INVESTIGATION OF TNFR2 REGULATORY T CELLS AND ASSOCIATED CYTOKINES ON THE PATHOGENESIS OF NASOPHARYNGEAL CARCINOMA

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by

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LIST OF ABBREVIATIONS

AICD	Activation-induced cell death
AJCC	American Joint Committee on Cancer
AML	Acute myeloid leukemia
ASR	Age-standardized rate
BCL-2	Regulator protein that regulate cell death by either inhibiting or inducing apoptosis
BSA	Bovine serum albumin
CAFs	Cancer-associated fibroblasts
CCL	Chemokine (C-C motif) ligand
CCRT	Concurrent chemoradiation therapy
CD-	Cluster of differentiation- e.g.: CD25, CD127
CD103	Type I transmembrane protein present on intestinal intraepithelial lymphocytes, some circulating leukocytes, and some T cells that facilitates adhesion to epithelia
CD127	IL-7 receptor alpha chain, found on human Tregs
CD25	Type I transmembrane protein found on T cells, involved in cellular activation or adhesion
CD3	Signaling component of the T cell receptor complex
CD4	Co-receptor for MHC Class II, found on T helper cells
CD45RA	Discriminates between naïve and TCR-triggered Tregs
cIAP	Complex inhibitors of apoptosis proteins
CR	Complete response
CT	Computerized tomography
CTAR-	C-terminal domains e.g.: CTAR1 and CTAR2
CTLA4	Cytotoxic T lymphocytes associated antigen 4
CXCR	C-X-C chemokine receptor
DD	Detectable difference
DFS	Disease free survival
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor

ELISA	Enzyme-linked immunosorbent assay
EMT	Endothelial-mesenchymal transition
ERKs	Extracellular signal-regulated kinases
FCS	Foetal calf serum
FDC	Follicular dendritic cells
FMO	Fluorescence minus one
FoxP3	Forkhead box P3
FSC	Forward-light scatter
HIF	Hypoxia inducible factor
HPE	Histopathology examination
IDC	Interdigitating dendritic cells
IFN	Interferon
IL	Interleukin
IUCC	International Union Against Cancer
JNK	c-Jun N-terminal kinase
kbp	kilobase pairs
Ki67	Nuclear protein associated with cellular proliferation
LCL	Lymphoblastoid cell line
LPDs	lymphoproliferative disorders
MAPK	Mitogen-activated protein kinase
MDSCs	Myeloid-derived suppressor cells
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
miRNA	microRNA
MMP	Matrix metalloprotease
MRI	Magnetic resonance imaging
NF-κβ	Nuclear factor-κβ
NKC	Non-keratinizing carcinoma, WHO Type II
NPC	Nasopharyngeal carcinoma
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PD	Progressive disease or recurrence
PD1	Programmed cell death protein 1

PDGF	Platelet-derived growth factor
PET	Positron emission tomography
PI3K/ AKT	Phosphoinositide-3-kinase0protein kinase B/ AKT
PMT	Photomultiplier tubes
PR	Partial response
PTLD	Post-transplant lymphoproliferative diseases
SCC	Squamous cell carcinoma, WHO Type I
SD	Standard deviation
SNP	Single nucleotide polymorphisms
SSC	Side-light scatter
STAT3	Signal transducer and activator of transcription 3
TAMs	Tumour-associated macrophages
Teff(s)	T effector cell(s)
TGF-β	Tumour growth factor-β
Th	T helper cells e.g.: Th1 and Th2
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor type I
TNFR2	Tumour necrosis factor receptor type II
Tr	T regulatory e.g.: Tr1
TRADD	Tumour necrosis factor receptor type 1-associated death domain protein
TRAF-	TNF receptor associated factor- e.g.: TRAF2, TRAF 6
Treg(s)	T regulatory cells
TSG	Tumour suppressor gene
UC	Undifferentiated carcinoma, WHO Type III
WGS	Whole-genome sequencing

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PENYIASATAN SEL T REGULATORI TNFR2 DAN SITOKIN BERKAITAN TERHADAP PATHOGENESIS KARSINOMA NASOFARINKS

ABSTRAK

Reseptor faktor nekrosis tumor jenis 2 (TNFR2) ialah penanda permukaan supresif secara maksimum subset sel T pengawalselia $CD4^+FoxP3^+$ (Tregs) pada manusia dan tikus. Kajian ini mengkaji ekspresi TNFR2 oleh Tregs daripada pesakit karsinoma nasofarinks (NPC) dan kawalan sihat. Proliferatif, migrasi, kemandirian Tregs $TNFR2^+$, dan hubungkait dengan ciri klinikopatologi dinilai dalam darah periferi (PB) (n = 29) dan mikropersekitaran tumor (TME) (n = 7) pesakit NPC berbanding dengan kawalan sihat (n = 29). Tahap ekspresi sitokin terpilih juga ditentukan. Sampel dinilai untuk Tregs ($CD4^+FoxP3^+$, $CD4^+FoxP3^+TNFR2^+$, dan $CD4^+CD25^+CD127^{low/-}FoxP3^+TNFR2^+$) menggunakan sitometri aliran berwarna pelbagai. Keputusan menunjukkan bahawa dalam PB (10.45 ± 5.71%) dan TME (54.38 ± 16.15%) pesakit NPC, Tregs mengekspresikan TNFR2 pada tahap yang lebih ketara berbanding sel T konvensional (Tconvs) (3.91 ± 2.62%, $p < 0.0001$), serupa dengan kawalan sihat. Ekspresi TNFR2 (1.06 ± 0.99%) berkolerasi lebih baik daripada $CD25^+$ (0.40 ± 0.46%) dan $CD127^{-/low}$ (1.00 ± 0.83%) dengan ekspresi FoxP3 dalam NPC PB (p = 0.0005). Walaupun tiada hubungkait yang signifikan antara ekspresi TNFR2 dengan kapasiti berfungsi (proliferatif, migrasi dan kemandirian) Tregs ($p > 0.05$), nisbah perkadaran $TNFR2^+$ Tregs PB dan TME pada pesakit NPC menunjukkan keupayaan proliferasi lebih tinggi, kapasiti migrasi yang lebih tinggi, dan kebolehan kemandirian yang lebih baik berbanding dengan kawalan sihat. Selain itu, Tregs $TNFR2^+$ daripada pesakit NPC mengekspresikan jumlah sitokin yang lebih tinggi secara signifikan, IL-6 (p = 0.0077), IL-10 (p = 0.0001), IFN-γ (p = 0.0105), dan TNF-

α (p < 0.0001) daripada kawalan yang sihat. Yang terpaling signifikan, ekspresi TNFR2 dalam populasi Tregs supresif secara maksimum berkaitan dengan kategori histologi WHO Jenis III, metastasis jauh, status penyakit progresif, dan prognosis yang buruk bagi pesakit NPC. Oleh itu, penyelidikan ini mengandaikan bahawa ekspresi TNFR2 oleh Tregs PB dan TME mungkin menjadi penanda-bio prognostik yang berguna dalam pesakit NPC.

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ABSTRACT

Tumour necrosis factor receptor type 2 (TNFR2) is a surface marker of highly suppressive subset of $CD4^+FoxP3^+$ regulatory T cells (Tregs) in humans and mice. This study examined the TNFR2 expression by Tregs of nasopharyngeal carcinoma (NPC) patients and healthy controls. The proliferation, migration, survival of $TNFR2^+$ Tregs, and association with clinicopathological characteristics were assessed in the peripheral blood (PB) (n = 29) and tumour microenvironment (TME) (n = 7) of NPC patients in comparison with healthy controls (n = 29). The expression levels of selected cytokines were also determined. Samples were evaluated for Tregs ($CD4^+FoxP3^+$, $CD4^+FoxP3^+TNFR2^+$, and $CD4^+CD25^+CD127^{low/-}FoxP3^+TNFR2^+$) using multicolor flow cytometry. The results demonstrated that in both PB (10.45 ± 5.71%) and TME (54.38 ± 16.15%) of NPC patients, Tregs expressed TNFR2 at noticeably greater levels than conventional T cells (Tconvs) (3.91 ± 2.62%, $p < 0.0001$), akin to healthy controls. Expression of TNFR2 (1.06 ± 0.99%) was correlated better than $CD25^+$ (0.40 ± 0.46%) and $CD127^{-/low}$ (1.00 ± 0.83%) with FoxP3 expression in NPC PB (p = 0.0005). Though there was no significant association between TNFR2 expression with the functional capacity (proliferation, migration and survival) of Tregs ($p > 0.05$), the proportions of PB and TME $TNFR2^+$ Tregs in NPC patients showed more proliferative, higher migration capacity, and better survival ability, as compared to those in healthy controls. Furthermore, $TNFR2^+$ Tregs from NPC patients expressed significantly higher amounts of IL-6 (p = 0.0077), IL-10 (p = 0.0001), IFN-γ (p =

0.0105), and TNF-α ($p < 0.0001$) than those from healthy controls. Most significantly, TNFR2 expression in maximally suppressive Tregs population were linked to WHO Type III histological type, distant metastasis, progressive disease status, and poor prognosis for NPC patients. Hence, this research implies that TNFR2 expression by PB and TME Tregs may be a useful predictive indicator in NPC patients.

CHAPTER 1

INTRODUCTION

1.1 Research background

Nasopharyngeal carcinoma (NPC), previously called as lymphoepithelioma, is an undifferentiated form of squamous cell carcinoma arising from the epithelium of the nasopharynx (Shah and Nagalli, 2022). It is the most common malignancy of the nasopharynx. Endemic to parts of Asia and Africa but found worldwide, the malignancy shows a variety rate of occurrence ranging from high incidence in the southern part of China (25 to 50 cases per 100, 000) to a low rate in European populations (1 case per 100,000) (Chang and Adami, 2006). A complex interplay of genetic susceptibility and Epstein-Barr virus (EBV) infection is responsible for these epidemiological patterns.

NPC is characterised by very high stromal and immune infiltration, which is likely due to proximity to lymphoid structures in the nasopharynx and the close association with EBV infection (Gong et al., 2021). A key feature of NPC is the immune contribution to pathogenesis, where the immunosuppressive microenvironment provides a favourable environment for tumour cells (Gong et al., 2021). Generally, $CD4^+$ conventional T cells (Tconvs) mediate adaptive immune responses, whereas regulatory T cells (Tregs) function in balancing the central and peripheral tolerance of immune homeostasis by controlling and suppressing the immune reactions to self- and nonself-antigens.

The opposing activities of Tconvs and Tregs depend on the stage of the immune response and their environment, with an orchestrating role for cytokine and costimulatory receptors. However, Tregs are contributed in cancer initiation and advancement in tumour immunity by hindering anti-tumour immunity (Ohue and Nishikawa, 2019). There are several Tregs immunosuppressive mechanisms, including inhibitory cytokines secretion, interleukin (IL)-2 utilisation by high-affinity IL-2 receptors with high CD25 expression, tryptophan and adenosine metabolic alteration, co-stimulatory signals inhibition by dendritic cells (CD80/ CD86) via cytotoxic T-lymphocyte antigen 4 (CTLA4), and direct killing of T effector cells (Teffs) (Ohue and Nishikawa, 2019). The intrusion of Tregs into the tumour microenvironment (TME) and secondary lymphoid organs appears in several murine and human cancers. Tregs are chemoattracted to the TME and surrounding lymphoid organs by chemokines, for instance CCR4-CCL17/22, CCR8-CCL1, CCR10-CCL28, CXCR3-CCL9/10/11, and CCR7-CCL19/21 (Paluskievicz et al., 2019). Tregs are then triggered and impede the anti-tumour immune responses.

Chemoradiation is currently the standard of care for NPC, but recurrence occurs in approximately 20% of patients at which point the 5-year overall survival drops from 70% to 41% (Howlett et al., 2021). Thus, significant focus has been placed on the use of novel strategies, such like immune checkpoint inhibitor (ICI) for immunotherapy and prognostic studies in NPC to improve the patient outcomes (Gong et al., 2021). It has been shown that subtypes of NPC delineated by immune features of the TME can predict survival and response to immunotherapy (Chen et al., 2021).

Tumour necrosis factor (TNF) is a powerful pro-inflammatory cytokine that includes various pathological processes. TNF binds to two distinct receptors, TNF receptor type I or p55 (TNFR1), that functions as a principal signalling receptor for majority of cells and responsible for majority of pro-inflammatory, cytotoxic and apoptotic outcomes. Meanwhile TNF receptor type II or p75 (TNFR2) receptor predominantly mediates lymphocyte activation, proliferation, and immunosuppressive effects. The presence of TNFR2 Tregs as maximally suppressive Tregs in NPC patients are presumed to be stronger in the acceleration of excessive functional activity (proliferation, migration, and survival) of the cancer cells compared to Tconvs as defined in previous studies (Chen et al., 2008; Chen et al., 2010). To date, no findings have been found that investigate the effects of $TNFR2^{+}$ Tregs expression and its association with clinicopathological characteristics in NPC.

Poor survival in cancers are correlated with a significant Tregs infiltration in TME. Thus, approaches for Tregs depletion and management of Tregs activities to boost the anti-tumour immune reactions are urgently needed in cancer immunotherapy. Numerous molecules highly expressed by Tregs comprising ICIs (e.g.: CTLA4, PD1, TIM3, LAG3, TIGIT), cytokines, chemokine receptors (e.g.: TNFR2, CXCR5, CCR4, CCR7, BCL-2, CD39, CD73), and metabolites, that are guided by antibodies or small molecules; though, different approaches required to adjust and enhance for boosting anti-tumour effects constrained in the TME while preventing autoimmunity (Santegoets et al., 2015; Ohue and Nishikawa, 2019). Therefore, by investigating the effects of $TNFR2^{+}$ Tregs expression and its association with clinicopathological characteristics in NPC, the findings of this study may lead to a potential prognosis biomarker in NPC management.

1.2 Rationale of study

The myriad of NPC features have can make the early diagnosis challenging. NPC may presents with a diversity of clinical symptoms, occasionally being non-specific. Clinically, NPC is asymptomatic at early stage and majority of NPC patients are initially identified at an advanced stage (stage III or IV) and regularly with nodal metastasis, leading to poor survival and prognosis.

The clinical importance of Tregs accumulation in human tumours has been extensively discussed in the literature. The capacity to distinguish the intra-tumoural cells from "non-inflamed" tumours with insufficient immune cells infiltration is currently, almost regularly obtained in pathology. NPC is categorized as an inflamed tumour based on its spatial localization of stromal cells with respect to tumour compartments (Binnewies et al., 2018). Stromal cells are in close proximity to and in contact with NPC cells, instead of being embedded in the surrounding regions away from the tumour core. Hence, cytokine secretion and ligand-receptor interactions are both involved in the bilateral tumour-stroma interplay. It is also noteworthy that the nasopharynx is one of the first defence organs against viral and bacterial entry and infection, which makes its underlying microenvironment highly heterogenous and immunogenic prior to malignant transformation (Gong et al., 2021).

The finding implies that Tregs are significantly existent amongst tumour-infiltrating lymphocytes (TILs) in tumours comprising a prominent immune cells infiltration but rarely seen in the poorly infiltrated of TME (Badoual et al., 2006). Thus, by defining the ratio of Tregs to Teffs of many cancers, it is possible to utilise the Treg/ Teff ratio to approximate the patient prognosis (Tanchot et al., 2013). The strategy is according

to the hypothesis that a redundant of Tregs facilitate an immune suppression in the TME and consequently stimulates tumour development, which later on related with adverse prognosis and poor overall survival (Tanchot et al., 2013). Still, the Treg/ Teff ratio does not convey Tregs function as pro-cancer or anti-cancer, however, Tregs with whichever characteristic can impact the prognosis.

When compiled data from several findings were observed, the redundant of Tregs (i.e., the high Treg/ Teff ratio) was associated with poor outcomes and worse survival in some cancers (e.g., NPC, pancreatic cancer, ovarian cancer, lung cancer, glioblastoma, and melanoma), whereas in other cancers (e.g., breast cancer, colorectal cancer, and bladder cancer), the high Treg/ Teff ratios are associated with promising prognosis and better overall survival (OS) (Curiel et al., 2004; Sato et al., 2005; Bates et al., 2006; Sinicrope et al., 2009; Frey et al., 2010; Shang et al., 2015; Baras et al., 2016; Zhou et al., 2021). The association of Tregs redundant with the enhanced OS is largely observed in chronically inflamed cancers, for example, colorectal cancer. It has been defined by Tregs ability to suppress and regulate the "tumour-promoting inflammation." Hence, the rationale for the prognostic use of the ratio is weak.

The difference in prognosticative importance of the Treg/ Teff ratio in many tumours could reveal Tregs functional heterogeneousness, which has not yet been recognised phenotypically. There are also questions concerning how thoroughly Tregs were identified in these investigations conducted. This issue is relevant as it is assumed that there are no specific marker for Tregs. The phenotype of $CD4^+CD25^+FoxP3^+$ Tregs which is commonly utilised to define Tregs, is likely to include $CD4^+$ T effector cells that are momentarily $FoxP3^+$ and exclude induced Tregs that may be $FoxP3^-$ (deLeeuw

et al., 2012; Devaud et al., 2014). Undoubtedly, without a marker that can specifically classify Tregs, especially the subset causing the immunosuppression in TME, the results of the correlative prognostic studies described in the current literature are always open to criticism.

TNFR2 affects cancer progression and metastasis in various ways. TNFR2 encourages tumour immune evasion by its capability to induce several suppressive cell types, for instance, Tregs and myeloid-derived suppressor cells (MDSCs) (Medler et al., 2022). The three primary mechanisms of tumour evasion are loss of antigenicity, immunogenicity, and an immunosuppressive microenvironment (Takeuchi and Nishikawa, 2016). High TNFR2-expressing Tregs exhibit the strongest suppressive action (Chen et al., 2008). The existence of $TNFR2^+$ Tregs as maximally suppressive Tregs in NPC patients are hypothesized to be more potent in accelerating over-proliferation, survival, and migration of NPC cells compared to $TNFR2^+$ Tconvs. Given that TNFR2 expression was positively correlated with larger tumour size, more advanced clinical stages, and higher pathological grades in breast cancer, it is possible that the TNFR2 expression influences the course and prognosis of the disease (Yang et al., 2017). In certain cancer types, elevated serum levels of TNFR2 act as a prognostic indicator with a poor clinical outcome (Heemann et al., 2012; Tarhini et al., 2014; Babic et al., 2016). It is interesting to note that Tregs in TME express more TNFR2 than they do in healthy and normal conditions.

Apart from that, TNFR2 is also expressed on Tconvs, where it commonly functions as a co-stimulatory molecule (Aspalter et al., 2003). Teffs expressed higher TNFR2 after T cell receptor stimulation, which is necessary for Teff proliferation and activation as

well as the induction of activation-induced cell death (AICD) (Chen et al., 2010). AICD can inhibits the Teff proliferative response, which depends on the downstream mediator TRAF2 of TNFR2 receptor (Li et al., 2022). Additionally, Teff's ability to fight tumours can be inhibited by the increased production of soluble TNFR2 (van Mierlo et al., 2008). Thus, back to the concern on the Treg/ Teff ratio issued above, TNFR2 could be utilised to specifically classify Tregs, especially maximally suppressive Tregs in TME, as a prognostic indicator for NPC patients.

Taken together, this study not only aimed to investigate the effects of $TNFR2^+$ Tregs expression and its association with clinicopathological characteristics in NPC. Additionally, the findings of this study may lead to the investigation of TNFR2 as a potential prognostic biomarker for NPC.

1.3 Hypothesis

It was hypothesized that the presence of $TNFR2^+$ Tregs, as maximally suppressive Tregs in NPC, would be more effective than $TNFR2^+$ Tconvs in accelerating proliferation, migration, and survival. In the therapeutic therapy of NPC, the TNFR2 receptor may be a good candidate as prognostic biomarker.

1.4 Research questions

The following research questions in this study were of particular interest to be answered:

1. Does the presence of TNFR2 marker influences in proliferation, migration, and survival ability of Tregs in NPC?

2. Do clinicopathological features of NPC patients, such as lymphatic invasion, histological type, distant metastases, patient status and prognosis, correlate with $TNFR2^+$ Tregs?

3. Does the TNFR2 receptor is more effective than CD25 and CD127 in correlating with the expression of FoxP3 in Tregs?

4. How do the $TNFR2^+$ Tregs expression relate to the onset and spread of NPC?

1.5 Research objectives

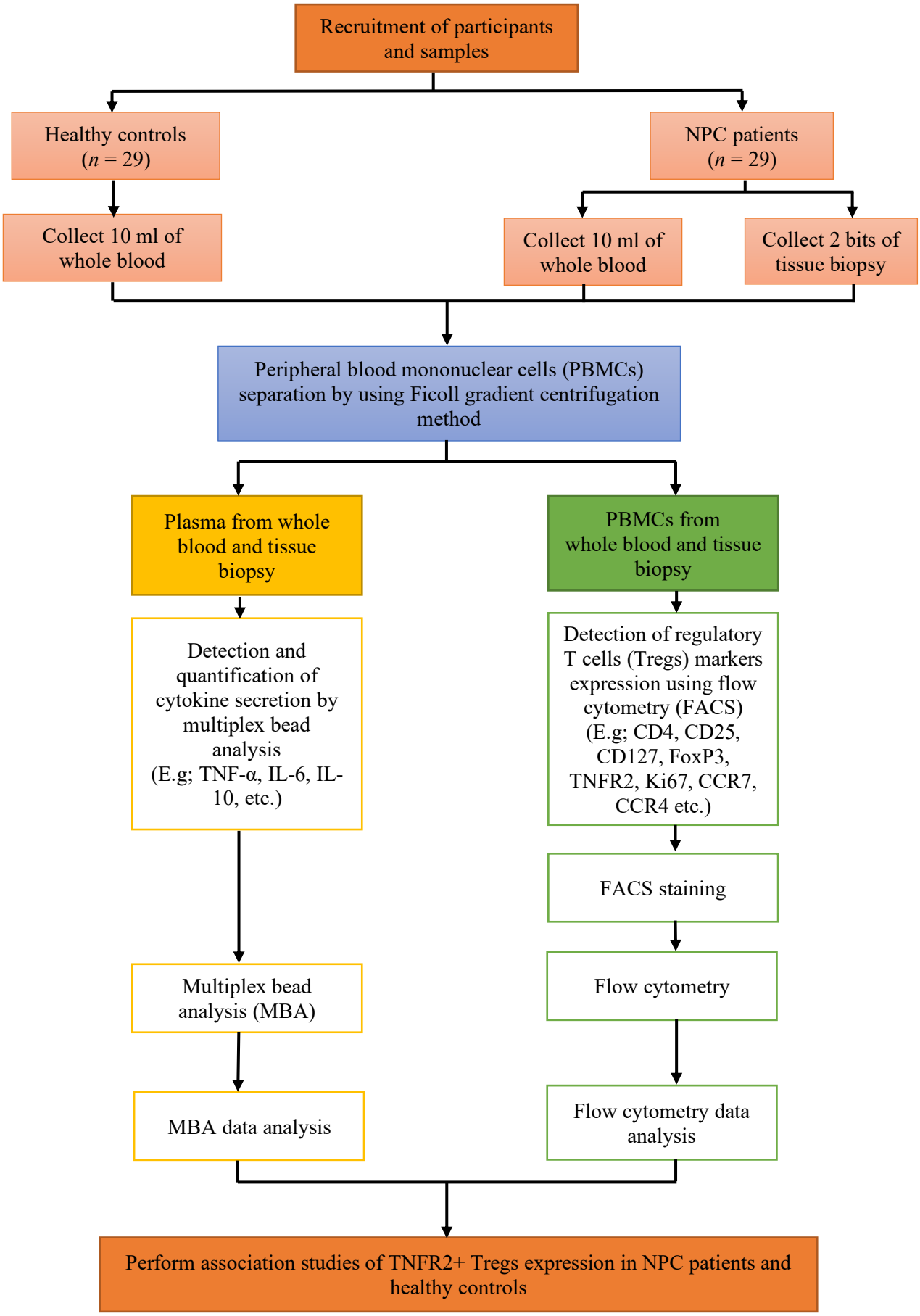
1.5.1 General objective

To compare NPC patients with healthy controls in terms of the TNFR2 Tregs functional activities (proliferation, migration and survival), cytokine production, and clinicopathological characteristics of NPC.

1.5.2 Specific objectives

1. To optimize the flow cytometry assay for investigating TNFR2 marker on the Tregs and Tconvs population.
2. To determine the proportion of total FoxP3$^+$ Tregs, TNFR2$^+$ Tregs and maximally suppressive Tregs as well as Tconvs in NPC patients and healthy controls.
3. To evaluate the expression of Ki67, CCR7, CCR4, and BCL-2 markers on TNFR2$^+$ Tregs and Tconvs in NPC patients and healthy controls.
4. To evaluate the cytokines secretion in plasma samples taken from NPC patients and healthy controls.
5. To conduct an association study on the TNFR2 expression on Tregs and Tconvs, associated cytokines and the clinicopathological characteristics of NPC.
    - To ascertain how the TNFR2, CD25, and CD127 receptors in the CD4$^+$ population, which are a superior marker in associating with FoxP3 expression.
    - To ascertain the relationship between the expression of TNFR2$^+$ Tregs, TNFR2$^+$ Tconvs and NPC clinicopathological traits.
    - To ascertain the TNFR2 expression with the expression of cytokines levels in NPC patients and healthy controls.

1.6 Flow-chart of the study



CHAPTER 2

LITERATURE REVIEW

2.1 Nasopharyngeal carcinoma (NPC)

2.1.1 Epidemiology and risk factors of NPC

According to estimates, there were 18.1 million cancer cases worldwide in 2018, of which 129 079 were NPC cases, giving rise to an age-standardized rate (ASR) of 1.5 per 100 000 people (Bray et al., 2020). 72 987 NPC fatalities were reported in the same year, accounting for 0.8% of all cancer fatalities. When compared to other types of cancer, NPC is a rare occurrence, and its global distribution is unbalanced (Figure 2.1). NPC is a rare cancer in the United States (ASR 0.45) and most of Europe (ASR 0.44), but it is much more common in Asia, especially in Southern (ASR 5.0) and Eastern Asia countries (ASR 2.7), where 27% and 50% of NPC cases have been reported, respectively (Ferlay et al., 2019).

As one of the top five cancers identified in Malaysia and the fifth-most common malignancy among Malaysian men, NPC poses a significant health burden on the nation (Yassin, 2017). According to the Malaysian National Cancer Registry Report 2012 – 2016, between 2007 and 2011, an average of 900 – 1000 NPC cases were reported yearly (Azizah et al., 2019). Males had more than two-fold higher incidence rate than females. The ASR for men was 5.2 per 100 000, whereas the ASR for women was 2.0 per 100 000 (Figure 2.2). Chinese men had a higher incidence, with an ASR of 11.0 per 100 000 population, compared to Malay men (3.3 per 100 000) and Indian men (1.1 per 100 000) (Azizah et al., 2019).

In the National Strategic Plan for Cancer Control Programme (NSPCCP) 2021 – 2025, NPC is one of the cancer types that the Ministry of Health Malaysia focuses on to address cancer care and management from a holistic viewpoint that spans primary intervention, screening, early detection, diagnosis, treatment, rehabilitation, and palliative care, as well as traditional and complementary medicine and research. In particular, there is an aim to develop a NPC research programme to identify biomarkers and therapeutic targets, as well as to understand NPC biology.

The prevalence is notably high among several native populations in Sarawak, which is particularly relevant (Azizah et al., 2019). The Bidayuh native group had the highest rate of NPC (31.5 per 100 000 men) compared to other Malaysian ethnicities or any ethnicity in around the globe, according to an ethic-based study on NPC conducted between 1996 – 1998 (Devi et al., 2004). In Pahang, the ASR for men is 2.4 per 100 000 people, while the ASR for women is 0.9 per 100 000 people (Ahmad et al., 2021).

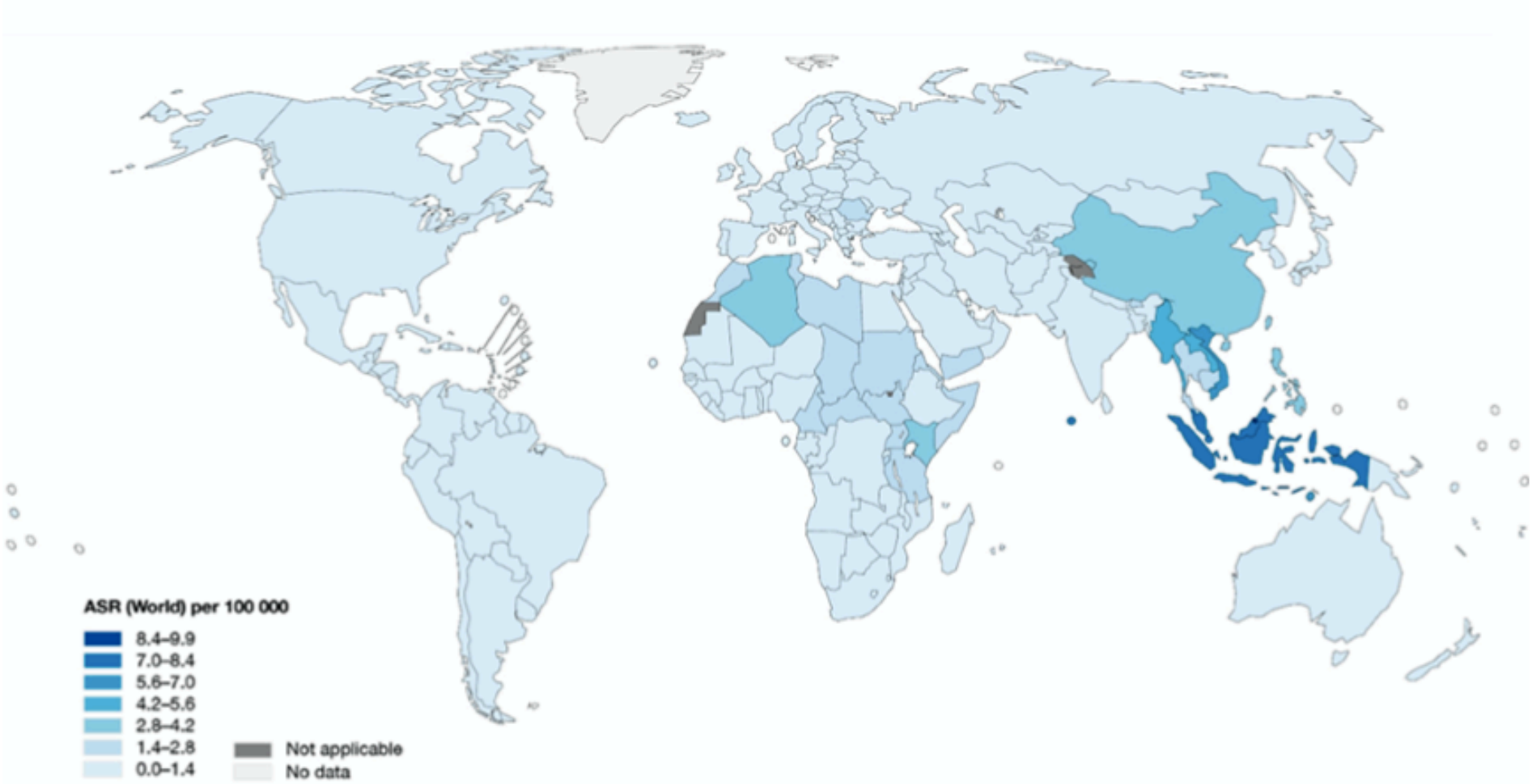


Figure 2.1 Global distribution of NPC. The map is obtained from International Agency for Research on Cancer (IARC) web-based cancer database, Global Cancer Observatory (GCO), and modified based on Globocan, 2018 (Adopted from Bray et al., 2020). NPC incidence rate is represented as 100 000 person/ year for all sex at all ages (0 - 85 years old).

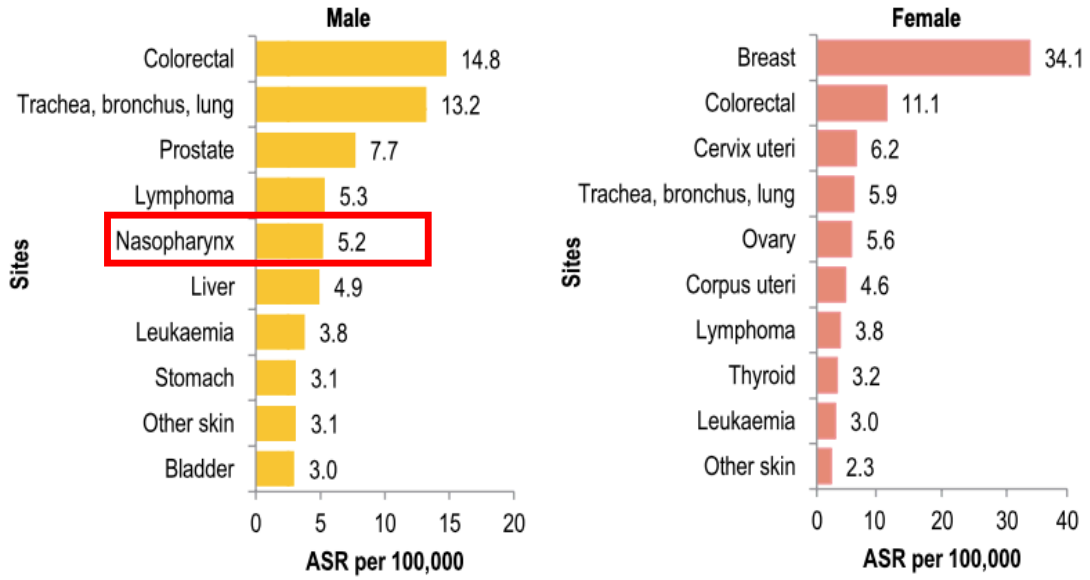


Figure 2.2 Age-standardised incidence rate for ten common cancers by gender, Malaysia, 2012 – 2016 (Adopted from Azizah et al., 2019).

The uneven distribution of NPC worldwide has drawn attention to ethnicity-related risk as a significant risk factor. Chinese descendants, for instance, typically have a higher incidence than other ethnic groups in the specific area, making them more at risk (Armstrong et al., 1979; Chang and Adami, 2006; Mak et al., 2015). In Chinese population, extensive case-control studies have shown a higher risk of developing NPC, with a greater familial risk among relatives and a younger age of onset in families with more affected cases (Xie et al., 2015).

Inflammatory-inducing diets have also been linked to an increased incidence of NPC (Shivappa et al., 2016). Non-preserved vegetable and fruit-eating, on the other hand, is inversely related to NPC and might be regarded as protective in effect (Jia et al., 2010; Polesel et al., 2013). Traditional nitrosamine-rich meals, such as salted fish and preserved vegetables, may contribute to NPC development (Jia et al., 2010; Okekpa et al., 2019). Lifestyle choices such as smoking (Okekpa et al., 2019), on the other hand, have been linked to NPC as well as a greater mortality rate in patients (Lin et al., 2015). Regardless of origin, familial risks of NPC appear to persist, with similar findings in non-Chinese populations in the United States, Europe, and Australia, underscoring the importance of genetic determinants in the development of NPC (Zeng and Jia, 2002).

The major histocompatibility complex (MHC) region on chromosome 6p21, which contains the human leukocyte antigen (HLA), is known as the important risk locus for NPC (Tse et al., 2009). SNPs found in DNA isolated from EBV-positive NPC tissues by whole-genome sequencing (WGS) may reflect mutations that occur during tumourigenesis rather than genetic alterations in the patients. Point mutations, MHC Class I rearrangements, and mutations in the MHC expression inducer NLRC5

accumulated during the development of tumour cells (Li et al., 2017). According to a recent cohort research, the incidence of NPC has been continuously reducing, despite the high prevalence of the condition in Asian nations (Yu and Hussain, 2009). NPC prevalence has been steadily decreasing over the previous few decades, possibly because of changes in lifestyle and advancements in healthcare services (Tang et al., 2016; Carioli et al., 2017).

2.1.2 Clinical features of NPC

NPC hardly arises to medical attention before it has spread to nearby lymph nodes. Changes in hearing (often linked to obstruction of the eustachian tube, but further extending into the ear is possible), cranial nerve palsies, and nasal blockade (e.g., congestion, nasal discharge, bleeding) are all possible symptoms of NPC enlargement and extension (commonly linked with the extension of the tumour into the skull base). Most NPC patients who undergo diagnostic testing in clinics exhibit locoregionally advanced (LA) illnesses (stage III and IV) and typically have cervical lymph node metastases (Petersson, 2015). Still, distant metastases to the lungs, brain, bone, mediastinum, and liver could also occur (Brennan, 2006). Males are more likely than females to develop NPC in areas with high and low incidence regions (about a 3:1 ratio).

Patients with NPC may present with symptoms from one or more of the following four categories; first, nasal symptoms caused by a bulk in the nasopharynx (epistaxis, nasal blockage, and discharge); second, otologic symptoms linked to eustachian tube dysfunction caused by the lateroposterior extension of NPC to the para nasopharyngeal space (reduced hearing, tinnitus); third, cranial nerve palsies, commonly in the 5th and

6th cranial nerves, related to the superior extension of NPC to the base of the skull (erosion, headache, diplopia, facial pain, numbness/ paresthesia), and fourth, neck mass that are commonly first show in the upper neck region (Wei and Sham, 2005; Wei and Kwong, 2010; Petersson, 2015). Regrettably, most NPC patients are diagnosed with advanced stage due to the impediment during nasopharynx clinical examination and the non-specific symptoms (weight loss and anorexia) associated with NPC (Wei and Sham, 2005; Wei and Kwong, 2010).

A study reported numerous initial symptoms during NPC clinical presentation are like neck mass (76%), enlarged neck node (75%), nasal malfunction (73%), aural (62%), headache (35%), cranial nerve palsies (20%), ophthalmic (diplopia, squint) (11%), facial numbness (8%), weight loss (7%), trismus (3%) and slurring of speech (2%), respectively (Lee et al., 1997). Although 81.9% of NPC patients reported having several symptoms, 17.8% only had one, and the rest were asymptomatic (Lee et al., 1997).

2.1.3 Clinical staging of NPC

The current TNM classification, created by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (IUCC), is the most accurate prognostic tool to stratify NPC patients for treatment and evaluate their treatment success. The three anatomical criteria listed are the local extent of the tumour at the site of origin (T), the degree of regional lymph node (N) metastasis involvement, and the presence or absence of illness from distant metastasis (M) (Guo et al., 2019). The TNM classification was updated in 2016 (8th edition), further refining primary and nodal disease staging and the stage groups (Table 2.1).

Clinical history, physical examinations, endoscopic examinations, tumour imaging studies, regional lymph nodes, distant metastases, primary tumour biopsies, and surgical exploration or other relevant pertinent examinations are used to define the NPC clinical staging (Wei and Kwong, 2010). Currently, the NPC clinical staging is determined based on advanced imaging studies. MRI enables a more accurate evaluation of local disease extension, particularly for ethmoid sinus, oropharyngeal extension, and parapharyngeal tumour extension (Poon et al., 2000). According to the 6th UICC/ AJCC staging system, these led to alterations in 49.8% of T-stage cases, 10.7% of N-stage cases, and 38.6% of clinical stage cases patients (Liao et al., 2008).

Additionally, chest radiography, abdominal ultrasound, and bone scintigraphy are less effective than ^{18}F-FDG-positron emission tomography (PET)/ CT scans at detecting distant metastasis, small cervical lymph node metastases, and local residual/ recurrent disease (Chen et al., 2016; Wei et al., 2016). According to research by Peng et al., (2017), 135 (28.7%) and 46 (9.8%) patients had their N categories and entire stages modified as result of their PET/ CT scans (Peng et al., 2017). According to a different study by Law et al. (2011), PET/ CT was useful in terms of influencing how 33% of patients with NPC were managed (significant effect in finding M1 illness in 8% of patients and medium effect by upstaging the N category or displaying the same lymph node in 25% of patients) (Law et al., 2011).

Table 2.1 The 8th edition of the UICC/ AJCC staging system for NPC (Adapted from Guo et al., 2019).

UICC/ AJCC Staging system for NPC		
Primary Tumour (T)	**TX**	Primary tumour cannot be assessed
	T0	No tumour identified, but EBV-positive cervical node(s) involvement
	T1	Nasopharynx, oropharynx or nasal cavity without parapharyngeal extension
	T2	Parapharyngeal extension, adjacent soft tissue involvement (medial pterygoid, lateral pterygoid, prevertebral muscles)
	T3	Bony structures (skull base, cervical vertebra) and/ or paranasal sinuses
	T4	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)	**NX**	Regional lymph nodes cannot be assessed
	N0	No regional lymph node metastasis
	N1	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 or less in greatest dimension, above the caudal border of cricoid cartilage
	N3	> 6 cm and/ or below caudal border of cricoid cartilage (regardless of laterality)
Distant metastasis (M)	**M0**	No distant metastasis
	M1	Distant metastasis
Stage group	**I**	T1 N0 M0
	II	T2 N0-1 M0, T0-1 N1 M0
	III	T3 N0-2 M0, T0-2 N2 M0
	IVA	T4 or N3 M0
	IVB	Any T, any N M1

2.1.4 Histopathological classification of NPC

NPC, nasopharyngeal papillary adenocarcinoma, salivary gland anlage tumour, hairy polyp, juvenile angiofibroma, and other tumours make up the current WHO categorization system for head and neck malignancies (Stelow and Wenig, 2017). Following that, these tumours are classified by the WHO as basaloid squamous cell carcinoma (SCC), non-keratinizing, and keratinizing types. Undifferentiated and differentiated SCC are other subcategories of non-keratinizing tumours (Table 2.2). Despite the designation's relative lack of specificity, "NPC" continues to be preferred diagnostic name for all SCCs of the nasopharynx (Stelow and Wenig, 2017).

In regions where the tumour is not considered endemic, EBV infection contributes substantially less to the emergence of keratinizing SCC (Niedobitek et al., 1991). Keratinizing SCC is likely locally advanced at presentation and less likely to disseminate to nearby lymph nodes than non-keratinizing SCC (Reddy et al., 1995). Keratinizing SCC appears to be connected to smoking, just like non-keratinizing SCC at other sites in the upper aerodigestive tract (Ji et al., 2011). In different geographical areas, keratinizing and non-keratinizing NPC progress to very different degrees, and they manifest in patients with varied ages and risk factors. Moreover, it has been difficult to show EBV infection or even keratinization as a standalone prognostic factor at specific clinical phases (Reddy et al., 1995). However, there has yet to be an agreement on the prognostic importance of NPC histological subtype or EBV-NPC status because more consistent studies are needed. The stage at presentation is currently the most reliable prognosis indicator for NPC (Sun et al., 2014).

Table 2.2 WHO classification for NPC in comparison with AJCC classification (Adapted from Manasan et al., 2019).

WHO Types	Cellular characteristics	AJCC classification
Type I	Keratinizing SCC	Keratinizing SCC
Type II	Non-keratinizing (transitional) carcinoma - *Without lymphoid stroma (intermediated cell)* - *With lymphoid stroma (lympho-epithelial)*	Non-keratinizing SCC, differentiated
Type III	Undifferentiated (anaplastic) carcinoma - *Without lymphoid stroma (clear cell)* - *With lymphoid stroma (lympho-epithelial)*	Non-keratinizing SCC, undifferentiated

The WHO did not advise categorizing the disease by keratinization of EBV status, maybe primarily because the term "NPC" has become entrenched (e.g., EBV-associated NPC). It conflicts with the oropharynx's high-risk human papillomavirus (HR-HPV) status. It is now well acknowledged that the HR-HPV status is a key prognostic factor at this site, which has sparked discussion about referring to such oropharyngeal tumours as HPV-associated carcinoma.

The last thing to note is that some SSCs in the nasopharynx are caused by HR-HPV infection. Given that HR-HPV infection causes a quarter of sinonasal carcinomas and the majority of oropharyngeal SCCs, it should not be shocking (Sharma et al., 2021). These SCCs can mimic tumours connected to EBV infection in the nasopharynx and are often non-keratinizing. Diagnosis of HPV or EBV is required for differentiation between these two viruses. For the purpose of differentiating between these two viruses, a diagnosis of HPV or EBV is necessary. Some have proposed that the prognosis of HR-HPV-associated malignancies may fall between that of EBV-related SCCs and those tumours not related to oncogenic viruses, despite the fact that the prognostic significance of EBV infection at this site is still debatable.

2.1.5. Screening and diagnosis of NPC

Due to the deep position of the suspected cancer and the lack of identifiable early signs, more than 80% of advanced NPC cases are detected too late. In NPC, having a late diagnosis reduces the chances of survival (Ren et al., 2017). The two-year survival rate for NPC patients with IUCC/ AJCC stage III or IV is barely 20 – 30% despite aggressive concurrent chemoradiation therapy, and lesions frequently develop to distant metastasis despite local control (Cheng et al., 1997; Cheng et al., 2000). Nevertheless, the 10-year survival rate for NPC patients with UICC/ AJCC stage I or II could approach 90% or even higher (Chua et al., 2003). Unfortunately, stage III or IV NPC affects most newly diagnosed patients. There are a number of diagnostic techniques for NPC, however the two that are most frequently used for screening are EBV serology and nasopharyngoscopy.

Human body can produce many EBV-related antigens early on that can be used for NPC screening, including EBV-DNA, early antigen IgA (EA-IgA), viral capsid antigen IgA (VCA-IgA), and EBNA1-IgA. EBV-DNA, which is also referred to as cell-free DNA, may be released from NPC cancer cells into the bloodstream after apoptosis, necrosis, or the EBV viral replication stage. It has been demonstrated that the levels of circulating EBV-DNA correlated with the phases, recurrence rate, and NPC screening (Tan et al., 2020). A meta-analysis study reported that VCA-IgA, EBV-DNA, EBNA1-IgA and Rta-IgG can be utilized for early diagnosis of NPC as they have high accuracy (Liu et al., 2021). Moreover, detection of NPC via EBV-DNA has the highest accuracy whereas early antigen IgA (EA-IgA) is suitable for the diagnosis but not NPC screening.

Prior investigations have shown that cell-free EBV-DNA detection has reasonable specificity and sensitivity in NPC diagnosis, which is confirmed by a large number of studies (Lin et al., 2004; Fung et al., 2016). A study found that locating cell-free EBV DNA facilitated the precise diagnosis of NPC patients without symptoms (Chan et al., 2013). Additionally, in the phase II enlarged trial, approximately 20 000 people were screened utilizing cell-free EBV DNA detection. The results showed excellent specificity and sensitivity (98.6% and 97.1%), respectively (Chan et al., 2017). Physical examination, radiology, CT and MRI imaging, as well as assessments of hearing and cranial nerve function, are further diagnostic techniques. However, these methods either lack the sensitivity or specificity needed to identify precursor lesions and early NPC cases, or they are too expensive or inconvenient to be used as screening assays. Furthermore, when the tumour is relatively advanced, a physical examination is reliable.

For the study of malignant neoplasms, proteomic techniques have been used. Biomarkers should be quantifiable in fluid body samples for practical use in tumour screening (Chang et al., 2010). Wei et al. (2008) used proteome analysis to examine serum samples from patients with NPC (Wei et al., 2008). They found that NPC patients could be distinguished by four protein peaks at 4,097, 4,180, 5,912, and 8,295 Daltons (Da) with a sensitivity of 94.5% and a specificity of 92.9%. Also, by using a three-marker panel (cystatin A, MnSOD, and MMP2) may aid in the detection of NPC (Chang et al., 2010). Potential diagnostic indicators for NPC include galectin-1, fibronectin, Mac-2 binding protein, and plasminogen activator inhibitor 1 (Wu et al., 2005; Tang et al., 2010). It is possible that incorporating these tests into routine NPC screening will help to improve early detection.