ELUCIDATING THE PROPORTION OF $CD4^+$ AND $CD8^+$ REGULATORY T-CELLS, ASSOCIATED CYTOKINES, AND EPSTEIN-BARR VIRUS' LMP1 *Xho*I MUTATION IN NASOPHARYNGEAL CARCINOMA

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by

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ABSTRAK

Sel T pengawalatur (Tregs) terlibat dalam imunopatogenesis pelbagai penyakit kanser termasuk kanser nasofarinks (NPC) yang berkait rapat dengan jangkitan virus Epstein-Barr (EBV). Kehilangan tapak pemotongan enzim *Xho*I pada protein membran pendam (LMP1) pada EBV 1 dilaporkan telah meningkatkan keupayaan strain EBV dalam pertumbuhan tumor. Di sebalik pemahaman tentang peranan Tregs dan mutasi EBV LMP1 *Xho*I dalam kanser, peranan mereka dalam patogenesis NPC masih belum jelas. Oleh itu, kajian ini bertujuan untuk mengelusidasi kadar Tregs, sitokin berkaitan, dan mutasi EBV LMP1 *Xho*I di dalam NPC bagi menyediakan data asas dan tambahan untuk memupuk langkah pendekatan imunoterapeutik semasa dan akan datang. Sampel darah dikumpulkan daripada pesakit NPC (*n*=23) dan individu yang sihat (*n*=23) sebagai kawalan. Biopsi tisu diperoleh daripada 7 pesakit NPC tersebut. Sampel digunakan dalam penilaian Tregs ($CD4^+FoxP3^+$, $CD4^+CD25^+FoxP3^+$, $CD8^+FoxP3^+$, and $CD8^+CD25^+FoxP3^+$) melalui kaedah sitometri aliran pelbagai warna, serta mutasi *Xho*I melalui tindak balas rantaian polimerase. Proliferasi Tregs ditentukan oleh pengekspresan Ki67, manakala proliferasi *ex vivo* dinilai selepas mengkultur PBMC dengan IL-2 dan anti-CD3/CD28 antibodi monoklonal. Tahap ekspresi lima sitokin (IL-2, IL-10, IL-6, TNF-α, dan IFN-γ) dinilai menggunakan analisis manik multipleks. Pada

umumnya, Tregs didapati meningkat dalam pesakit NPC. Mutasi *Xho*I dikesan di dalam pesakit (39%) dan individu yang sihat (30%). Korelasi di antara ciri-ciri klinikal-patologi dengan frekuensi peredaran Tregs bagi kedua-dua $CD4^+$ dan $CD8^+$ dalam pesakit NPC tidak menunjukkan hubungan yang signifikan, walaupun kadar Tregs didapati sedikit meningkat dalam kalangan pesakit kanser peringkat IV berbanding dengan peringkat II/III. Kadar $CD4^+$ dan $CD8^+$ Tregs juga meningkat dalam kultur PBMC secara *ex vivo* berbanding dengan sel yang tidak dirangsang. $CD4^+$ dan $CD8^+$ Tregs yang diperoleh daripada PBMC individu sihat bertindak balas dengan lebih baik terhadap rangsangan *ex vivo* berbanding dengan pesakit NPC. Perubahan yang ketara dapat diperhatikan pada paras ekspresi sitokin IL-10 dan TNF-α di dalam plasma pesakit NPC berbanding dengan kawalan. Kepekatan IL-10 adalah jauh lebih rendah di dalam pesakit NPC (10.47 ± 16.17 *versus* 16.12 ± 33.38, $p = 0.0157$). Manakala, paras TNF-α adalah lebih tinggi di dalam plasma pesakit ($p < 0.0001$) serta didapati berkorelasi dengan Tregs $CD4^+$ di dalam kawalan, tetapi tidak berkolerasi pada pesakit NPC. Terdapat kolerasi antara IL-2 dan Tregs $CD8^+CD25^+FoxP3^+$ ($r = -0.4670$, $p = 0.0438$). Data daripada kajian ini menunjukkan bahawa sasaran atau tumpuan pada pembangunan terapi yang khusus terhadap modulasi bilangan Tregs, peredaran atau aktiviti yang berkaitan dengannya dapat membantu dalam kajian lanjut tentang NPC.

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ABSTRACT

Regulatory T-cells (Tregs) are thought to be involved in the immunopathogenesis of many cancers including nasopharyngeal carcinoma (NPC) which is known to be strongly associated with Epstein-Barr virus (EBV) infection. Loss of EBV latent membrane protein 1 (LMP1) *Xho*I restriction site has been suggested to define EBV strain associated with increased tumourigenicity. Despite the perceived roles of Tregs and EBV LMP1 *Xho*I mutation in cancers, their roles in the pathogenesis of NPC are yet to be fully elucidated. Hence, this study aims to elucidate the proportion of Tregs, associated cytokines, and LMP1 *Xho*I mutation in NPC in order to provide baseline and additional data to foster the strides of current and future immunotherapeutic approaches. Blood samples were collected from NPC patients (n=23) and healthy controls (n=23). Tissue biopsy was available for 7 of the NPC patients. Samples were assessed for Tregs ($CD4^+FoxP3^+$, $CD4^+CD25^+FoxP3^+$, $CD8^+FoxP3^+$, and $CD8^+CD25^+FoxP3^+$) using multicolour flow cytometry, and *Xho*I mutation using polymerase chain reaction. Tregs proliferation was determined by Ki67 expression, and *ex vivo* proliferation was assessed after culturing PBMCs with IL-2 and anti-CD3/CD28 mAbs. The expression levels of five cytokines (IL-2, IL-10, IL-6, TNF-α, and IFN-γ) were evaluated using multiplex bead analysis. Tregs were generally found to be elevated

in NPC patients. *Xho*I mutation was detected in both patients (39%) and controls (30%). The correlation between clinicopathological features and the frequency of circulating Tregs in the patients revealed no significant correlation for both $CD4^+$ and $CD8^+$ Tregs, even though Tregs levels were fairly higher in patients with stage IV compared with stage II/III cancer. In comparison with unstimulated cells, both $CD4^+$ and $CD8^+$ Tregs proportion heightened following the *ex vivo* culture of PBMCs. $CD4^+$ and $CD8^+$ Tregs in the PBMCs obtained from healthy controls responded better to *ex vivo* stimulation compared with those from patients. Of the cytokines, significant expression levels were observed for plasma IL-10 and TNF-α in patients compared with controls. The concentration of IL-10 was significantly lower in patients (10.47 ± 16.17 versus 16.12 ± 33.38, $p = 0.0157$). Meanwhile, plasma levels of TNF-α were higher in patients ($p < 0.0001$) and were found to correlate with $CD4^+$ Tregs in controls but not in patients. Correlation between IL-2 and $CD8^+CD25^+FoxP3^+$ ($r = -0.4670$, $p = 0.0438$) Tregs was also observed. Data from this study demonstrates that targeting or developing therapies that specifically modulate Tregs number, trafficking, or activity, will be promising in NPC treatment.

CHAPTER 1

INTRODUCTION

1.1 Background of research

Globally, infectious agents account for about 2 million cancer cases annually (de Martel et al., 2020, 2012). The first human tumour virus to be identified, Epstein-Barr virus (EBV), is considered to be the cause of at least 0.2 million instances of cancer per year (de Martel et al., 2012). EBV-attributable malignancies are estimated to cause 1.8% of all cancer deaths (Khan and Hashim, 2014). In addition, about 95% of people worldwide are infected by EBV (Montgomery, 2014). Nasopharyngeal carcinoma (NPC), a tumour that emanates from the epithelial cells of the nasopharynx, is largely linked to EBV infection (Li et al., 2011; See et al., 2008). Although a rare type of malignancy in most regions of the world, NPC is endemic in southeast Asia (Chan et al., 2002a; Mahdavifar et al., 2016). It is the fifth most prevalent malignancy among Malaysian males, and one of the five most prevalent cancers among the Malaysian population (NCR, 2019).

The subtle signs and symptoms and/or asymptomatic presentation of NPC contribute to the commonly observed advanced stage NPC in diagnosed patients (Aziz et al., 2017). Usually, patients who present with late-stage NPC disease respond poorly to conventional therapy (Li et al., 2011). In non-metastatic NPC, the mainstay of treatment is radiotherapy. Meanwhile, except for patients with stage I disease, chemotherapy is typically recommended (Wong et al., 2021). These days, the intensity-modulated radiotherapy (IMRT) with or without chemotherapy is used to cure almost all

NPC patients with stage I-II disease: at 5 years, the estimated overall survival (OS) for stage I and II NPC is 98% and 92%, respectively; locoregional failure-free survival (FFS) is 98% and 94%; and distant FFS is 98% and 91% (Pan et al., 2016). An earlier report showed that upon administering conventional two-dimensional (2D) radiotherapy alone, patients with stage III-IVA NPC had poor prognoses due to the high incidence of distant metastases. The 5-year OS and progression-free survival (PFS) were 58.6% and 52.1%, respectively (Chan et al., 2005). Early in the new millennium, the inclusion of concomitant chemotherapy to radiotherapy (Blanchard et al., 2015) and a gradual transition from conventional radiotherapy techniques to IMRT (Chen et al., 2019a) marked two significant shifts in the treatment paradigm, with concomitant chemotherapy/radiotherapy raising 5 year OS and PFS up to 70.4% and 61.1%, respectively (Blanchard et al., 2015; Wong et al., 2021). In the last ten years, induction chemotherapy has attracted renewed interest, with many trials showing OS benefit when combined with concurrent chemoradiotherapy. As the intricate interrelationship between EBV and NPC are still being unravelled, the immunotherapeutic approach would likely hold sway in standard clinical practice and provide long-lasting remissions in NPC patients with advanced disease, who are, until now, incurable (Jain et al., 2016a).

EBV infection has been associated with many human tumours, with the closest association being the undifferentiated histological type of NPC which is endemic in Southeast Asia and southern China (Lo et al., 2004; Tsao et al., 2014). The detection of high titres of anti-EBV serum antibodies, including anti-VCA (anti-viral capsid antigen), as well as early antigen diffuse (EAd/BMRF1) in patients marked the first discovery of the association between NPC and EBV infection (Gunvén et al., 1970). Although EBV

infection occurs in nearly all NPC cells and virtually all undifferentiated NPCs, other than in salivary gland tumours, the virus is not found in other cancers of the head and neck (Young et al., 2016; Young and Dawson, 2014; Young and Rickinson, 2004). EBV is mostly transmitted through bodily fluids, especially saliva. Upon entry, it launches a lytic or latent infection in healthy host cells (e.g., epithelial and B-cells).

EBV latent membrane protein 1 (LMP1), an EBV protein capable of regulating its own expression and the expression of human genes (Pratt et al., 2012), is an oncoprotein of the EBV latent gene products as it is expressed in the majority of EBV-associated human cancers (Ersing et al., 2013). A mutation (single nucleotide polymorphism) in EBV *LMP1* gene that results in the loss of *Xho*I restriction site (*Xho*I-loss) arising from G $\rightarrow$ T mutation at position 169425 (Hu et al., 1991) have been suggested to define EBV variant that is linked with heightened tumourigenicity or with disease among people of a specific geographical location (Li et al., 1996; Trivedi et al., 1994). *In vitro* investigations showed that the LMP1 variant lacking the *Xho*I cleavage site in its amino terminus is more tumourigenic than the B95.8 cell prototype LMP1 (Chen et al., 1992). Compared with healthy controls, *Xho*I-loss is associated significantly with Chinese NPC, and it is considered a potential tumour marker (Chen et al., 1992; Hu et al., 1991; Jeng et al., 1994). However, the exact mechanism by which EBV LMP1 *Xho*I mutation enhances NPC pathogenesis and its modulation of immune responses in NPC remains to be substantiated in the literature.

Diverse cells, including almost all immune cells ($CD4^+$ T-cells, $CD8^+$ T-cells, regulatory T-cells (Tregs), natural killer cells, dendritic cells, etc.), constitute a typical tumour microenvironment (Liu et al., 2016a). In this study, however, the Tregs

component of the adaptive immunity of NPC patients alongside common cytokines implicated in NPC pathogenesis was explored. Tregs are a minor T-cells subset with a strong suppressive ability and function essentially in maintaining immune homeostasis (Liu et al., 2016a). While this suppressive function favours self-tolerance and helps avert autoimmune disease, the cytotoxic roles of immune cells are also repressed, thus, allowing the proliferation of cancer cells. Both $CD4^+$ and $CD8^+$ Tregs have been described, with $CD4^+$ Tregs being well documented. Tregs are characterised by the expression of the transcription factor FoxP3 (Forkhead box protein 3) (Chan et al., 2002a), which is considered the most potent marker for Tregs. Researches have demonstrated $CD4^+CD25^{hi}CD127^{low/-}$ cells as $FoxP3^+$ (Kondělková et al., 2010). Numerous subsets of $CD8^+$ Tregs have been defined using CD8αα, CD25, CD38, CD56, CD103, CD122, LAG-3, CD45RA, CD45RO, FOXP3, CXCR3, and/or HLA-G expression, and the absence of CD28 and CD127 (Churlaud et al., 2015). In this study, both $CD4^+$ and $CD8^+$ Tregs were investigated.

$CD4^+$ Tregs are known to exert their suppressive functions in four main ways (Vignali et al., 2008). First, by the secretion of inhibitory cytokines such as interleukin-10 (IL-10), IL-35 and transforming growth factor-β (TGF-β), which affects effector T-cell proliferation and function. Second, via Tregs-cell-mediated target-cell killing by the secretion of granzymes and perforins. Third, through disruption of metabolic processes including high-affinity CD25-dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A ($A_{2A}R$)-mediated immunosuppression. Lastly, via mechanisms that modulate the maturation and/or functions of dendritic cells (Vignali et al., 2008). On the

other hand, soluble factors such as TNF-α and CCL-4 are thought to be involved in the suppressive mechanism of $CD8^+$ Tregs (Ablamunits et al., 2010; Mahic et al., 2008). There have also been reports of costimulatory molecule downregulation on DCs cocultured with mouse or human $CD8^+FoxP3^+$ Tregs (Correale and Villa, 2010; Sun et al., 2019). Additionally, it has been demonstrated that the adoptive transfer of $CD8^+FoxP3^+$ Tregs induces the expansion of $CD4^+$ Tregs (Lerret et al., 2012; Sun et al., 2019). However, direct cytotoxicity or competition for IL-2 do not appear to be significant factors (Ablamunits et al., 2010) for $CD8^+FoxP3^+$ Tregs. The levels of $CD4^+CD25^+FoxP3^+$ Tregs is reported to be higher in NPC patients compared with controls (Bi et al., 2017). Although data on $CD8^+CD25^+FoxP3^+$ Tregs population in NPC is lacking, their frequencies have also been found to increase in the blood and tissues of colorectal cancer patients in comparison to controls (Chaput et al., 2009a).

Several cytokines, including pro- and anti-inflammatory cytokines (e.g., 1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, TNF-α, etc.) have been evaluated in connection with NPC disease (Beck et al., 2001; Chang et al., 2011; Huang et al., 1999; Lo et al., 2021). Cytokines such as IL-2, IL-10, and TGF-β are important for the proliferation and functions of Tregs. Further, IFN-γ is crucial to the function of CD8 effector T-cells. In comparison with controls, the levels of theses cytokines appear to vary. In this present study, the levels of some of these cytokines were also assessed.

Over a decade ago, an attempt was made to assess the proportion of $CD8^+$ Tregs in Malaysian patients (Yip et al., 2009). However, flow cytometry, the gold standard for immunophenotyping of peripheral blood leukocytes (PBLs) (Gerstner et al., 2006), was not utilised, and Tregs were assessed from paraffin-embedded tissues. Thus, it is

imperative to elucidate the distribution of this T-cell subset both in peripheral blood cells and tissue microenvironment with flow cytometry technique using fresh patient samples. To decipher the true proportion of these regulatory T-cells in NPC, this study hopes to address limitations of the previous research and ultimately provide baseline data on the actual distribution and functional roles of both $CD4^+$ and $CD8^+$ Tregs in NPC.

1.2 Justifications of the study

As generally established, NPC and EBV infection are closely associated. This virus has been shown to infect greater than 90% of people worldwide, emphasising the risk of developing the cancer. In addition, mild and severe cases of NPC are frequently reported among the Malaysian population. Moreover, some cases are diagnosed at a late stage of the cancer, thus, dwindling the life expectancy of the patient.

Late-stage NPC remains a major menace in the clinical treatment of NPC as current conventional therapy appears ineffective against it. The race for immunotherapy, including the utilisation of adoptive T-cell transfer and immune checkpoint inhibitors as alternative therapeutic approaches, relies on detailed insight into the immunological status of patients. While several studies have focused on $CD4^+$ Tregs, there is a dearth of data on $CD8^+$ Tregs in NPC patients. Like $CD4^+$ Tregs, $CD8^+$ Tregs possess suppressive ability, and both cells have been shown to be present in cancer and autoimmune disease conditions. Furthermore, even though $CD8^+$ Tregs express more proliferative marker (Ki67) than other $CD8^+$ T-cells (Churlaud et al., 2015), their expansion in the peripheral blood and tissue microenvironment of NPC patients remains unelucidated. The

combined presence of $CD4^+$ and $CD8^+$ Tregs could influence overall immune suppression in a disease condition. However, no study currently investigates the combined presence of these Tregs, particularly the $FoxP3^+$ Tregs populations, in NPC. In addition, some cytokines, including pro- and anti-inflammatory cytokines, have been implicated in Tregs function and the pathogenesis of NPC. Expounding the distribution and function of these T-cell subsets alongside the associated cytokines in NPC patients will bolster the stride towards developing effective and durable immunotherapy against NPC. Ultimately, data from this study would not only be vital to the understanding of EBV's pathogenesis and future treatment of NPC but would also be helpful in developing immunotherapy for other cancers and autoimmune diseases.

1.3 Hypothesis

This study hypothesised that:

1. An increased level of Tregs is associated with NPC.
2. Tregs exhibit a non-anergic phenotype.
3. EBV LMP1 *Xho*I mutation is peculiar to NPC and can distinguish NPC from non-NPC.
4. Pro- and anti-inflammatory cytokines are involved in the pathogenesis of NPC.

1.4 Research questions

The research questions for this study are:

1. Is the proportion of $CD4^+$ and $CD8^+$ Tregs increased in NPC patients?
2. Do $CD4^+$ and $CD8^+$ Tregs proliferate *in vivo* and *ex vivo*?

3. Is EBV LMP1 *Xho*I mutation present only in NPC?
4. What are the expression levels of IL-2, IL-6, IL-10, TNF-α, and IFN-γ in NPC, and which correlates with the frequencies of Tregs in NPC?

1.5 Aim and objectives

The study aims to elucidate the proportion of regulatory T-cells, associated cytokines, and EBV LMP1 *Xho*I mutation in NPC.

Specific objectives include:

1. To determine the proportion of $CD4^+$ and $CD8^+$ Tregs in the peripheral blood and tissues of NPC patients in comparison with healthy controls.
2. To determine the ratios of $CD4^+$ to $CD8^+$ Tregs in the peripheral blood and tissues of NPC patients in comparison with healthy controls.
3. To assess the correlation between clinicopathological features of NPC patients and the proportion and ratios of $CD4^+$ and $CD8^+$ Tregs.
4. To assess the proportion of EBV LMP1 *Xho*I mutation in NPC patients and healthy controls.
5. To evaluate the *in vivo* proliferative status and the *ex vivo* proliferative competence of $CD4^+$ and $CD8^+$ Tregs.
6. To assess cytokine (IL-2, IL-6, IL-10, TNF-α, IFN-γ) levels in NPC patients and healthy controls.
7. To determine the correlation between the frequencies of Tregs and cytokine levels in NPC patients and healthy controls.

CHAPTER 2

LITERATURE REVIEW

2.1 Nasopharyngeal carcinoma (NPC)

Nasopharyngeal carcinoma (NPC), also known as nasopharyngeal cancer or nasopharynx cancer or simply NPC, is a rare head and neck tumour which arises mainly from the nasopharynx's mucosa epithelium. The tumour is commonly seen at the fossa of Rosenmüller (pharyngeal recess) in the nasopharynx. It is a type of head and neck malignancy, and it originates from the squamous cells (Gaillard and El-Feky, 2021). Despite arising from comparable cell or tissue lineages, NPC and other epithelial tumours of the head and neck are clearly distinct (Chen et al., 2019a). Of the primary malignancies of the nasopharynx, NPCs are the most common, as they account for nearly 70% of the malignancies (Gaillard and El-Feky, 2021).

2.1.1 Epidemiology

Compared with other types of cancers, NPC is relatively uncommon. According to estimates from the International Agency for Research on Cancer for 2020, there were about 133,354 new cases of NPC, although accounting for only 0.7% of carcinomas diagnosed in 2020 (Sung et al., 2021). NPC is unevenly distributed globally. The cancer is more prevalent in south China, southeast Asia, and north Africa. In Hong Kong and the areas of southern China, incidence rates of up to 50 per 100,000 individuals have been reported (Smith and Khanna, 2012). According to reports, NPC is the primary cause of death among Hong Kong, Guangxi, and Guangdong people, and ranks as

China's eighth foremost cause of cancer-related deaths (Guo et al., 2009). In southeast Asia, it is the sixth most dominant malignancy in males (Tay et al., 2014). In Malaysia, it is the fifth most prevalent cancer and the fifth most common among males (NCR, 2019). The incidence of NPC is approximately 2 – 3 times greater in males than females. For China, a male-to-female ratio of about 2.5 was reported in 2015. In Malaysia, a ratio of around 2.7 was recorded in the 2012-2016 national cancer registry report (NCR, 2019). Emigration of people from endemic areas of Asia into regions like the United States and Australia has led to a gradual increase in the cases of NPC in those areas (Hamid, 2021).

2.1.2 Aetiology and risk factors

Like in many other epithelial cancers, the non-keratinizing kind of epithelial cancer has been described as progressing gradually from dysplasia to carcinoma in situ (Shanmugaratnam, 1967; Yeh, 1967). Preinvasive lesions are linked to allelic losses on chromosomes 3p and 9p, which supposedly render tumour suppressors such as p14, p15, and p16 inactive (Chan et al., 2000; Lo et al., 2000b, 2001, 1996). It is interesting to note that southern Chinese nasopharyngeal epithelia with histologically normal chromosomes 3p and 9p loss have been found (Chan et al., 2002b, 2000). Preinvasive NPC lesions have also been reported to harbour EBV in situ (Lo et al., 2004; Pathmanathan et al., 1995; Yeung et al., 1993).

While the carcinogens exactly responsible for NPC are largely unknown, smoking and the consumption of alcohol are possible causative elements for keratinizing NPC. High consumption of foods preserved with salt, as well as fermented foods

containing a high amount of nitrosamine, has been implicated in non-keratinizing NPC, especially in populations where non-keratinizing NPC is endemic. As far back as 1972, the aetiology of non-keratinizing NPC was suggested to be multifactorial, including genetic factors, EBV infection and the intake of salted fish for several years (Ho, 1972). The involvement of genetic susceptibility has been widely investigated. Lu et al (Lu et al., 1990) published the seminal study associating human leukocyte antigen (HLA) loci to heightened risk of non-keratinizing NPC in 1990. Among Chinese residing in the Asian region, the HLA alleles A2 and B46 are linked to greater NPC risk (Goldsmith et al., 2002; Hu et al., 2005). Two genome-wide studies reported about a decade ago found that certain HLA loci were linked with predisposition to NPC occurrence in Taiwanese and Cantonese people (Bei et al., 2010; Tse et al., 2009). In contrast to the prevalent Caucasian HLA-A*0201 subtype, a high-resolution genotyping investigation discovered a steady link between NPC and the common Chinese A2 subtype HLA-A*0207 (Hildesheim et al., 2002). In addition, DNA repair enzymes encoded by *hOGG1* and *XRCC1* genes as well as genes *CYP2E1* and *GSTM1* that encode metabolic enzymes have all been related to polymorphisms that enhance the likelihood of NPC development (Hildesheim et al., 1997, 1995). Other investigations suggest that chromosomes 3, 4, and 14 harbour the susceptibility loci for NPC (Bei et al., 2010; Cheng et al., 1997; Feng et al., 2002; Shao et al., 2002; Zeng et al., 2006).

Certain changes in genomic constituents capable of stimulating NPC's development and advancement have been identified. These include mutations related to multiple loss-of-function in NF-kB pathway's negative regulators, mutations associated

with the cell cycle (TP53), PI3K/MAPK signalling pathways, chromatin modification, and DNA repair (Chen et al., 2019a) (Table 2.1).

Table 2.1: Major genetic mutations in NPC

Pathway/cellular process	Gene	Gene type
NF-κB pathway	*TRAF3*	Tumour suppressor
	TNFAIP3	
	NLRC5	
	NFKBIA	
	FBXW7	
	CYLD	
PI3K or MAPK pathway	*PIK3CA*	Oncogene
	NRAS	
	KRAS	
	FGFR2	
	ERBB3	
	ERBB2	
	PTEN	Tumour suppressor
	NF1	
Cell cycle	*MED12L*	Oncogene
	CCND1	
	TP53	Tumour suppressor
	CDKN2A	
Chromatin modification	*TSHZ3*	Tumour suppressor
	KMT2D	
	KMT2C	
	EP300	
	BAP1	
	ARID1A	
DNA repair	*MLH1*	Tumour suppressor
	MSH6	

This table was adapted from Chen et al (2019) (Chen et al., 2019a).

One environmental element that is considered to have the biggest correlation with non-keratinizing NPC is salted food (Armstrong et al., 1998, 1983; Yu et al., 1986, 1981). Studies revealed that by feeding rats with Chinese salted fish, NPC can indeed be induced (Huang et al., 1978b). Intriguingly, these salted diets have also been shown to contain EBV-activating elements and volatile carcinogenic nitrosamines or their precursors (Huang et al., 1981, 1978a; Shao et al., 1988; Zou et al., 1994). Other causative or contributing environmental factors that have been reported to constitute potential risks for NPC include work-related exposure to formaldehyde, wood dust, smoke, dust, heat, and chemical fumes (Armstrong et al., 2000; Ning et al., 1990; Yu et al., 1986). The burning of incense sticks containing polycyclic aromatic hydrocarbon with well-established carcinogenic properties has also been associated with non-keratinizing NPC (Schoental and Gibbard, 1967; Yang et al., 2007; Yu et al., 1990).

2.1.3 Screening for NPC with plasma EBV DNA

EBV genome is harboured by the tumour cells in nearly all of the NPC cases in endemic areas (Lam and Chan, 2018; Wolf et al., 1973). Due to the great link with EBV, viral nucleic acids (Lo et al., 1999) or the host antibody response to the virus (Henle et al., 1970; Ho et al., 1976) have been investigated as potential NPC biomarkers. Plasma EBV DNA that are in circulation have been thoroughly investigated as a biomarker of cancer for the purpose of tracking and predicting NPC. For monitoring recurrence following radical treatment, it is utilised in addition to endoscopy and imaging (Hong et al., 2004). The prognostication relevance of plasma EBV DNA levels before, during, and after treatment (Leung et al., 2006, 2014; Lin et al., 2004; Lo et al., 2000a) have likewise been assessed in NPC patients. Of recent, the plasma EBV DNA's role in the

assessment of NPC was confirmed in a major prospective investigation constituting 20,174 asymptomatic individuals in an endemic area (Chan et al., 2017). The term "screen-positive" was used to define participants with any evidence of plasma EBV DNA by real-time PCR assay twice in a row (i.e., two successive occasions). These "screen-positive" individuals subsequently underwent magnetic resonance imaging and endoscopy for diagnosis confirmation. A larger percentage of early NPC cases (stage I and II) among the screened group compared to the historical cohort illustrated the importance of early detection. Furthermore, compared with the historical cohort, patients whose NPC status was detected by screening demonstrated superior progression-free survival (PFS).

2.1.4 Diagnosis

An in-depth examination of the head and neck, including nasopharyngoscopy, is done when diagnosing NPC. The major methods of diagnosis include: (i) clinical assessment of the position and size of the cervical lymph nodes; (ii) assessment of primary tumour using indirect nasopharyngoscopy; (iii) cranial nerves neurological testing; (iv) magnetic resonance imaging (MRI) or computed tomography (CT) scan of the head and neck until areas under the clavicles to evaluate base of skull erosion; (v) radiotherapy of the chest (lateral, as well as anteroposterior) to assess possible lungs metastasis of the NPC; (vi) bone scintigraphy by Tc 99m diphosphonate to evaluate bone metastasis of the tumour; (vii) full blood count; (viii) creatinine, electrolyte, urea, liver function, PO_4, Ca, alkaline phosphate; (ix) EBV DNA and EBV viral capsid antigen; (x) histological examination of biopsy of the primary tumour or the lymph nodes (Brennan, 2006).

Differential diagnosis includes other malignant conditions like melanoma, lymphoma, adenoid cystic carcinoma, and extramedullary plasmacytoma, and several benign conditions like nasal polyp, tuberculosis, and serous otitis media (Chen et al., 2019a). In some cases, it is hard to distinguish lymphoma from undifferentiated NPC. Specific lymphoma immunohistochemical markers and EBER in situ hybridisation can supplement haematoxylin-eosin staining in such cases. Liquid biopsies such as plasma EBV serology and especially plasma EBV DNA can also help confirm NPC diagnosis (Chan et al., 2017; Coghill et al., 2014).

2.1.5 Classification and staging of NPC

Three histological subtypes of NPC are categorised by the World Health Organization (WHO): keratinizing squamous cell carcinoma (WHO type I), differentiated non-keratinizing carcinoma (WHO type II), and undifferentiated non-keratinizing carcinoma (WHO type III) (Sinha and Gajra, 2021). The keratinizing subtype is responsible for lower than 20% of the global cases, and it is infrequent in areas where it is endemic (e.g., southern China). On the other hand, the non-keratinizing subtype is the most prevalent in endemic areas, responsible for over 95% of cases, and it has a strong correlation with EBV infection. (Chen et al., 2019a). Types II and III are the most responsive to treatments such as radiotherapy and chemotherapy (Sinha and Gajra, 2021). A newer, rarer histological category is NPC with basaloid features and is known to behave more aggressively (Sinha and Gajra, 2021).

The tumour-node-metastasis (TNM) staging system of the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) (Table 2.2) is

often used for the classification and staging of the tumour. In 2016, the scheme was updated to the eighth edition, and the new edition is increasingly being adopted for the staging of NPC worldwide.

Table 2.2: AJCC/UICC staging system for NPC

	7th Edition	8th Edition
Primary tumour (T-category)		
TX	Primary tumour cannot be assessed	Primary tumour cannot be assessed
T0	–	No tumour identified, but there is EBV-positive cervical node(s) involvement
T1	Nasopharynx, oropharynx, or nasal cavity without parapharyngeal extension	Nasopharynx, oropharynx, or nasal cavity without parapharyngeal extension
T2	Parapharyngeal extension	Parapharyngeal extension, adjacent soft tissue involvement (medial pterygoid, lateral pterygoid, prevertebral muscles)
T3	Bony structures of skull base and/or paranasal sinuses	Bony structures (skull base, cervical vertebra) and/or paranasal sinuses
T4	Intracranial, cranial nerves, hypopharynx, orbit, infratemporal fossa or masticator space	Intracranial extension, cranial nerve, hypopharynx, orbit, extensive soft tissue involvement (beyond the lateral surface of the lateral pterygoid muscle, parotid gland)
Regional lymph nodes (N-category)		
NX	Regional lymph nodes cannot be assessed	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis	No regional lymph node metastasis
N1	Unilateral cervical, unilateral, or bilateral retropharyngeal lymph nodes, above the supraclavicular fossa; ≤6 cm	Unilateral cervical, unilateral, or bilateral retropharyngeal lymph nodes, above the caudal border of cricoid cartilage; ≤6 cm
N2	Bilateral metastasis in lymph node(s), ≤6 cm in greatest dimension, above the supraclavicular fossa	Bilateral metastasis in lymph node(s), ≤6 cm in greatest dimension, above the caudal border of cricoid cartilage
N3a	Bilateral metastasis in lymph node(s), ≤6 cm in greatest dimension, above the caudal border of cricoid cartilage	>6 cm and/or below caudal border of cricoid cartilage (regardless of laterality)
N3b	Supraclavicular fossa	–
Distance metastasis (M-category)		
M0	No distant metastasis	No distant metastasis
M1	Distant metastasis	Distant metastasis
Stage group		
I	T1 N0 M0	T1 N0 M0
II	T2 N0–1 M0, T1 N1 M0	T2 N0–1 M0, T0–1 N1 M0
III	T1–3 N2 M0, T3 N0–1 M0	T3 N0–2 M0, T0–2 N2 M0
IVA	T4 N0–2 M0	T4 or N3 M0
IVB	Any T, N3 M0	Any T, any N, M1
IVC	Any T, any N, M1	–

This table was adapted from Chen et al (2019) (Chen et al., 2019a).

2.1.6 Clinical presentations

The clinical signs and symptoms of NPC correlate with the anatomic regions involved (Figure 2.1). NPC often starts from the nasopharynx's lateral wall, which includes the fossa of Rosenmüller (Brennan, 2006) (Figure 2.2). The pattern of spread follows established routes: anteriorly into the nasal cavity, paranasal sinuses, and orbits; laterally into the levator and tensor veli palatini muscles, parapharyngeal space, and infratemporal spaces; superiorly and posteriorly into the base of the skull, clivus, and intracranial structures; and inferiorly into the oropharynx (Chen et al., 2019a). In most cases (about 75-90%), NPC patients develop locoregionally advanced disease, usually with metastasis to cervical lymph node (Petersson, 2015). The typical symptoms NPC patients present with can be categorised into four main groups: (I) association with nasopharyngeal mass (nasal obstruction, discharge, and epistaxis); (II) related to dysfunction of the Eustachian tube (tinnitus, decreased hearing); (III) involvement of the skull base (erosion) with impairment of the fifth and sixth cranial nerves (facial pain, diplopia, headache, and paresthesia/numbness) and; (IV) neck mass (Petersson, 2015). In about 70% of all patients, lymphatic spread to the neck is common. The most popular presenting symptom that prompts a patient to seek medical care is the development of cervical mass (Chen et al., 2019a). While skipped metastasis is uncommon, the most commonly involved nodal stations are the retropharyngeal and level II neck nodes (Chen et al., 2019a).

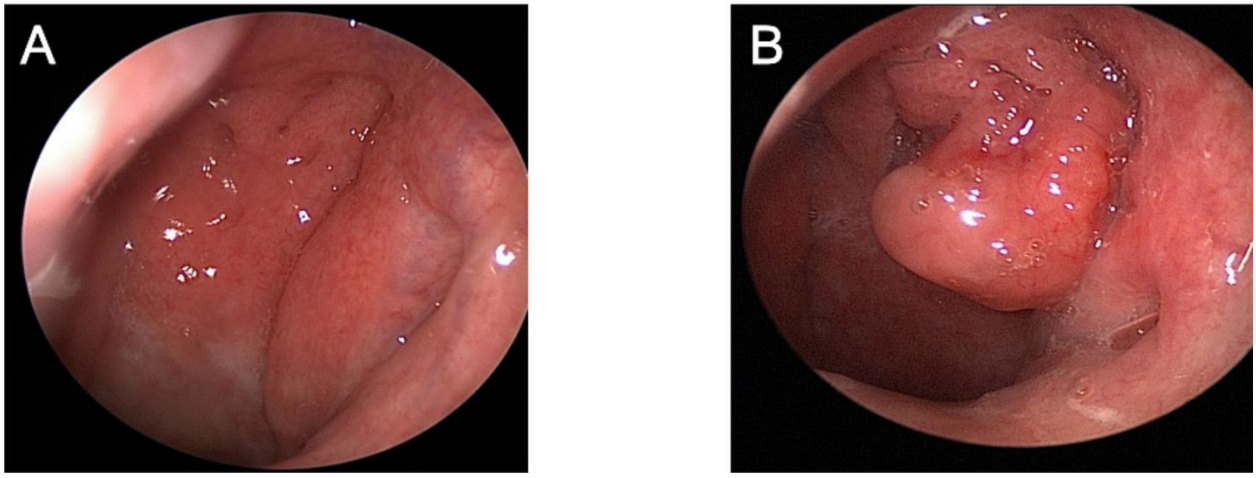

Figure 2.1: Endoscopic view of (A) normal nasopharynx and (B) nasopharyngeal carcinoma.

This figure was adapted from Chen et al. (Chen et al., 2019a)

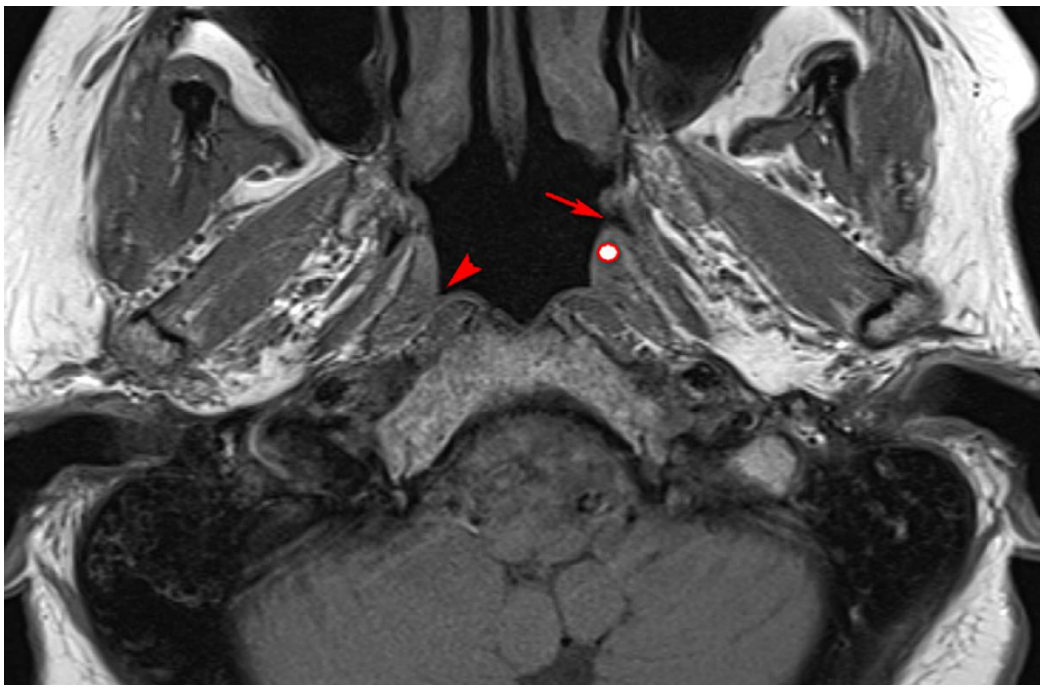

Figure 2.2: Axial non-fat-saturated T1-weighted MRI scan of the nasopharynx.

"The fossa of Rosenmüller (arrowhead) is a deep recess lying posterior to the torus tubarius (circle), a prominent elevation on the lateral wall of the nasopharynx formed by the cartilaginous end of the Eustachian tube. The Eustachian tube opens anteriorly (arrow)." This figure was adapted from Petersson, 2015 (Petersson, 2015).

2.1.7 Treatment

Radiotherapy (RT) is apparently the mainstay for treating NPC, and it is the primary treatment option for people with stage I disease (Petersson, 2015). While the control of NPC primary tumour can be achieved with radiotherapy (Deutsch et al., 1978; Jenkin et al., 1981; Ng et al., 2022; Pick et al., 1974), it does not hinder the emergence of distant metastasis (Chen et al., 2021; Deutsch et al., 1978; Jereb et al., 1980). Chemotherapy is also employed in the treatment of NPC, and can be used together with radiation therapy (chemoradiotherapy) as the main treatment for more advanced NPC, or as an initial treatment prior to chemoradiation (induction chemotherapy) or after radiation/chemoradiation (adjuvant chemotherapy) (ACS, 2022). The chemotherapeutic drug often used to treat NPC is cisplatin. The drug may be combined with other drugs like gemcitabine or 5-fluorouracil (5-FU) (ACS, 2022). In cases where cisplatin is not a good choice, carboplatin might be used. Other helpful drugs include epirubicin, paclitaxel, methotrexate, docetaxel, and capecitabine (ACS, 2022). For patients with stage II disease, chemoradiotherapy has been suggested, however, randomised trials are lacking (Petersson, 2015). Chemoradiotherapy have been used to treat patients with stage III disease since 1998, with a record 30% rise in 3-year survival compared with treatment using only radiotherapy (Al-Sarraf et al., 1998). The addition of adjuvant cisplatin and fluorouracil to chemoradiotherapy appears not to be advantageous (Chen et al., 2012). Patients with stage IVA/B NPC are likewise treated with chemoradiotherapy, meanwhile stage IVC patients only receive chemotherapy. As for neck (regional) recurrence, the preferred method is surgery (unless contraindicated, e.g., a case with the involvement of the carotid). Similarly, surgery is usually employed for low-T-stage

local recurrences (rT1 and rT2) if the tumour is deemed resectable (Petersson, 2015). In situations where surgery is not practical, either re-irradiation (re-RT) or chemoradiotherapy is utilised. Re-RT is faced with the problem of toxicities. Even though chemotherapy is utilised, there is a substantial chance of therapy failure in NPC patients suffering from locoregional advanced disease (particularly due to metastasis) (Cheng et al., 1998). Generally, patients suffering from stage IV disease often exhibit poor prognosis (Cheng et al., 2000). Meanwhile, as for patients suffering from advanced locoregional disease, a cure can be anticipated (Petersson, 2015).

Immunotherapy for NPC is a burgeoning area with rising evidence for potential future utilisation in routine treatment for NPC. Since non-keratinizing EBV^+ NPC is frequently associated with extensive lymphocyte infiltration of the tumour stroma, it is a desirable immunotherapeutic target (Le et al., 2019). Nonetheless, NPC is known for its ability to evade immune surveillance by its host. Past immunotherapeutic approaches concentrated on the use of EBV-specific vaccines based on peptides or dendritic cells, treatment involving the use of autologous cytotoxic T-cell, and initiation of EBV lytic infection. Available evidence suggests that the utilisation of adoptive transfer of EBV-specific cytotoxic T-cells is capable of initiating a long-lasting immunogenic response, producing favourable objective responses and durable disease control (Li et al., 2015; Louis et al., 2010; Smith et al., 2017; Straathof et al., 2005).

Recent researches utilising immune checkpoint inhibitors, principally leveraging the PD1 or PD-L1 pathway in conditions of recurrence or metastatis, have revealed promising therapeutic effects (Fang et al., 2018; Hsu et al., 2017; Ma et al., 2018). A minimum of two ongoing trials employ immune checkpoint inhibitors for locoregionally

advanced NPC. One is a phase 2 single-arm investigation (NCT03267498) of chemoradiotherapy in the presence or absence of adjuvant nivolumab at various dose schedules for up to 3 months. Another is a phase 3 randomised trial (NCT03427827) to assess 1-year adjuvant camrelizumab following chemoradiotherapy versus observation of NPC in stage III–IVA. The immune checkpoint is a sort of immunosuppressive molecule that is involved in the developmental process of malignant tumours (Abril-Rodriguez and Ribas, 2017; Lim et al., 2017; Wei et al., 2018). Studies have proven immune checkpoints effective targets for the suppression of tumour cells (Gubin et al., 2014; Topalian et al., 2016). The discovery of immune checkpoints like PD-1, PD-L1, and CTLA-4 (Constantinidou et al., 2019; Rowshanravan et al., 2018) is crucial to the development and advancement of tumour immunotherapy. Inhibitors of the PD-1/PD-L1 immune checkpoint have particularly been of immense interest in anti-tumour NPC therapy (Jain et al., 2016b; Masterson et al., 2020). In a two-arm investigation involving sixty-seven NPC patients having recurrent and metastatic disease, the patients' 6-month progression-free survival (PFS) and overall survival (OS) rates were markedly increased when chemotherapy was used to complement anti-PD1 inhibitor compared with the use of anti-PD1 inhibitor alone for treatment (Jin et al., 2021). The PD-1 inhibitor, tislelizumab, was shown to elicit anti-tumour responses in NPC patients with solid tumour, although anaemia was reported as the most common adverse event (Shen et al., 2020). Another PD-1 inhibitor, nivolumab, was proven to have anti-tumour effects in patients having various kinds of recurrent and metastatic squamous cell carcinoma of solid tumour (Ferris et al., 2016). In an investigation carried out by Sato et al. (Sato et al., 2020), a high 1-year survival rate of 75.8% was recorded