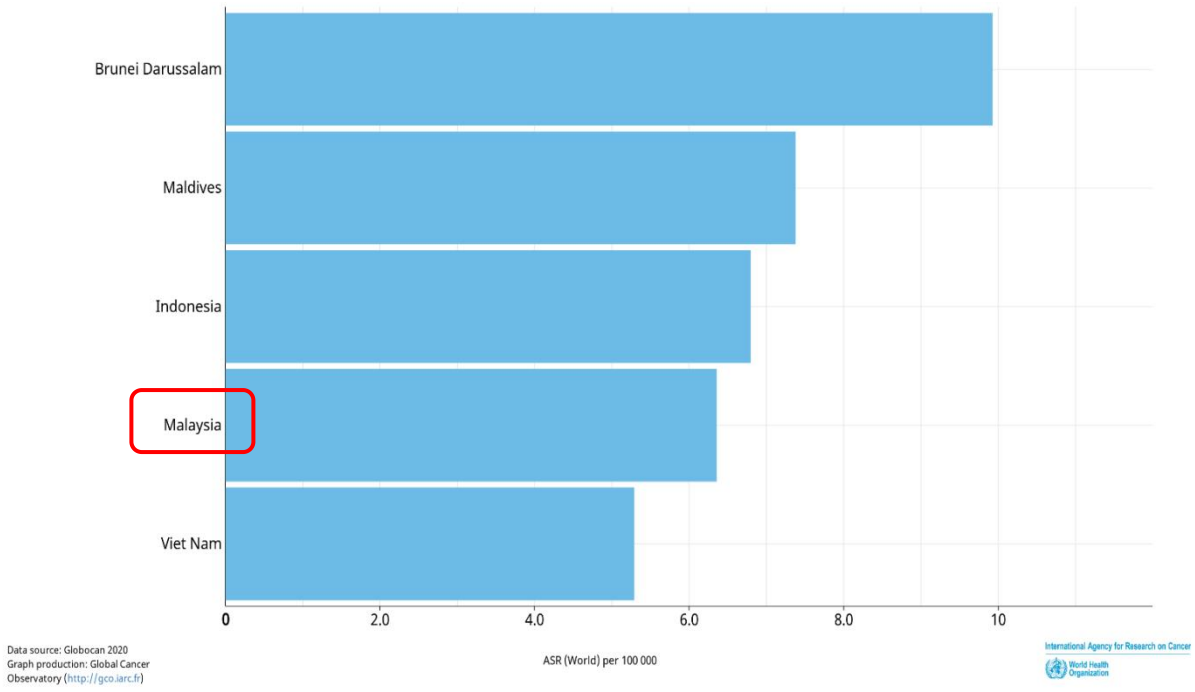

Figure 2.3: Estimated age-standardization incidence rates (ASR) for NPC by countries in the world. Malaysia (see red box) is ranked fourth with the highest ASR for NPC after Brunei, Maldives and Indonesia. Figure adapted from GLOBOCAN 2020, IARC.

According to the most recent Malaysia National Cancer Registry (MNCR) report, NPC is ranked as the fifth most common cancer in Malaysian after breast, colorectal, trachea, bronchus and lung (trachea, bronchus and lung grouped together) and lymphoma (Figure 2.4). MNCR reported that on average 900–1,000 cases of NPC reported each year in 2007–2011 (MNCR 2012-2016).

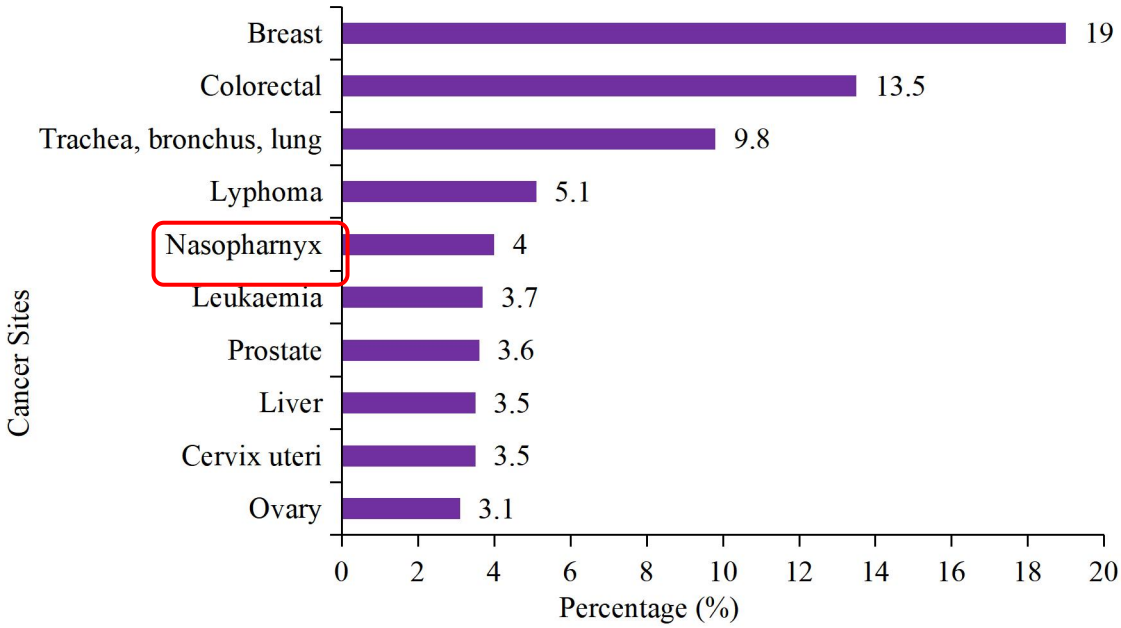

Figure 2.4: The ten most common cancers in Malaysia. NPC (see red box) is the fifth most common cancer among Malaysians. Figure adapted from MNCR 2012-2016, Ministry of Health, Malaysia.

NPC is predominantly diagnosed in males. According to the MNCR report, it is the third most common cancer among males who were between 25-59 years old (Figure 2.5). Males of Chinese descent are more susceptible to this cancer compared to males from the other two major ethnic groups in Malaysia. Although the incidences of NPC among females are lower than males, the incidences are higher in females of Chinese descent compared to females from the other two major ethnic groups (MNCR 2012-2016) (Figure 2.6). The NPC incidences in males increased after the age of 25 and peaked between 50-54 years of age (MNCR 2012-2016). Similarly, although incidences are lower, females displayed similar age pattern as males (Figure 2.7). Regardless of gender, majority of NPC cases are detected at late stages (stage III and IV) (MNCR 2012-2016) (Figure 2.8). Heredity, lifestyle and Epstein-Barr virus (EBV) infection are

among the causative factors of the cancer. Family history of NPC increases the risk of the disease, and frequent consumption of preserved food such as salted vegetables and dried fish also contributes to the high incidences among the Chinese descent (Li *et al*., 2007).

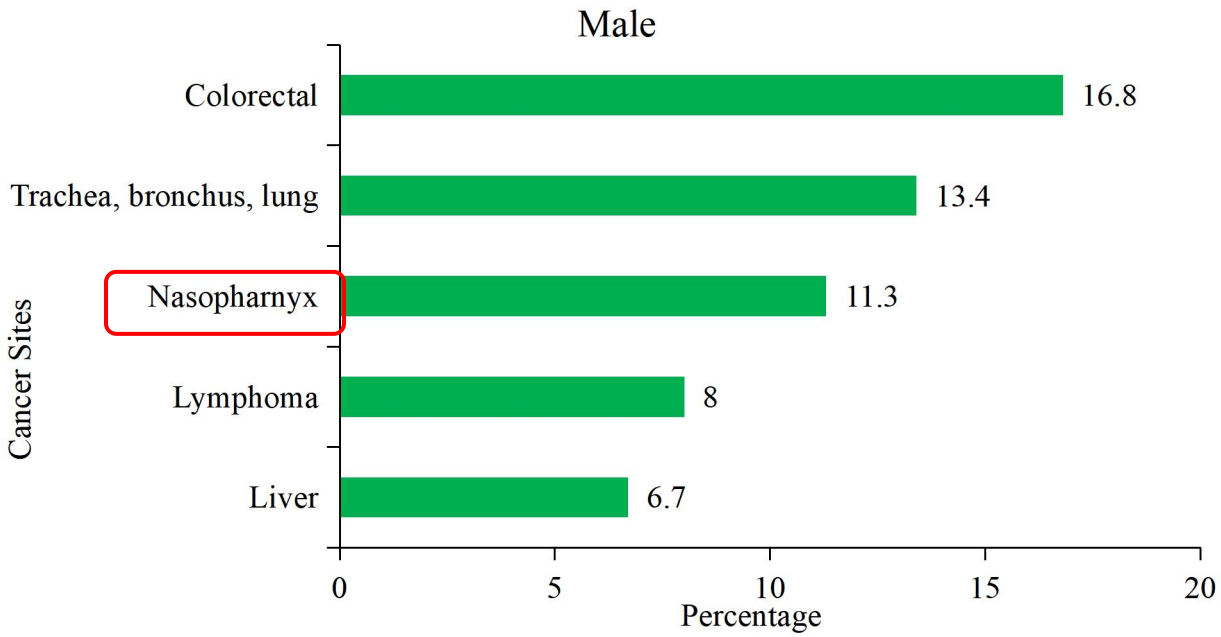

Figure 2.5: Common cancers among males between 25-59 years of age. NPC is (see red box) third most common cancer in Malaysian males. Figure adapted from MNCR 2012-2016, Ministry of Health, Malaysia.

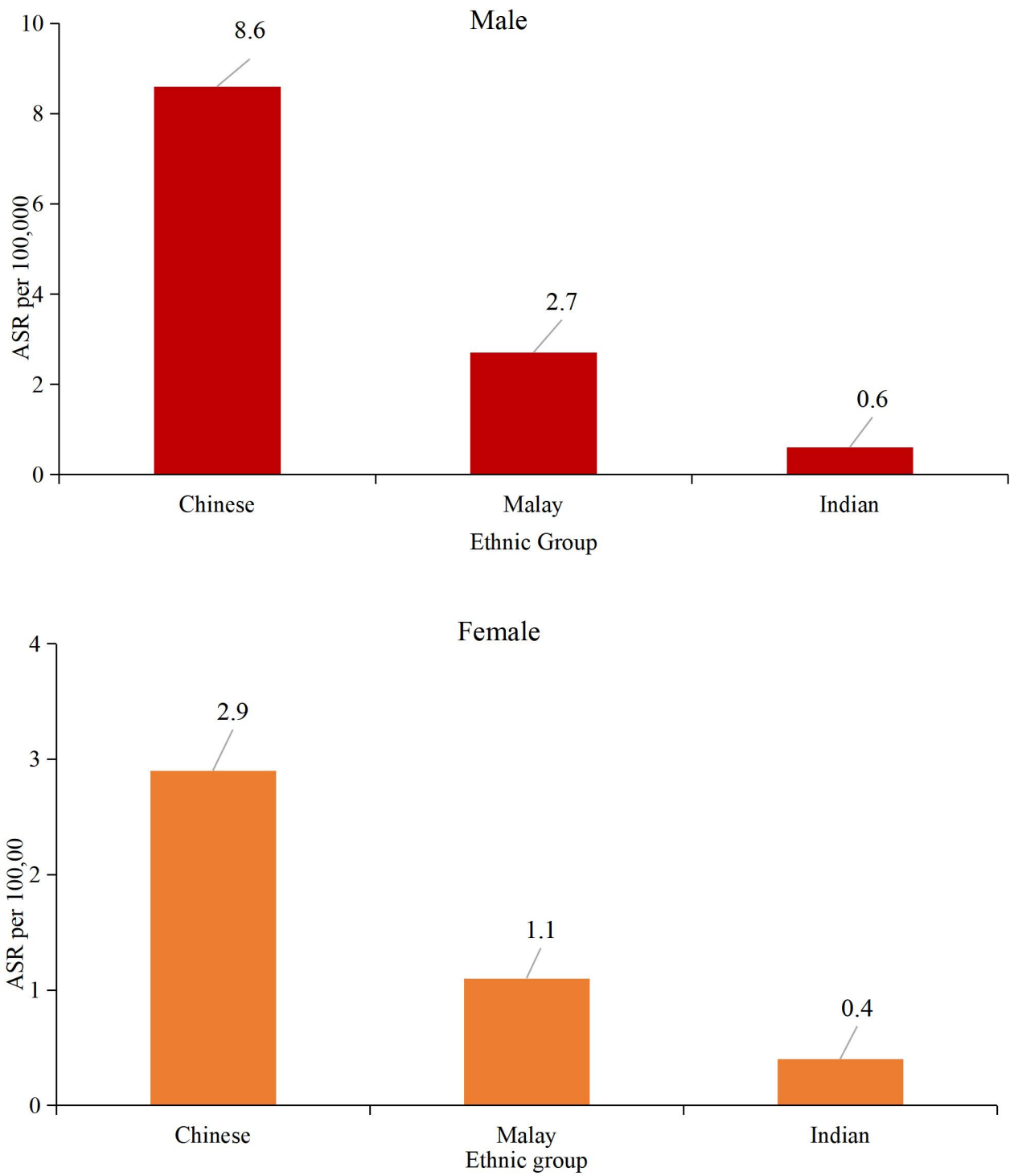

Figure 2.6: NPC age-standardized incidence rates (ASR) according to ethnic background and gender. NPC is predominantly diagnosed in Chinese compared to the Malays and Indians. Figure adapted from MNCR 2012-2016, Ministry of Health, Malaysia.

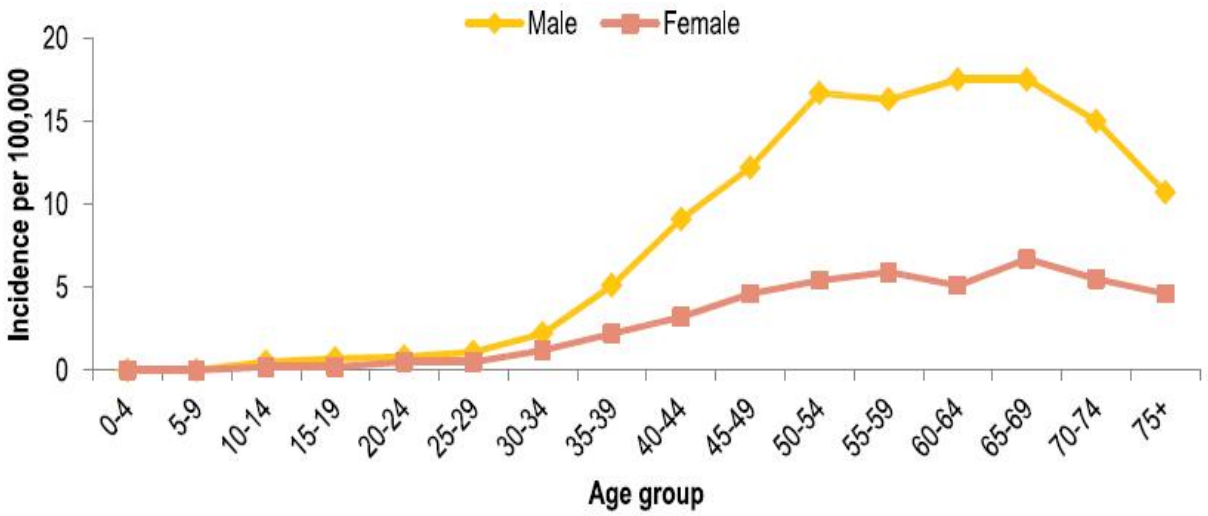

Figure 2.7: Age-specific incidence rates for NPC by gender. The incidences of NPC in males increased after the age of 25 and peaked between 50-54 years of age. Figure adapted from MNCR 2012-2016, Ministry of Health, Malaysia.

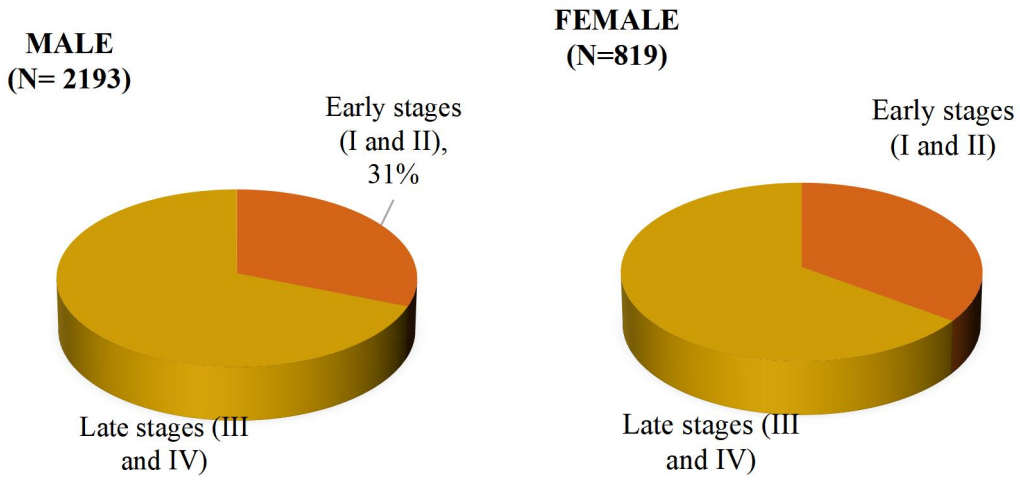

Figure 2.8: NPC staging according to gender. Majority of NPC cases are detected at the late stages (stage III and IV). Figure adapted from MNCR 2012-2016, Ministry of Health, Malaysia.

2.3 Pathogenesis of Nasopharyngeal carcinoma

Nasopharyngeal carcinoma has multifactorial aetiology namely Epstein-Barr virus (EBV) infection, environmental factors such as exposure to nitrosamines and genetic susceptibility (Li *et al*., 2007). EBV has been associated with a number of other malignant carcinomas including Hodgkin's lymphoma, Burkitt's lymphoma, post-transplant lymphoproliferative disease (PTLD), and diffuse large B cell lymphoma (Tsao *et al*., 2017). EBV rarely contributes to manifestation of epithelial cancers but epithelial cancers such as EBV associated gastric carcinoma (EBV-GC) and NPC, are exceptions (Young *et al*., 2017). The viral infection is detected in almost all undifferentiated NPC cells, the most common histologic subtype diagnosed in Southeast Asia (Chen *et al*., 2019).

EBV also known as the human herpes virus 4 (HHV-4), is a DNA virus (Tsao *et al*., 2017). Approximately 95% of the world's population sustain a life-long asymptomatic infection of this virus (Cohen, 2000). The proposed pathogenesis of NPC and the involvement of EBV in NPC pathogenesis is shown in Figure 2.9. Long term exposure to environmental carcinogens such as nitrosamines as a result of salted fish and preserved food consumption, lead to DNA damage and various somatic mutations in the nasopharynx epithelia (Li *et al*., 2007). The inactivation of tumour suppressor genes such as *Ras* association domain family 1A *(RASSF1A)* and cyclic dependent kinase inhibitor 2A *(p16/CDKN2A)* and also loss of heterozygosity (LOH) of chromosomes 3p and 9p are some of the initial events in the onset of NPC pathogenesis (Chan *et al*., 2002). These series of events lead to manifestation of low-grade and pre-invasive lesions which render the epithelial cells more vulnerable to EBV infection (Young & Dawson,

2017). Over-expression of *cyclin D1* or p16-resistant-*Cdk4* assist in persistent EBV infection in human telomerase reverse transcriptase-immortalized premalignant nasopharyngeal epithelial (NPE) cells (Tsang *et al*., 2012).

Upon infecting the epithelial cells, EBV established latent infection in the cells by expressing a set of latent genes namely the latent membrane proteins (LMP-1, LMP-2A and LMP-2B), EBV-determined nuclear antigens (EBNA) namely EBNA-leader protein (EBNA-LP), EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, two EBV-encoded small RNA, (EBER-1 and EBER-2) and the non-transcript BART (*Bam*HI-A region rightward 12 transcript). These viral proteins facilitate persistent infection of EBV in the epithelial cells – a key step in NPC pathogenesis (Tsao *et al*., 2017).

LMP-1 is present in 80-90% of NPC tissues and is the principal oncoprotein of EBV (Werner *et al*., 2007). LMP1 induces methylation of the *CDH-1* promoter to repress E-cadherin production (Tsai *et al*., 2002). Furthermore, LMP1 also drives the expansion of EBV-infected clones at early stages of NPC development which converts normal epithelia to high-grade dysplastic lesions (Pathmananthan *et al*., 1995). LMP1 activates the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway which leads to upregulation of survivins that support cell survival (Tsao *et al*., 2002). The activation of NF-κB also induces the expression of Interleukin-6 (IL6) for the activation of the IL6 receptor (IL6R) and the STAT3 signalling pathway, which result in the upregulation of B-cell lymphoma 3 (Bcl3) in NPC cells (Zhang *et al*., 2013). Moreover, LMP1 inactivates pro-apoptotic protein BAD via the P13K/Akt signalling pathway (Dawson *et al*., 2012). Meanwhile, the LMP2A viral protein activates the P13K/Akt signalling pathway which results in the inhibition of cellular

differentiation and increased cell proliferation through upregulation and stabilization of ΔNp63 (Fotheringham & Mazzucca, 2010). In advanced NPC cases, somatic mutations of TP53, RAS and other genes may lead to rapid expansion of sub-clones of NPC which are more resistant to chemo- and radio-therapy. The sub-clones emerge as local recurrent disease as well as distant metastasis (Tsang *et al*., 2020).

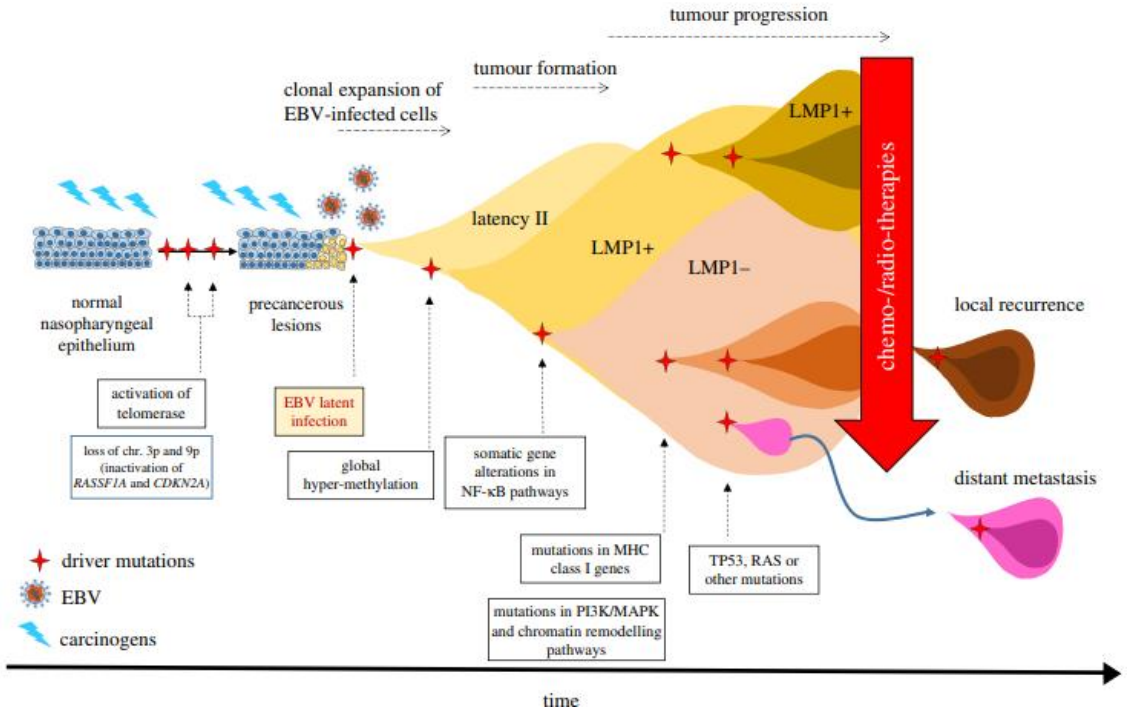

Figure 2.9: Proposed schematic presentation of NPC pathogenesis. Persistent EBV infection in genetically aberrant epithelial cells and clonal expansion of these infected cells are postulated to initiate tumorigenic transformation of the nasopharynx. Continuous exposure of the nasopharyngeal mucosa to carcinogens such as nitrosamines causes DNA damage which can lead to various somatic mutations in the epithelial cells. Driver mutations such as inactivation of tumour suppressor proteins *RASSF1A* and *p16* on chromosomes 3p and 9p lead to genomic instability and EBV infection in histologically normal cells. Persistent EBV infection and clonal expansion of the EBV infected cells are postulated to trigger tumorigenic transformation of the epithelia. EBV induces latency II gene products such as *EBNA1, LMP1, LMP2A, EBERs, and BART*s to facilitate cell proliferation, clonal expansion of EBV-infected pre-invasive nasopharyngeal epithelial cells and global hyper-methylation. Global hyper-methylation and mutations in NF-κb signalling pathway enhance tumour heterogeneity. In order to

compensate for the loss of LMP1, somatic mutations such as *TP53, RAS* and other mutations are acquired during the advance stages of the disease to facilitate sub-clones to progress and result in local recurrence and distant metastasis. These sub-clones are resistant to conventional treatment modalities such as chemo- and radiotherapy. Figure adapted from Tsao *et al*., Philosophical Transactions of the Royal Society B: Biological Sciences, 2017. ***Note:*** EBV: Epstein-Barr virus; *EBNA1*: EBV-determined nuclear antigens 1; *LMP1*: latent membrane protein 1; *LMP2A*: latent membrane protein 2A; *EBERs*: EBV-encoded small RNA; *BART*: *Bam*HI-A region rightward 12 transcript.

2.4 Existing Treatment Approaches for NPC

2.4.1 Conventional treatment modalities for NPC – Radio- and Chemotherapy

Nasopharyngeal carcinoma is a very radio- and chemo-sensitive tumour. Surgery is not a mainstream approach for NPC treatment due to its deep-seated anatomic location. Radiotherapy (RT) has been the mainstay and primary curative treatment modality for non-metastatic NPC (Tan *et al*., 2016). At present, the therapeutic decisions and strategies for NPC are predominantly based on the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) tumour-node-metastasis (TNM), staging system. Patients diagnosed with stage I NPC are treated with RT alone. Concurrent chemo-radiotherapy (CCRT) followed by adjuvant chemotherapy (AC) or induction chemotherapy (IC) followed by CCRT are treatment strategies for stage II to stage IVB NPC (Li *et al*., 2021). The five-year survival rate is more than 90% for patients who are diagnosed early. However, the survival rates decline to 50– 70% with locally advanced disease (Lee *et al*., 2005). Advanced RT technique, namely the intensity-modulated RT (IMRT), reported to decrease treatment-related toxic effects in patients (Lee *et al*., 2009). However, 50% to 75% of patients treated with IMRT, experience acute grade 3 or 4 adverse events and 10 to 20% of surviving patients

experience serious late complications, such as feeding tube dependency and tissue necrosis (Lee *et al*., 2009; Li *et al*., 2021).

Cisplatin is the standard-of-care chemotherapeutic drug for NPC and it is commonly used drug in CCRT. Cisplatin in combination with other chemotherapeutic drugs and/or radiotherapy have resulted in clinical benefits for patients diagnosed with NPC. For example, patients diagnosed with stage III-IVB non-keratinizing NPC, were randomly assigned to radiotherapy-alone (RT_i group: 218 patients) or cisplatin-based chemo-radiotherapy (CRT_i group: 223 patients), followed by cisplatin plus fluorouracil for three cycles. Findings of the study demonstrated that a significant improvement in overall failure-free rate (FFR), locoregional-FFR and cancer-specific survival ($p \leqslant 0.019$) were achieved in the CRT_i group (Lee *et al*., 2011). A phase 3 randomized controlled trial was performed to determine the survival benefits of adding docetaxel, cisplatin, and 5-fluorouracil (known as TPF) as induction chemotherapy prior to concurrent chemo-radiotherapy (CCRT), for treating patients diagnosed with locally advanced nasopharyngeal carcinoma (LA-NPC) with nodal stage N2–3. Patients were assigned to either the CCRT group (n = 16) or induction TPF followed by CCRT group (n = 12). Findings report that TPF followed by CCRT reduced distant metastasis in patients, indicating that this combination may be feasible for the treatment of LA-NPC with nodal stage N2–3. However, the TPF induction regime resulted in more side-effects compared to CCRT alone, especially in relation to neutropenia (low level of neutrophils) and leukopenia (low white blood cells count) (Kawahira *et al*., 2017). A retrospective study analysed the effect of gemcitabine and cisplatin (GP) on LA-NPC patients. All newly diagnosed NPC patients without any distant metastasis were subjected to either GP or

Fluorouracil + Cisplatin (FP) treatment arm. Patients whom received the GP regime, demonstrated improved three- and five-year overall survival (92.3% and 83.9%, respectively) and improved distant metastasis-free survival (DMFS) (92.3%) compared to patients whom received the FP regime. The three- and five-year OS for patients whom received the FP regime were 73.0% and 60.3%, respectively, while DMFS was 78.3%. Collectively, findings suggest that the GP regime was more effective for treating locoregionally advanced NPC (Zheng *et al*., 2015). Similarly, in another study, the GP regime displayed a higher OS rate compared to the FP regime in patients diagnosed with stage III-IVB NPC (Zhao *et al.,* 2017).

In events where patients either present with borderline renal function or experience adverse toxicities associated with high dose cisplatin, carboplatin is opted as a substitute for cisplatin. A phase III randomized trial demonstrated equivalent survival outcomes with either cisplatin or carboplatin administered concurrently with RT, followed by adjuvant chemotherapy. Moreover, tolerability to carboplatin was better compared to cisplatin. Patients treated with cisplatin experienced more renal toxicity, leukopenia, anaemia, nausea and vomiting. A higher percentage of patients in the carboplatin arm completed the planned CRT as well as the planned adjuvant therapy compared to patients in the cisplatin arm (Chitapanarux *et al*., 2007). Patients diagnosed with LA-NPC received carboplatin-based CCRT for three cycles, followed by adjuvant chemotherapy of carboplatin plus 5-fluorouracil for two cycles. The 3-years OS rate and PFS rates were 89.7% and 72.7%, respectively. The study suggested that CCRT with carboplatin followed by carboplatin and 5- fluorouracil could serve as an alternative treatment regime to cisplatin, for locally advanced NPC patients (Dechaphunkul *et al*.,

2011). A more recent study analysed case records of recurrent or metastatic (R/M) NPC patients treated with combination of paclitaxel, carboplatin and cetuximab (PCE). Study analysis reported response rate of 58.3% and 4.1-month PFS in patients treated with PCE. The most common grade 3 or 4 adverse events were neutropenia and skin reaction. Findings showed that PCE was feasible and may be potentially effective for patients with recurrent and/or metastatic NPC. Given that the study population was small, further evaluation using a larger study population is warranted (Ueda *et al.,* 2020).

Chemo- and radiotherapies have immensely contributed to treating NPC patients but it is often related to adverse events. Moreover, treating patients with local recurrence and distant metastasis with these treatment modalities remain a huge challenge as patients eventually develop resistance, thus making subsequent treatment with the same treatment regimens difficult. Recent understanding of the genomic landscape of NPC has resulted in testing of targeted therapies for NPC. The following section elaborates on targeted therapies.

2.4.2 Targeted therapies for NPC

Targeted therapies block signal transmission, and in some cases, activate cell death by targeting specific molecules, whilst minimizing toxicity and sparing normal cells. Unlike other solid tumours, there are only a limited number of targeted therapies that have undergone clinical trials for the treatment of NPC. Majority of drugs tested target either the Epidermal Growth Factor Receptor (EGFR), Vascular Endothelial Growth Factor (VEGF) or Vascular Endothelial Growth Factor Receptor (VEGFR) (Table 2.1). Clinical studies demonstrated that targeted therapies when tested alone only

yielded overall survival of less than a year. However, combination of these targeted therapies with chemotherapeutic drugs and/or radiotherapy displayed improved overall survival of more than a year (Table 2.1). However, there are outstanding issues remained to be addressed in testing targeted therapies for NPC; (1) Patient numbers enrolled in clinical trials are often low which undermine the efficacy of the drugs tested; (2) There are number of other potential therapeutic targets and miRNAs remain to be tested for NPC treatment. Expanding the list of targets may open new treatment avenues for NPC as there are limited number of second-line treatment options for patients who are suffering from advance NPC and (3) Most of the clinical studies are limited to phase I and II studies. More phase III trials at an accelerated phase are needed.

Table 2.1: List of targeted therapies which have undergone phase I and II clinical trials for the treatment of NPC. Table adapted from Kang *et al*., Signal Transduction and Targeted Therapies, 2020.

Targeted therapies	Mechanism/ Target	Trial Phase	Treatment Schedule	OS	Reference
Gefitinib	EGFR	II	Single drug	16 months	Chua *et al*., 2008
Famitinib	VEGFR, PDGFR, SCFR	I	Single drug	-	Chen *et al*., 2018
Sunitinib	Multi-kinase inhibitor	II	Single drug	10.5 months	Hui *et al*., 2011
Sorafenib	Multi-kinase inhibitor	II	Single drug	11.8 months	Xue *et al*., 2013
Axitinib	VEGFR	II	Single drug	10.4 months	Hui *et al*., 2018
Mk-2206	Akt	II	Single drug	10 months	Ma *et al*., 2015
Bevacizumab	VEGF	II	-	2 years	Lee *et al*., 2012
Cetuximab	EGFR	II	Cetuximab-radiotherapy	3 years	Xu *et al*., 2015
Aflibercept	VEGF	I	Aflibercept plus docetaxel	-	Huang *et al*., 2014
Endostar	VEGF, VEGFR,	II	Combination of gemcitabine	1 year	Jin *et al*., 2014

	PDGFR				
Nimotumab	EGFR	II	Combined with cisplatin and 5-Fluorouracil	16.3 months	Zhao *et al*., 2019
Nimotuzumab	EGFR	II	Induction chemotherapy, sequential Nimotuzumab plus concurrent chemoradiotherapy	3 years	Zhang *et al*., 2018
Cetuximab	EGFR	II	Concurrent HT with cetuximab, followed by chemotherapy (docetaxel and cisplatin)	2-3 years	Zhang *et al*., 2016

Note: "–" = not clear; OS = overall survival; EGFR = epidermal growth factor receptor; HT = helical tomotherapy; PDGFR = platelet-derived growth factor receptor; SCFR = stem cell factor receptor; VEGF = vascular endothelial growth factor; VEGFR = VEGF receptor

As shown in Table 2.1, most of the recent treatment efforts for NPC are focused on targeting the receptor kinases. Despite several studies reporting on high expressions of the BCL-2 anti-apoptotic proteins in NPC tissues and cells, efforts to target these molecules were minimal and the findings were unsatisfactory (Sheu *et al.,* 1997; Yu *et al.,* 2003; Fan *et al*., 2006) . These proteins could be potentially attractive targets for the treatment of NPC but the question is which of these proteins that NPC cells are addicted for survival? The broad aim of this work is to address that question. The following sections elaborate in detail on the apoptosis pathway, the BCL-2 family of proteins and BH3-mimetics that specifically target these proteins.

2.5 Apoptosis Pathway

Apoptosis is a biological process that is essential for normal physiological functions and maintenance of homeostasis in an organism (Quinn *et al*., 2012). Generally, apoptosis occurs during development and aging process and also as a defence mechanism which include immune response and cell damage via disease (Carneiro and El Deiry, 2020). Apoptosis can be triggered by various stress factors namely activation of oncogenes, ultraviolet radiation, growth factor deprivation and DNA damage caused by drugs (Carneiro and El Deiry., 2020). Apoptosis is characterized by cell shrinkage followed by the formation of apoptotic bodies, membrane- enclosed cell fragments that are rapidly cleared by phagocytes after they recognize the “eat- me” signals found on the outer surface of the plasma membrane of cells undergoing apoptosis (Dai *et al*., 2016). Apoptosis can occur through two distinct but interlinked pathways that include the extrinsic pathway activated by death-receptors and their respective ligands or the intrinsic or mitochondrial pathway (Quinn *et al*., 2012).