EFFECT OF EPSTEIN-BARR VIRUS (EBV) ENCODED LATENT MEMBRANE PROTEIN-1 (30 BP DELETION MUTANT) ON IMMUNE CHECKPOINT REGULATION IN NASOPHARYNGEAL AND EBV-ASSOCIATED NON-NASOPHARYNGEAL CANCERS

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by

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Thank you!

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LIST OF SYMBOLS

+	Plus
-	Minus
×	Multiplication
÷	Division
±	Plus-minus
~	Approximately
%	Percentage
<	Less than
>	More than
≤	Less than or equal
≥	More than or equal
℃	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
™	Trade mark sign
®	Registered sign
μ	Micro sign
λ	Wavelength

LIST OF ABBREVIATIONS

3D	3 Dimensional
µL	Microliter
µm	Micromole
A260/A230	Absorbance at 260 nm per absorbance at 230 nm
A260/A280	Absorbance at 260 nm per absorbance at 280 nm
Ab	Antibody
Ag	Antigen
Atto-g	Atto-gram
AIDS	Acquired immunodeficiency syndrome
AJCC	American Joint Committee on Cancer
BART	BamHI-A rightward transcripts
BL	Burkitt's lymphoma
bp	Base pair
BZLF1	BamHI Z fragment leftward open reading frame 1
CCRT	Concurrent chemoradiation therapy
Cq	Cycle threshold
CT	Computed Tomography
DFS	Disease-free survival
DMFS	Distant metastasis-free survival
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
EA	Early antigen
EBER	EBV-encoded small RNAs
EBNA	EBV nuclear antigen
EBV	Epstein–Barr virus
FDG-PET	Fluorodeoxyglucose-positron emission tomography
FFS	Failure-free survival
fg	Femtogram
FNA	Fine-needle aspiration
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen

HPV	Human papillomavirus
IAC	Internal amplification control
IM	Infectious mononucleosis
IMRT	Intensity-modulated radiation therapy
i-qPCR	Innovated real-time polymerase chain reaction
JNK	c-Jun N-terminal kinases
KSCC	keratinizing squamous cell carcinoma
L	Liter
LAG-3	Lymphocyte activation gene-3
LA-NPC	Locoregionally advanced-Nasopharyngeal carcinoma
LMP	Latent membrane protein
LRFFS	Locoregional failure–free survival
M	Molar
MAPK	Mitogen-activated protein kinases
mg/mL	Milligram per milliliter
MHC	Major histocompatibility complex
min	minute
mm-NPC	Metachronous metastasis-Nasopharyngeal carcinoma
MRI	Magnetic resonance imaging
MT	Mutant type
NAC	Nasopharyngeal adenocarcinomas
NF-κB	Nuclear factor κB
ng	Nanogram
NHL	non-Hodgkin’s lymphoma
NK	Natural killer
NKC	non-keratinizing carcinoma
NKDC	non-keratinizing differentiated carcinoma
NKUC	non-keratinizing undifferentiated carcinoma
nm	Nanometer
NPAC	Nasopharyngeal papillary adenocarcinoma
NPC	Nasopharyngeal carcinoma
OHL	Oral hairy leukoplakia
ORR	Objective response rate
OS	Overall survival

PFS	Progression-free survival
pg	Picogram
PTLD	Posttransplant lymphoproliferative disorder
qPCR	Real-time polymerase chain reaction
RARβ2	Retinoic acid receptor beta2
RASSF1A	Ras association domain family protein1 isoform A
RFS	Recurrence–free survival
RT	Radiotherapy
SCC	Squamous cell carcinoma
Sec	Second
SmNPC	Synchronous metastasis NPC
Taq	Thermus aquaticus
TIM-3	T cell immunoglobulin and mucin domain-containing protein-3
TIGIT	T cell immunoglobulin and ITIM domain
TME	Tumor microenvironment
UC	Undifferentiated carcinoma
UCNT	Undifferentiated carcinoma nasopharyngeal type
USM	Universiti Sains Malaysia
UV	Ultraviolet
v/v	Volume per volume
VCA	Viral capsid antigen
w/v	Weight per volume
WB	Whole blood
WHO	World Health Organization
WT	Wild type

LIST OF APPENDICES

KESAN VIRUS EPSTEIN-BARR (EBV) YANG MENGECODKAN PROTIN MEMBRAN TERPADAM-1 (30 BP DELETAN MUTAN) TERHADAP KAWALAN *CHECKPOINT* IMUN DALAM KANSER NASOFARINKS DAN EBV-BERKAITAN BUKAN-NASOFARINKS

ABSTRAK

Virus Epstein-Barr (EBV) dikaitkan dengan beberapa jenis kanser, termasuk nasofarinks, payudara, gastrik, dan paru-paru. Walaupun kajian sebelumnya menunjukkan perhubungan diantara perkembangan karsinoma nasofarinks (NPC) dan penyaringan mutasi pemadaman 30 bp LMP1 EBV dalam pesakit NPC, beberapa persoalan masih belum terjawab. Dalam penyelidikan ini, perbandingan tahap ekspresi reseptor cekpoint imun dalam 29 pesakit dengan NPC yang dikaitkan dengan EBV, 29 pesakit dengan kanser bukan NPC, dan 29 kawalan sihat (HC) telah ditentukan dan dinilai untuk perhubungan mereka dengan mutasi pemadaman 30 bp LMP1 EBV. Tahap ekspresi reseptor cekpoint imun (CD3, CD4, CD25, CD127, FoxP3, LAG-3, TIM-3, dan TIGIT) diakses pada Tregs dengan menggunakan asai sitometri aliran. Sementara itu, pengesanan kepekatan sitokin dan kemokin (IL-10, IL-2, IFN-γ, TNF-α, IL-8, dan IL-6) telah dilakukan dengan menggunakan ujian plex mudah. Selain itu, pengesanan mutasi pemadaman 30 bp LMP1 EBV (varian jenis mutan) dan LMP1 (varian jenis liar) daripada sampel darah penuh telah dilakukan dengan menggunakan PCR masa nyata berasaskan proba (qPCR). Analisis statistik akhir untuk kajian perhubungan telah dilakukan dengan menggunakan instrumen statistik yang berbeza. Kebanyakan pesakit NPC merupakan etnik Melayu (79.31%), diikuti dengan Cina (20.68%). WHO jenis III (62.06%) adalah diagnosis yang paling umum untuk pesakit NPC, diikuti dengan WHO jenis II (27.58%) dan WHO jenis I

(10.34%). Ekspresi LAG-3, TIM-3, dan TIGIT pada Tregs adalah lebih tinggi dalam pesakit NPC dan bukan NPC berbanding dengan HC (p<0.05). Berbanding dengan bukan NPC, ekspresi LAG-3 dan TIGIT adalah lebih tinggi secara signifikan dalam pesakit NPC (p<0.05). Walaupun tahap ekspresi TIM-3 adalah sedikit lebih tinggi dalam NPC berbanding dengan pesakit bukan NPC, tetapi ia tidak signifikan secara statistik (p=0.053). Terdapat perbezaan statistik yang diperhatikan apabila membandingkan tahap IL-2, IFN-γ, TNF-α, IL-6 dan IL-8 (p<0.05) dalam pesakit NPC dengan HC. Walau bagaimanapun, tiada perbezaan statistik untuk IL-10 (p=0.054). Dalam kes perbandingan bukan NPC dengan HC, signifikan diperhatikan untuk IL-10 dan IL-2 (p<0.05), manakala tiada perbezaan statistik yang dicatatkan untuk IFN-γ, TNF-α, IL-8, dan IL-6 (p>0.05). Sitokin yang paling dominan dalam pesakit NPC adalah TNF-α (10.70 ± 4.00 pg/mL); dalam pesakit bukan NPC, ia adalah IL-8 (13.36 ± 16.41 pg/mL). Manakala IL-2 adalah sitokin yang paling kurang dirembeskan dalam pesakit NPC (0.36 ± 0.25 pg/mL) dan pesakit bukan NPC (0.21 ± 0.14 pg/mL). Varian jenis mutan telah dikesan dalam 27.58% pesakit NPC, manakala tiada sampel dari bukan NPC dan HC yang didapati positif untuk varian jenis mutan. Varian jenis liar telah dikesan dalam 89.65% NPC, 65.51% bukan NPC, dan 3.44% HC. Tiada hubungan (p<0.05) ditemui untuk perbezaan dalam ekspresi biomarker cekpoint imun dalam kes varian jenis mutan dengan kes jenis bukan mutan. Penemuan ini meningkatkan pemahaman kita tentang kesan LMP1 EBV terhadap tahap ekspresi reseptor cekpoint imun dalam pesakit kanser yang dikaitkan dengan EBV. Ini boleh membantu mengenal pasti potensi ubat perencat cekpoint imun dan biomarker peramal bagi pemantauan dalam pesakit kanser yang dikaitkan dengan EBV (NPC dan bukan NPC). Selain itu, profil sitokin yang berbeza

menonjolkan perbezaan potensi dalam tindak balas imun antara individu NPC dan bukan NPC.

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ABSTRACT

Epstein-Barr virus (EBV) is associated with several types of cancer, including nasopharyngeal, breast, gastric, and lungs. Despite previous studies showing a link between the development of Nasopharyngeal carcinoma (NPC) and the identification of EBV's LMP1-30 bp deletion in NPC patients, several questions remain unanswered. In this research, a comparison of immune checkpoint receptor's expression levels in 29 patients with EBV-associated NPC, 29 patients with non-NPC cancers, and 29 healthy controls (HC) was determined and evaluated for their association with EBV's LMP1-30 bp deletion mutation. The expression levels of immune checkpoint receptors (CD3, CD4, CD25, CD127, FoxP3, LAG-3, TIM-3, and TIGIT) were accessed on Tregs using flow cytometry assay. While, the detection of cytokine and chemokine concentrations (IL-10, IL-2, IFN-γ, TNF-α, IL-8, and IL-6) were performed using the simple plex assay. Furthermore, the detection of EBV's LMP1-30 bp deletion mutation (mutant type variant) and LMP1 (wild type variant) from whole blood samples were performed using probe-based real-time PCR (qPCR). The final statistical analysis for association study was performed using different statistical tools. Most of the NPC patients were Malay (79.31%) ethnic, followed by Chinese (20.68%). WHO type III (62.06%) was the most common diagnosis for NPC patients, followed by WHO type II (27.58%) and WHO type I (10.34%). The expression of LAG-3, TIM-3 and TIGIT on Tregs was higher in NPC

and non-NPC patients when compared with HC (p<0.05). As compared to non-NPC, the expression of LAG-3 and TIGIT was significantly higher in NPC patients (p<0.05). Although, the expression level of TIM-3 was slightly higher in NPC comparing to non-NPC patients but it was statistically not significant (p=0.053). There was a statistical significance observed when comparing the levels of IL-2, IFN-γ, TNFα, IL-6 and IL-8 (p<0.05) in NPC patients with HCs. However, there was no statistical difference for IL-10 (p=0.054). In case of non-NPC comparison with HCs, the significance was observed for IL-10, and IL-2 (p<0.05), while there was no statistical difference noted for IFN-γ, TNFα, IL-8 and IL-6 (p>0.05). The most dominant cytokine in NPC patients was TNF-α (10.70 ± 4.00 pg/mL); in non-NPC patients, it was IL-8 (13.36 ± 16.41 pg/mL). While IL-2 was found to be the least secreted cytokine in NPC (0.36 ± 0.25 pg/mL) and non-NPC patients (0.21 ± 0.14 pg/mL). The mutant type variant was detected in 27.58% of NPC patients, while none of sample from non-NPC and HC were found positive for the mutant type variant. The wild type variant was detected in 89.65% NPC, 65.51% non-NPC, and 3.44% of HCs. There was no association (p<0.05) found for differences in the expression of immune checkpoint biomarkers in mutant type variant cases with non-mutant type cases. These findings improve our understanding of the effects of EBV's LMP1 on immune checkpoint receptor expression levels in EBV-associated cancer patients. This could help identify potential immune checkpoint inhibitor drugs and predictive biomarkers for monitoring in EBV-associated cancer patients (NPC and non-NPC). Additionally, distinct cytokine profiles highlighted potential differences in the immune response between NPC and non-NPC individuals.

CHAPTER 1

INTRODUCTION

Epstein-Barr virus (EBV) is a well-known human herpes virus and a member of the Herpesviridae family that belongs to double-stranded DNA viruses. The herpes virus family is surrounded by a lipid envelope containing peplomers, that is why EBV causes infectious mononucleosis (IM) (it is unstable or has an increased amount of monocyte in the blood) and spreads worldwide (Li *et al*., 2023). The virus is spread generally through various body fluids such a saliva, blood, semen, etc., and thus is easily transmitted from person to person (Tonoyan *et al*., 2024).

The EBV contains a linear genome, double-stranded DNA and encodes around 85 genes and 1,72,000 base pairs (Xiao *et al*., 2024). It has icosahedral symmetry and contains the projections glycoprotein for attachment (Jiang *et al*., 2024). The EBV contains the envelope made of lipids and the glycoprotein gp 350/220 for the virus's attachment to the host cell's surface. Also, it contains the receptor for the C3d complement component (Pardoll, 2012). EBV, with the help of glycoproteins, attaches itself to the surface of the host cell, and after this, the process of endocytosis takes place in which the viral envelop starts to fuse with the cell membrane host cell by using three glycoproteins gp85, gp25, gp42 where gp42 attach to the major histocompatibility complex (MHC) class-2 and cause infection in B lymphocyte. However, this virus causes the infection, particularly in B cells, but in certain conditions, it is also attached to epithelial cells as well as T cells (Tao *et al*., 2006, Linke-Serinsöz *et al*., 2017).

The EBV is usually present on intimate objects like toothbrushes and glass wherever moisture is present. If a healthy person uses these objects that are used by an infected person, then the virus will easily enter the healthy host and causes

infection (Soldan and Lieberman, 2023). Another way by which the virus enters the host body is through sexual contact, e.g., through semen (Salnikov *et al*., 2023). When the EBV virus enters the body, it usually becomes latent, which means inactive, but in patients who have a weak immune system, the virus becomes active in those patients and causes various symptoms such as fever, enlarged spleen, swelled liver and lymph nodes (Rezk *et al*., 2018). Two types of epidemiology patterns were seen in relation to EBV. The first pattern is found in children aged from 1 to 6 years, and the second in individuals aged from 14 to 20 years. In many developed countries, EBV infections occur at earlier ages, and 90% of children get positive for EBV by the age of 2 years (Su *et al*., 2023).

Because the virus could be present in saliva, it can be transmitted by kissing an infected person. It can also be contracted by drinking from the same glass or brushing your teeth with an infected person. It can be contracted through a blood transfusion, or an organ transplant because it's present in blood. (Chung *et al*., 2013). When the EBV enters the body, it primarily targets epithelial cells and various immune cells, including B cells, natural killer (NK) cells, and T cells. Upon infection, EBV employs distinct mechanisms to manipulate these host cells, leading to different pathological outcomes. The specific interaction patterns between EBV and these cells influence the type of malignancy that may develop (Lun *et al*., 2012).

The EBV is known to infect humans primarily in two forms: EBV-1 and EBV-2 (also called type-A and type-B). These two forms correspond to various genetic strains of the virus that exist among humans (Smatti *et al*., 2018). In order to investigate the diversity and molecular features of the virus and gain important insights into its prevalence, transmission, and possible consequences in various types of EBV-associated diseases, it is imperative to comprehend the existence of these

two types of EBV (Hassan *et al*., 2024). These types exhibit distinct genetic variations, highlighting the dynamic nature of the virus and its ability to adapt to the genetic landscape of its human hosts. The EBV-1 and EBV-2 share a common viral genome but they differ in specific regions such as the EBV nuclear antigen (EBNA)-2 and EBNA-3 genes. These genetic variations have been instrumental in classifying the virus into two types, providing a framework for understanding its global distribution and evolutionary history (Smatti *et al*., 2018).

Cancer is defined as an enormous spectrum of diseases that originate from uncontrolled cellular growth. The majority of identified cancer cases originates from oncogenic viral infections. Approximately 12% of cancers globally are attributed to viral infections, with the predominant majority (over 85%) occurring in developing countries (Soldan and Lieberman, 2023). These causative agents may induce tumor development accompanied by other risk factors such as chronic infections, continuous exposure to environmental hazards, lifestyle, genetic susceptibility to host factors, and even epigenetics. In 2010, it was estimated that around 10-15% of human malignancies globally are caused by EBV, human T-lymphotropic virus-I (HTLV-I), hepatitis C virus (HCV), hepatitis B virus (HBV), human papillomaviruses (HPV) 16 and HPV 18, Merkel cell polyomavirus (MCV), and Kaposi’s sarcoma herpesvirus (KSHV) (Campion *et al*., 2021, Li *et al*., 2021).

The EBV was reported to be associated with 38% of all virus-associated cancers (Chung *et al*., 2013). This virus was the first human cancer virus that has been identified (Argirion *et al*., 2023). The EBV has been associated with a variety of malignancies in humans, including lymphoid and epithelial tumors. The nasopharyngeal carcinoma (NPC), Burkitt lymphoma (BL), gastric cancer, breast cancer, non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma (HL), as well as

EBV-associated illnesses in individuals with impaired or suppressed immune systems, have all been related to EBV (Ali *et al*., 2019).

The NPC is a cancer that develops in the nasopharynx, the area behind the nose and above the back of the throat. The EBV is strongly related to the oncogenesis of NPC (Lee *et al*., 2021, Song *et al*., 2021). Undifferentiated NPC has the strongest link to EBV infection. Early detection of NPC is difficult. This is most likely because the nasopharynx is notoriously challenging to study, and the symptoms and signs of NPC are similar to those of other more prevalent diseases (He *et al*., 2012).

According to the 4th edition of World Health Organization (WHO) classification (2017) (Bossi *et al*., 2021), there are three subtypes of NPC:

Type-1: keratinizing squamous cell carcinoma (typically found in older adults)

Type-2: non-keratinizing carcinoma, differentiated type (most common NPC)

Type-3: non-keratinizing carcinoma, undifferentiated type (most common in teenagers)

While type 2 cases are relatively rare, type 3 malignancies are more frequently observed in adolescents and young children. Both types 2 and 3 are associated with high titers of EBV, unlike type 1, which has not yet been linked to EBV (Soldan and Lieberman, 2023, Tonoyan *et al*., 2024).

The proximity of linking the development of NPC lies within the EBV latency genes, particularly latent membrane protein 1 (LMP1) (Zebardast *et al*., 2021). A chronic and prolonged EBV latent infection can lead to tumorigenesis via latency transcription programs at cellular mutation in EBV-infected B cells (Vanshika *et al*., 2021).

The onset of NPC pathogenesis is thought to begin with idle EBV infection in pre-intrusive nasopharyngeal (NP) epithelium (Soldan and Lieberman, 2023). Multiple somatic mutations in the upstream negative NF-κB signalling have been discovered in a recent genomic analysis of NPC. The development of latent EBV infection in NPC may be aided by dysregulated NF-κB signalling. Through numerous pathways, stable infections of EBV and latent EBV gene expression are thought to induce the transition of pre-invasive NP epithelial cells to cancer cells (Chung *et al*., 2013).

The immune checkpoint biomarkers play a crucial role in regulating the immune response, ensuring that it is effective against pathogens and cancer cells while preventing autoimmunity (Chen and Chen, 2014). Among these biomarkers, regulatory T cells (Tregs) are of particular interest due to their immunosuppressive functions (Fourcade *et al*., 2018). Tregs are characterized by the expression of several surface and intracellular markers, including CD3, CD4, CD25, CD127, and FoxP3 (Hou *et al*., 2015). CD3 and CD4 are common markers found on all T cells, but Tregs can be further identified by the high expression of CD25 (the alpha chain of the IL-2 receptor) and the low expression of CD127 (Engku Abd Rahman *et al*., 2023). FoxP3 is a transcription factor that is considered the master regulator of Treg development and function, and its expression is a hallmark of Tregs (Hariyanto *et al*., 2022).

The Tregs help maintain immune homeostasis, but in the tumor microenvironment, they often contribute to immune evasion by suppressing anti-tumor immune responses (Gupta *et al*., 2012). This suppression is mediated through various mechanisms, including the expression of immune checkpoint molecules such as LAG-3, TIM-3, and TIGIT (Cai *et al*., 2023). These checkpoints are critical in

dampening the immune response, allowing cancer cells to evade detection and destruction by the immune system (Anderson *et al*., 2016).

Lymphocyte Activation Gene-3 (LAG-3) is an immune checkpoint receptor that negatively regulates T cell function. Its expression on Tregs enhances their suppressive capabilities, contributing to the immune-suppressive environment in tumors (Camisaschi *et al*., 2010). T cell Immunoglobulin and Mucin domain-containing protein-3 (TIM-3) is another checkpoint molecule that, when expressed on Tregs, can inhibit the activation and proliferation of effector T cells (Du *et al*., 2017). T cell Immunoreceptor with Ig and ITIM domains (TIGIT) similarly plays a role in immune suppression, with its expression on Tregs being associated with the inhibition of NK cell and T cell responses (Jiang *et al*., 2022). The upregulation of LAG-3, TIM-3, and TIGIT on Tregs in NPC and other EBV-associated cancers underscores their potential as targets for immunotherapy (Cai *et al*., 2023).

1.1 Study rationale

Cancer is a disease that has many unresolved long-standing controversies in terms of clinical diagnosis, management, and advance molecular research. Multiple etiological factors have been identified as predisposing factors for cancers (such as obesity, tobacco smoking, lack or exercise, poor diet, etc.). The treatability of cancer depends on various factors, including the type and stage of cancer, individual patient characteristics, and available treatment options. Many cancers, when detected early and localized, can be effectively treated or even cured.

The EBV-associated cancers, particularly NPC, impose a substantial burden on public health. Investigating the underlying mechanisms and risk factors associated with EBV-related cancers can inform public health interventions, screening programs, and treatment protocols. One of the viral proteins implicated in the

oncogenic process is the LMP1. Specifically, a 30 bp deletion in the LMP1 gene has been linked to an increased risk of cancer. Previous investigations have explored the identification of a 30 bp deletion in the EBV's LMP1 gene in patients with NPC, establishing its link to NPC development. However, many questions persist, especially regarding the implications of this mutation on the modulation of immune checkpoint receptors on immune cells.

Immune checkpoint receptors play a crucial role in maintaining immune homeostasis and preventing autoimmunity, but their dysregulation can lead to immune evasion by cancer cells. Regulatory T cells (Tregs), known for their immunosuppressive functions, play a critical role in maintaining immune homeostasis and preventing autoimmunity. However, in the context of cancer, Tregs can suppress anti-tumor immune responses, allowing cancer cells to evade the immune system. Despite the established link between EBV and NPC, the mechanisms by which the LMP1-30 bp deletion mutation contributes to immune evasion and tumor progression are not fully understood.

The Tregs, which express markers such as CD3, CD4, CD25, CD127, FoxP3, LAG-3, TIM-3, and TIGIT are particularly important in this context, as they can suppress anti-tumor immune responses. Assessing the expression of immune checkpoint receptors on Tregs and measuring cytokine levels may provide a comprehensive profile of the immune environment in EBV-associated cancers. This can reveal how Tregs contribute to immune evasion and tumor progression, offering insights into potential combination therapies that modulate Treg activity.

The existing body of research has yet to fully address these questions, leaving a gap in our understanding of the broader impact of the EBV LMP1-30 bp deletion on immune regulatory mechanisms across various cancer types. The identification of

specific EBV variants associated with cancers in the Malaysian population can have clinical implications. It may lead to the development of targeted therapies or personalized treatment approaches that are more effective for the local population.

Tregs are essential in maintaining the central and peripheral tolerance for immune homeostasis. Meanwhile, Immune checkpoints are natural components of the immune system, and their purpose is to prevent an overly aggressive immune response from destroying healthy cells. To the best of my knowledge, there is no studies regarding LAG-3, TIM-3, and TIGIT in NPC patients.

1.2 Research objectives

1.2.1 General objective

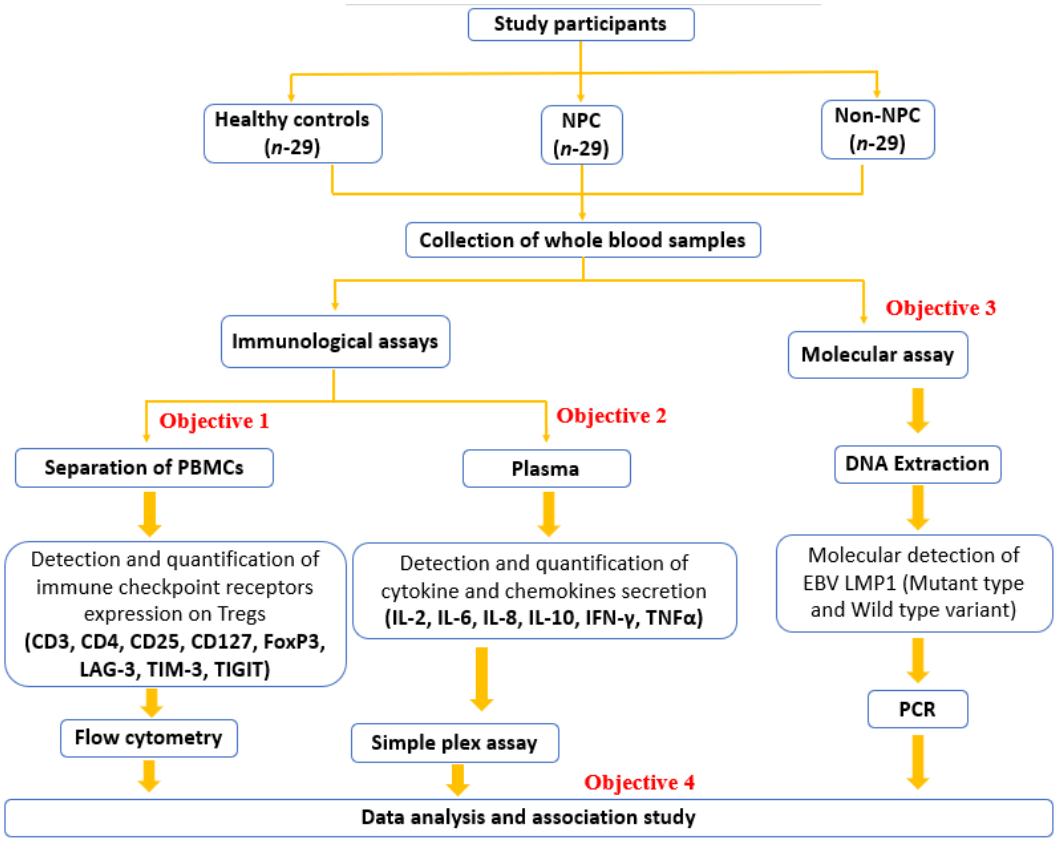
To access the effects of EBV LMP1-30 bp deletion on the expression levels of immune checkpoint receptors in EBV-associated NPC and non-NPC cancers.

1.2.2 Specific objectives

1. To assess the expression of immune checkpoint receptors on the Tregs population using flow cytometry analysis (Markers selection: CD3, CD4, CD25, CD127, FoxP3, LAG-3, TIM-3, and TIGIT) on collected blood samples of healthy controls and EBV-associated (NPC and non-NPC) cancer patients.
2. To assess the immune responses by measuring the cytokine levels (for example, IL-10 and TGF-α, etc.) and soluble immune checkpoints (for example, TNF, interferon-gamma, etc.) on collected blood samples of healthy controls and EBV-associated (NPC and non-NPC) cancer patients.
3. To perform molecular detection of EBV LMP1-30 bp deletion (mutant type variant) and LMP1 (wild type variant) using qPCR assay on collected whole blood samples of healthy controls and EBV-associated (NPC and non-NPC) cancer patients.

4. To determine the association between the expression levels of immune checkpoint receptors in the Tregs population with EBV LMP1-30 bp deletion status in EBV-associated (NPC and non-NPC) cancers.

1.3 Overview of the study



CHAPTER 2

LITERATURE REVIEW

2.1 Epstein–Barr virus (EBV)

The EBV belongs to the herpesvirus family, representing one of the most common human viruses. This virus was first discovered in 1964 in human cancer cells (Vockerodt *et al*., 2015). EBV is a double-stranded DNA virus with a substantial genome approximately 176 kb in length. This genome can code for around 80 proteins and encompasses 46 untranslated RNAs (Lee *et al*., 2009). Notably, EBV's ability to persist throughout an individual's lifetime sets it apart as a unique viral pathogen. The virus is widespread, with virtually everyone worldwide experiencing infection at least once in their lifetime. The primary transmission mode is through saliva, and initial infection typically occurs during childhood, often manifesting as an asymptomatic condition (Macedo *et al*., 2011).

The EBV can induce infectious mononucleosis (IM) during adolescence. A distinguishing feature of herpesviruses is their ability to enter a latent state, allowing gene expression within host cells for extended periods without leaving residual viral protein. EBV predominantly targets B lymphocytes and, on occasion, epithelial cells (Hutt-Fletcher, 2014, Rostgaard *et al*., 2019). Notably, if the initial infection does not occur in early childhood, a later infection, known as IM, can provoke an exaggerated immune response with pronounced symptoms. The underlying reasons for the heightened immune response in IM compared to primary infection in early childhood pose a complex puzzle (Hutt-Fletcher, 2014). Ongoing discussions center around the potential influence of Human Leukocyte Antigen (HLA) in shaping IM development. Additionally, studies have suggested that a substantial initial viral dosage might contribute to the onset of IM (Munz, 2017).

Approximately 56 years ago, the link between cancer and EBV was unveiled through a biopsy of BL, prevalent in Africa. This groundbreaking discovery marked the first direct association of a virus with human cancer (Pattle and Farrell, 2006). The extended persistence of the virus is believed to occur predominantly in B lymphocyte cells, and in carriers, a mere 0.1% of cells are typically infected. However, in the context of EBV-infected cancer cells, every malignant cell tends to be affected, establishing a robust association between EBV and cancer. This distinctive characteristic underscores the significant role of EBV in the pathogenesis of certain malignancies (Munz, 2021).

The onset of cancer is often a gradual process that unfolds over several years, and the prolonged presence of EBV may contribute to its association with cancer. EBV-induced differentiation of B lymphocyte cells is implicated in developing EBV-related malignancies. The virus has been linked to the development of various cancer types in humans, including HL, BL, and lymphomas, as well as certain immunocompromised disorders, gastric cancer, and NPC (Munz, 2021). The connection between EBV and the pathological characteristics of these cancers is a subject of investigation. Additionally, EBV is associated with malignancies such as NK/T cell lymphomas and methotrexate-associated lymphomas, further emphasizing the virus's potential role in the development of diverse forms of cancer (Pattle and Farrell, 2006).

2.1.1 EBV structure and genome

The EBV stands as a significant player in the realm of viral pathogens, particularly due to its intricate genome structure. The EBV genome is characterized by a linear double-stranded DNA, an expansive molecular entity spanning around 172 kilobase pairs (kb) (Dolan *et al*., 2006). This genetic blueprint is not

homogenous; instead, it is intricately partitioned into several sequence domains, each differing significantly in length (Zeng *et al*., 2005). The demarcation between these domains is facilitated by a series of terminal direct repeats (TRs) and internal repeat sequences (IRs), both measuring approximately 0.5 kb. This structural organization holds key information about the virus's biology, replication, and interaction with its host (Dolan *et al*., 2006).

In the landscape of virology, the infectious B95-8 strain of EBV, derived from IM, holds a pivotal position. A previous study achieved a groundbreaking feat by successfully cloning and sequencing the entire genome of this strain, marking a historic moment in the study of herpesviruses (Dolan *et al*., 2006). This milestone not only contributed substantially to the understanding of EBV but also set the stage for subsequent advancements in the field of molecular virology.

Building upon this initial breakthrough, recent years have witnessed the elucidation of the complete genomic sequences of two additional EBV strains: AG876 (accession number DQ279927) and GD1 (accession number AY961628) (Dolan *et al*., 2006). The AG876 strain was derived from a patient with a Ghanaian BL case, while the GD1 strain was isolated from a patient with a Chinese NPC case. These genomic sequences provide invaluable insights into the genetic diversity of EBV and its association with distinct clinical manifestations across diverse populations (Ali *et al*., 2015).

The genome of AG876, originating from a Ghanaian BL case, offers a glimpse into the viral factors potentially contributing to the pathogenesis of BL. Understanding the unique genetic makeup of this strain can shed light on the molecular events underlying EBV-associated oncogenesis in the context of BL, a malignancy prevalent in certain regions of Africa (Ayee *et al*., 2020). On the other

hand, the GD1 strain, linked to a Chinese NPC case, unravels genomic features that may be intricately tied to the development of NPC. This type of cancer, particularly prevalent in Southeast Asia, is characterized by its association with EBV. By deciphering the complete genomic sequence of the GD1 strain, researchers aim to unravel the specific viral elements that contribute to the oncogenic processes leading to NPC (Buettner *et al*., 2012).

The comprehensive genomic characterization of EBV strains, including B95-8, AG876, and GD1, has ushered in a new era in the study of this medically significant virus (Busson *et al*., 2004). These efforts have not only deepened our understanding of EBV's genetic architecture but have also paved the way for targeted investigations into the virus's role in distinct malignancies. The ongoing exploration of EBV genomics holds the promise of uncovering novel therapeutic targets, diagnostic markers, and preventive strategies against the diverse array of diseases associated with this ubiquitous virus (Ali *et al*., 2015).

About 80 proteins with the potential to be coded by the virus have not been described or identified, and characterized gene products are presented. The examination of the GD1 and AG876 sequences has led to the discovery of four ORFs that were previously unidentified. Additionally, the B95-8 sequence has been corrected. The open reading frames mentioned are BVLF1 and BDLF3.5. The roles of these open reading frames are currently not understood. Another open reading frame, BFRF1A, is likely involved in DNA packaging since it has similarities with other Herpesviruses. Lastly, BGLF3.5 is most probably a tegument protein, as suggested by (Dolan *et al*., 2006). Open reading frames, genes, and transcription or RNA processing sites are often associated with particular BamHI fragments labelled from A to Z based on their decreasing size. The original B95-8 genome was

sequenced using a library of cloned EBV DNA fragments cut with BamHI (Petersson, 2015).

2.1.2 EBV transmission and life cycle

The EBV life cycle's lytic phase consists of three regulated gene expression phases: immediate-early, early, and late. The immediate-early gene products are trans-activator proteins that initiate the expression of early genes and stimulate the production of enzymes necessary for viral DNA replication (Ahmed *et al*., 2022). The amplification of EBV's DNA determines the differentiation between early and late gene expression. Before infectious virions are released from the virus, viral structural proteins are formed and produced during the late stage of the cycle (Busson *et al*., 2004).

The virus can also infect epithelial cells when attached to B cells. However, the B cell infection is assumed to be the pathway through which this EBV (herpesvirus) chronic infection is established. Latent EBV infection in vivo is typified by three unique patterns or phases, including gene expression, referred to as the growth, default, and latency phases **(Figure 2.1)**. These separate viral gene expression programs have developed to drive infected B cells to differentiate by delivering the growth and survival signals necessary for B cell activation and memory formation and preserve the virus in a latent state in the periphery (Mrozek-Gorska *et al*., 2019).

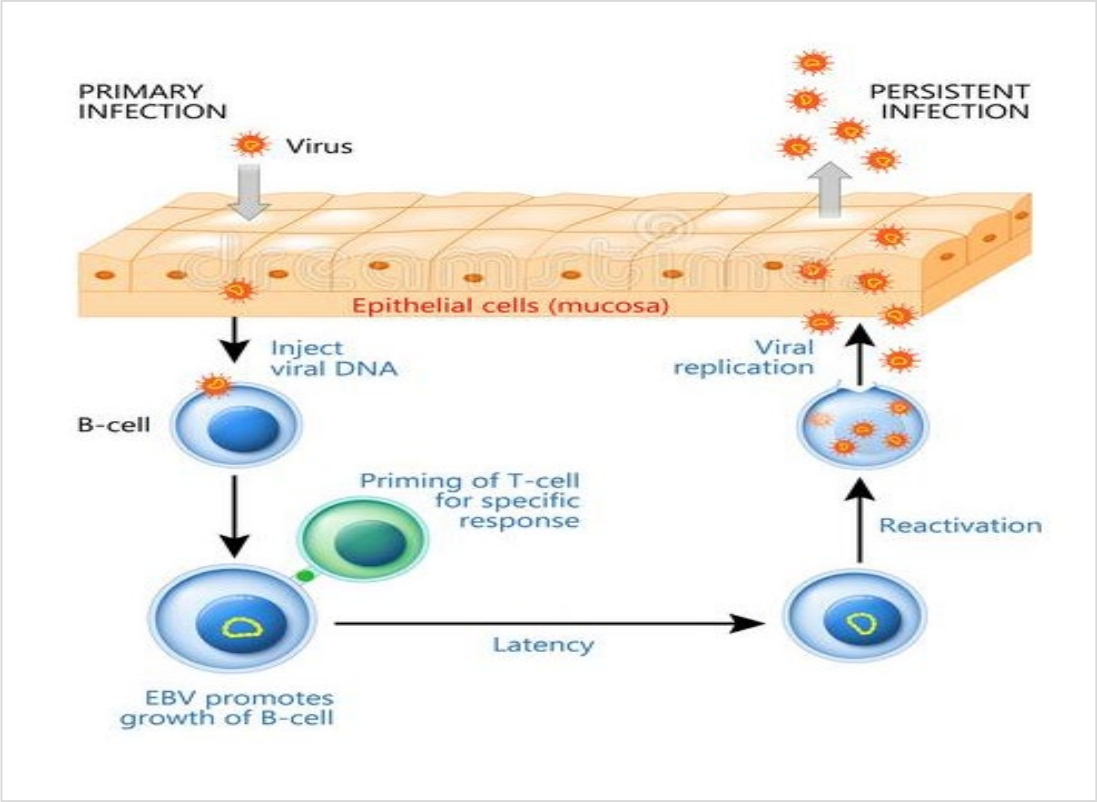


Figure 2.1. Life cycle of EBV. (Adopted from Mrozek-Gorska *et al*., 2019).

The intricate life cycle of the EBV involves a precisely orchestrated activation of specific genes, and among them, the immediate-early genes BZLF1 and BRLF1 play pivotal roles, particularly during the transition from latent to productive infection (Busson *et al*., 2004). These immediate-early genes are strategically positioned to serve as master regulators, initiating a cascade of events that lead to the lytic cycle of EBV. BZLF1, also known as Z-EBV replication activator (ZEBRA), is a transcription factor that holds the key to the initiation of lytic replication. Its expression marks the switch from latency to the lytic cycle, triggering the reactivation of the virus. BZLF1 acts by binding to specific DNA sequences in the EBV genome, promoting the transcription of early lytic genes essential for viral DNA replication and subsequent virion production (Ahmed *et al*., 2022).

Similarly, BRLF1, or Replication and transcription activator (Rta), is another immediate-early gene crucial for the induction of lytic replication. BRLF1 collaborates with BZLF1 in orchestrating the intricate dance of gene expression during the lytic cycle (Ali *et al*., 2015). Like BZLF1, BRLF1 also functions as a transcription factor, binding to specific viral promoters and kickstarting the expression of genes necessary for the progression of the lytic cascade (Ayee *et al*., 2020). The activation of BZLF1 and BRLF1 is tightly regulated, ensuring that the transition from latency to productive infection occurs under specific conditions (Argirion *et al*., 2023). Various stimuli, including environmental cues or host factors, can trigger the expression of these immediate-early genes. Once activated, BZLF1 and BRLF1 set in motion a complex molecular program, leading to the replication of viral DNA, assembly of new virions, and ultimately, the release of infectious particles (Buettner *et al*., 2012).

2.1.3 EBV infection stages

Prior studies on EBV chronic infection included biological and immunological investigations to identify and measure infected cells' populations and assess the immune response to these populations. Consequently, a biological model was established whereby EBV infects and activates naïve B-cells, which then transition via the germinal center to quiescent memory B-cells, serving as the virus's dwelling place (Awasthi *et al*., 2022). Occasionally, the virus may reactivate inside these memory cells to produce infectious virions. A fraction of this virus invades newly formed, inexperienced B-cells, finishing the infection cycle. The absence of understanding lies in the comprehension of the dynamic interplay among these elements and how their control by the immune response generates the observed viral persistence pattern. The current deficiency is in understanding the dynamic interplay among these constituents and how their control by the immune response generates the observed pattern (Chung *et al*., 2013). Epstein-Barr virus undergoes two distinct phases during its lifecycle: latent infection and lytic infection. While latent infection involves the persistence of the viral genome within host cells without active replication, lytic infection is characterized by the active production of infectious viral particles (Smatti *et al*., 2018).

2.1.3 (a) EBV lytic infection

During the lytic infection phase, specific viral genes are expressed, producing new virus particles that can infect neighbouring cells. This process involves a carefully orchestrated series of events. Early in the lytic cycle, immediate-early genes are activated, initiating the synthesis of regulatory proteins (Awasthi *et al*., 2022). These regulatory proteins, in turn, stimulate the expression of early genes responsible for viral DNA replication. Subsequently, late genes are expressed, encoding

structural proteins essential for assembling new viral particles. One hallmark of the lytic infection is the formation of distinctive viral structures within the host cell, such as replication compartments and viral factories. Replication compartments serve as sites for viral DNA replication, while viral factories facilitate the assembly of viral components into mature infectious particles (Chan *et al*., 2003).

The lytic infection is not only crucial for the production of new virions but also plays a significant role in the pathogenesis of EBV-associated diseases. Lytic replication contributes to the spread of the virus within the host and the transmission to new hosts. Furthermore, it has been implicated in the development of certain EBV-associated malignancies (Gupta *et al*., 2021). Understanding the intricacies of EBV lytic infection is essential for unravelling its complex interactions with the host and its role in diseases. Researchers explore various aspects of the lytic cycle to identify potential targets for antiviral therapies and gain insights into the mechanisms that govern EBV-associated pathologies. The study of lytic infection opens avenues for therapeutic interventions that could disrupt the virus's lifecycle and mitigate its impact on human health (McKenzie and El-Guindy, 2015).

In addition to being necessary for horizontal transmission of EBV among hosts, the lytic cycle also plays a role in initial infection and latency establishment. Several papers propose a scenario for initial infection in which EBV particles attack oral epithelial cells and go through lytic replication, producing new viral progeny (Heussinger *et al*., 2004). The initial infection was established when released virus particles spread to the oral submucosa and infected circulating B cells. EBV goes through a crucial temporary period of lytic gene expression after entering B cells. The induction and maintenance of latency in newly infected B cells depends on the expression of this small group of early lytic genes. Additionally, the pool of latently

infected B cells continually being hunted down by immune surveillance is replenished by spontaneous lytic reactivation from latency (Huynh and Rong, 2012).

2.1.3 (b) EBV latent infection

Latent infection is defined by the persistence of the viral genome within host cells without active viral replication. EBV establishes latent infection in B lymphocytes, where the viral genome becomes a stable component of the host cell's DNA (Gupta *et al*., 2021). During latent infection, EBV expresses a limited set of viral genes that play crucial roles in maintaining the viral genome, evading the host immune response, and promoting cell survival (Hassan *et al*., 2024). The viral genome typically exists as a circular episome in the nucleus of infected cells. One of the key factors contributing to the stealthy nature of EBV's latent phase is the minimal expression of viral proteins, which helps the virus evade detection by the host's immune system (Heussinger *et al*., 2004).

EBV has multiple latency programs, each associated with specific patterns of viral gene expression. These latency programs are called latency 0, I, II, and III (Li *et al*., 2022). Latency 0 involves no expression of viral proteins, latency I consist of the expression of EBNA-1, latency II includes additional viral proteins such as LMP1 and LMP2A, and latency III involves the expression of the full spectrum of latent viral proteins, including EBNA-1, LMP1, LMP2A, and others (Liu *et al*., 2021).

The maintenance of latent infection allows EBV to persist in the host for the entirety of the individual's life. While latent infection is generally asymptomatic, the virus can reactivate into the lytic cycle, producing infectious viral particles (Li *et al*., 2023). Latent infection is associated with EBV's role in various diseases, including infectious mononucleosis and the development of certain cancers, such as BL, NPC, and HL (El-Guindy *et al*., 2007). Understanding the dynamics of EBV latent

infection is crucial for comprehending the virus's complex interactions with host cells and its implications in diseases. Researchers aim to decipher the mechanisms underlying viral latency to identify potential targets for therapeutic interventions and develop strategies for controlling EBV-associated pathologies (Linke-Serinsöz *et al*., 2017).

2.1.3 (c) EBV latency patterns

A recent study has shown many connections between the process of terminal cell differentiation and the activation of lytic replication in gamma herpesviruses such as EBV. The Zp activity was increased when squamous epithelial cells underwent terminal differentiation. The XBP1 and BLIMP1, two factors governing the reactivation of EBV in B lymphocytes and epithelial cells, play crucial roles in cellular differentiation. In individuals with HIV, experiencing oral hairy leukoplakia, EBV undergoes replication exclusively in differentiated epithelial cells expressing BLIMP1 (Buettner *et al*., 2012). The activation of BLIMP1 induces the lytic cycle in epithelial cells by triggering Zp and Rp. Meanwhile, in plasma-differentiated cells, XBP1, once activated, binds to Zp's ZID/ZII elements, initiating the process of lytic replication (Lee *et al*., 2021).

Other biological components that help to preserve the B cell lineage, like EBF1 and Pax5, have been identified as repressors of lytic reactivation. Pax5 expression is regulated by the transcription factor EBF1. Lytic viral reactivation occurs in lymphoblastoid or BL cell lines when endogenous EBF1 or Pax5 expression is lost. Pax5 protein levels are also decreased by triggering the lytic cycle utilizing inducing agents (McKenzie and El-Guindy, 2015).

2.2 Clinical Significance of EBV

Although several clinical complications including splenic infarction and rupture, upper airway obstruction, and neurological issues, are possible, the majority of patients recover from EBV related infections without any long-term effects. Even in those without obvious immune deficiencies, EBV can result in chronic infections (Lee *et al*., 2021). Children and young adults are typically affected by Chronic active EBV infection (CAEBV) infection, which is characterized by chronic or recurrent symptoms similar to IM (Linke-Serinsöz *et al*., 2017). According to Traus' classic explanation, CAEBV infection meets the following three requirements: Histological evidence of major organ involvement, such as interstitial pneumonia, hypoplasia of some bone marrow components, uveitis, lymphadenitis, persistent hepatitis, and/or splenomegaly; increased amounts of EBV in affected tissues, which can be accurately assessed with the peripheral blood specimen by a quantitative polymer assay; and severe illness lasting more than six months that started as a primary EBV infection (Maeda *et al*., 2009).

The CAEBV presents a severe and protracted form of EBV infection, marked by persistent viral replication and a range of debilitating symptoms (Linke-Serinsöz *et al*., 2017). The most prevalent and distinctive symptom is a persistent high-grade fever that endures over an extended period. Individuals with CAEBV often experience significant lymphadenopathy, where lymph nodes enlarge, reflecting the continuous immune response against the virus (Li *et al*., 2022). Liver involvement is common, leading to hepatitis and resulting in jaundice, elevated liver enzymes, and abdominal discomfort (Li *et al*., 2023). Splenomegaly, or an enlarged spleen, contributes to abdominal pain and discomfort. CAEBV can progress to hemophagocytic syndrome, a severe condition characterized by overactivated

immune cells attacking blood cells, causing anaemia, thrombocytopenia, and coagulopathy (Gupta *et al*., 2021). Respiratory symptoms, including cough and shortness of breath, may occur due to the virus affecting the respiratory system, potentially leading to interstitial pneumonia in severe cases. Neurological manifestations such as headaches, seizures, and altered mental status can also be present. Skin rashes, ulcers, or petechiae may manifest as part of CAEBV-related symptoms (Heussinger *et al*., 2004).

2.3 EBV-associated malignancies

The immunodeficiency-associated immunocompromised patients have an increased risk of developing lymphomas and lymphoproliferative diseases that EBV causes. The virus has been identified in the tumor cells of these malignancies, and its role in promoting cellular proliferation and inhibiting apoptosis contributes to their pathogenesis. Understanding the complex interplay between EBV and different cancer types is crucial for developing targeted therapeutic strategies (Argirion *et al*., 2023). The EBV-associated non-NPC cancers include mesenchymal tumors, EBVaGC, BL, HL, pyothorax-associated lymphoma, lymphomatoid granulomatosis, etc. Certain immunocompromised disorders are among the non-NPC cancers linked to EBV (Ahmed *et al*., 2022). Ongoing research seeks to elucidate the molecular mechanisms by which EBV contributes to oncogenesis, aiming to advance treatment options and improve outcomes for individuals affected by EBV-associated cancers.

Primary EBV infections typically happen in young children and are symptomless or cause mainly generalized signs. However, EBV can cause a variety of disorders in people (Smatti *et al*., 2018). Acute IM syndrome can develop from primary infections in late teens or adults. EBV infection that is currently active very seldom becomes chronic. After a primary infection, EBV persists in lymphocytes in

the peripheral blood as a latent form (Tao *et al*., 2006). In most situations, a lifelong latent infection has no adverse effects, although it can occasionally result in different types of lymphoid, mesenchymal, or epithelial cancers, especially when there is immunodeficiency. Early identification of these potentially fatal conditions is possible by understanding the spectrum of lymphoproliferative disorders in immunocompromised patients (Vanshika *et al*., 2021).

The first human tumor virus to be identified was the EBV (Cohen, 2000). Other human pathogenic herpes viruses, including Herpes simplex viruses 1 and 2 (HSV 1 and 2) (Looker *et al*., 2017, Koganti *et al*., 2019), varicella zoster virus (VZV) (Pergam *et al*., 2009), cytomegalovirus (CMV) (Kotton, 2013), and HSV 6, 7, and 8 (Blauvelt, 2001), and HSV-4 (Ali *et al*., 2015). In BL cell lines, electron microscopy revealed it. Impaired host immunity may play a significant role in the pathogenesis of EBV-associated cancers because the incidence of endemic BL's is correlated with Human Immunodeficiency Virus (HIV) and malaria (Gardner *et al*., 2022).

The ability to recognize their clinical manifestations and imaging range is a crucial factor in the early diagnosis and treatment of conditions that may pose a risk to one's life (Maeda *et al*., 2009). Over time, advanced technologies have led to a greater understanding of the genetic and epigenetic characteristics, the environment around tumors, and the disease-causing roles of EBV in NPC. **Figure 2.2** depicts the pathogenic model of NPC.