EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 (LMP-1) EXPRESSION AND ITS ASSOCIATION WITH EXPRESSION OF DNA DAMAGE REPAIR PROTEINS IN NASOPHARYNGEAL CARCINOMA (NPC) – AN EVALUATION

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS & SYMBOLS

NPC	Nasopharyngeal Carcinoma
EBV	Epstein-Barr virus
HPV	Human Papilloma Virus
DNA	Deoxyribonucleic acid
bp	Base pair
TBE	Tris/Borate/EDTA
EDTA	Ethylenediaminetetraacetic acid
DAB	Diaminobenzidine
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
LSAB	Labelled Streptavidin Biotin
PCR	Polymerase Chain Reaction
LMP	Latent Membrane Protein
DNA-PKcs	DNA Protein Kinase catalytic subunit
ATM	Ataxia Telangiectasia Mutated
dH_2O	Distilled water
^oC	Degree Celsius
nm	nanometer
g	gram
l	Litre
V	Volts
Pos/+ve	Positive
Neg/-ve	Negative
HNSCC	Head and neck squamous cell carcinoma
EBER	Epstein-Barr encoded RNA

OD	Optical density
ASR	Age standardized incidence
KSCC	Keratinizing squamous cell carcinoma
UNCT	Undifferentiated carcinoma type
RT	Radiotherapy
CT	Chemotherapy
TNM	Tumor, lymph nodes, metastatsis
HSPGs	heparan sulfate proteoglycans
BM	basement membrane
DNA-PK	DNA protein kinase
DSB	Double strand break
IR	Ionizing radiation
A-T	Ataxia Telangiectasia
SCID	Severe combined immunodeficiency
shRNA	Small hairpin RNA
RNA	Ribonucleic acid
NHEJ	Non-homologous end joining
ssDNA	Single stranded DNA
MMTV	Mouse Mammary Tumor Virus
DNase	Deoxyribonuclease
DPX	Distrene, Plasticiser, Xylene
dNTP	Deoxyribonucleotide
DDB1	DNA damage binding protein 1
WHO	World Health Organization
EBNA	Epstein-Barr nuclear antigen

PENILAIAN HUBUNGAN ANTARA EKSPRESI LATENT MEMBRANE PROTEIN 1 (LMP-1) VIRUS EPSTEIN-BARR DENGAN PROTEIN PEMULIHAN DNA DALAM KANSER NASOFARINKS

ABSTRAK

Kanser nasofarinks (NPC) adalah kanser ketiga tertinggi di kalangan lelaki di Malaysia. Kanser ini adalah unik dari pelbagai aspek terutamanya hubung kait antara penyakit ini dengan virus seperti virus Epstein-Barr (EBV). LMP-1 adalah protein onkogen utama EBV. LMP-1 mengawal rangkaian ekpresi yang menyebabkan ketidakaturan pertumbuhan sel. Oleh sebab itu, LMP-1 telah dianggapkan berpotensi dijadikan sasaran terapi kanser. Bukan kesemua kanser nasofarinks akan menunjuk ekspresi LMP-1 kerana ekspresi LMP-1 dipercayai diregulasi dalam kanser. Kajian menunjukkan protein pemulihan DNA adalah dikaitkan dengan proses karsinogenesis maka protein pemulihan DNA dianggap mustahak dalam konteks ini. Kajian dari merata dunia telah menunjukkan kes koinfeksi Human papillomavirus (HPV) dalam kanser nasofarinks tetapi status di Malaysia masih tidak diketahui. Dalam kajian ini, kepentingan koinfeksi dalam kanser nasofarinks telah dikaji. Lima puluh tujuh (57) kes kanser nasofarinks dan dua puluh tujuh (27) kes normal dari Kelantan telah dimasukkan dalam kajian ini. Ekspresi protein pemulihan DNA seperti ATM, DNA-PKcs, Ku70, Ku86 dan caspase-3 telah dikaji dengan kaedah immunohistokimia. Status infeksi virus pula dikaji dengan menggunakan tindak balas rantai polimerase (PCR). Hasil kajian menunjukkan korelasi positif diantara eskpresi LMP-1 dengan ATM dan caspase-3 aktif. Hasil kajian juga menunjukkan bahawa HPV adalah dikaitkan dengan peningkatan ekspresi ATM dan penurunan ekpresi Ku86. Selain itu, hasil kajian juga menunjukkan bahawa LMP-1 dan HPV

mungkin bekerjasama dalam mengawalatur ekpresi protein. Kajian yang menunjukan infeksi EBV dalam kes normal dan pengaruh infeksi HPV menunjukkan kemungkinan EBV hanya memainkan peranan yang utama atau sambilan dalam kanser nasofarinks. Oleh itu, hasil kajian mencadangkan LMP-1 mempengaruhi ekspresi protein pemulihan DNA.

EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 (LMP-1) EXPRESSION AND ITS ASSOCIATION WITH EXPRESSION OF DNA DAMAGE REPAIR PROTEINS IN NASOPHARYNGEAL CARCINOMA (NPC) – AN EVALUATION

ABSTRACT

Nasopharyngeal carcinoma (NPC) is the third most occurring cancer in Malaysian males, and is unique in its association with Epstein-Barr virus (EBV). LMP-1 is the major oncogenic protein of the EBV. EBV LMP-1 acts by constitutive regulation of host signalling pathways leading to dysregulated cell growth and has been considered a potential therapeutic target for LMP-1 expressing tumours. LMP-1 is thought to be regulated in its expression as it is not expressed in all NPC tumours. DNA repair proteins are of importance in this context as the status of the function of these proteins has been linked to the process of carcinogenesis. Studies world-wide have shown co-infection of NPC by Human papilloma virus (HPV). The status in Malaysia is not known. In the present study, the importance of viral co-infection in NPC and how it influences the expression of the EBV and host proteins has been evaluated. Fifty seven (57) NPC and twenty seven (27) benign adenoid samples from State of Kelantan in Malaysia were included in this study. Expression of DNA repair proteins such as ATM, DNA-PKcs, Ku70, Ku86, pro and active caspase-3 protein were evaluated immunohistochemically. The viral status of the lesions was evaluated using PCR on extracted DNA from the tumour/benign tissues. Results showed that LMP-1 expression showed a positive correlation with the expression of ATM, and active caspase-3. No correlation was observed with the expression of other DNA repair proteins. In the present study, it was observed that HPV infection was

associated with increased ATM expression and decreased Ku86 expression. It was also observed that LMP-1 and HPV may work synergistically in altering the host protein interaction as seen in the different groups of lesions. The observation of a number of EBV positive non-cancerous lesions in this study and the influence of HPV infection give credence to the hypothesis that EBV might only play a main or casual role in NPC. These results thus suggest that LMP-1 influences the expression of host DNA damage repair proteins.

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1 Cancer

Cancer, also known as malignant neoplasm, refers to disease characterized by an uncontrolled growth and division of cells, and the ability of cells to invade and metastasise locally or at the other parts of the body. Cancer formation can be due to multiple causes. One major reason for formation of cancer is the damage and mutation of DNA, on the gene coding for protein which is essential for monitoring cell growth and death (proto-oncogene), with certain genes transforming into oncogenes. Oncogene activation causes unusually high expression of normal or altered proteins (oncoprotein), resulting in uncontrolled cell growth and death. Cancer can also be caused by infectious agents such as bacteria and viruses. Viruses have the ability to alter the genetic configuration of the cells. Viruses are known to activate oncogenes which increase the risk of cancer formation. Viruses have been accepted widely as the second most important risk factor for human cancer formation, surpassed only by tobacco use (zur Hausen, 1991). The transformation of a cell into a cancer cell is accompanied by alterations in protein expression, behavior, and morphological features such as size and shape of cell and nucleus. At this stage, the cancer cell is distinguishable from its normal counterpart.

1.1.1 Cancer and viruses

In 1908, Oluf Bang and Vilhelm Ellerman demonstrated that avian erythroblastosis could be transmitted by cell-free extracts, initiating the theory that cancer can be caused by viral particles (Ellermann and Bang, 1909). Since then, many viruses have been identified to be associated with cancer formation. In 2002, it was estimated that

17.8% of cancer worldwide was caused by infections, with around 12% being caused by human tumour viruses. The frequency was higher in developing countries (26.3%) as compared to that in developed countries (7.7%) further supporting the hypothesis of a relationship of cancers with infections (Parkin, 2006).

Viral carcinogenesis follows several direct mechanisms that involve the introduction of viral oncogenes, enhancement of cell proto-oncogenes, and the inactivation of tumour suppressor genes. Viruses can also contribute to oncogenicity indirectly, by reducing host immunity (such as in the case of HIV), inactivating host proteins (HPV and EBV associated cancers) and chronic nonspecific inflammation occurring over long time span of infection, such as in the case of HCV-induced liver cancers. In the context of DNA virus, virus transforms the cell by integration of viral genome into the host genome. Introduced viral oncogenes modify cellular gene expression patterns to suit the proliferation and spread of the virus. In the process, a number of host protective mechanisms are affected which may lead to cancers.

Recent studies show that in the case of most virus-induced cancers, infection by a single virus *per se* is not sufficient for induction of the cancer but requires promoting and interacting effects of either other viruses or factors related to environment, genetic susceptibility or habits (Danaei et al., 2005). For example, HIV-HBV and HIV-HCV co-infection cause liver cancer development (Hu and Ludgate, 2007). Besides this, it has also been found that EBV and *Plasmodium falciparum* co-infection can cause endemic Burkitt's Lymphoma (Moormann et al., 2011). HHV-8 and HIV co-infection were also implicated in the development of Kaposi's sarcoma (Pyakurel et al., 2007). In the case of EBV-associated cancers, it is clear that EBV infection alone is not sufficient to cause nasopharyngeal cancers (Chang and Adami, 2006). It is a known fact that more than 90% of the adults worldwide are infected

with EBV with a very small proportion of them developing NPC. This point towards the role of other factors in the causation of NPC. HPV has been reported to be a candidate with highest probability in this process at least in a proportion of NPCs.

This study mainly focuses on effect of Epstein-Barr virus (EBV), the virus known to be associated with Nasopharyngeal Carcinoma, the LMP-1 protein which is the major oncogenic protein of the EBV and co-infection by Human Papilloma Virus (HPV) in NPCs from Kelantan state.

1.2 Nasopharyngeal carcinoma

Nasopharyngeal carcinoma is a tumour that arises from the epithelial cells that covers the surface and line the nasopharynx. NPCs are classified into three subtypes by the World Health Organisation, i.e. Type I: Keratinizing squamous cell carcinoma, Type II: non-keratinizing carcinoma and lastly Type III: undifferentiated carcinoma.

Nasopharyngeal carcinoma occurs in all age groups, with increasing incidence after the age of 30 years. It is a rare tumour in most parts of the world (Table 1.1). However, it is endemic in parts of Asia, especially Southern China and South East Asia. In Peninsular Malaysia, it is the third most frequent male cancer found, accounting for up to 8.4% of total cancers (Figure 1.1). As per the National Cancer Registry data, the Chinese had the highest incidence with an age standardized incidence (ASR) of 10.9 in males and 3.5 in females, followed by the Malays and the Indians. In comparison with other countries, ASR of Malaysian Chinese males and females ranked second after Hong Kong (Omar and Tamin, 2011). The hospital record in USM Hospital shows the cancer to be also prevalent in individuals belonging to Malay and Indian ethnicity (unpublished data). The report on

investigations from Sarawak in Malaysia shows the incidence in the people belonging to the Bidayuh community to be the highest in the world (Devi et al., 2004).

Treatment of NPC includes chemotherapy and radiotherapy (Brennan, 2006). Although NPC is highly sensitive to radiotherapy with good control of the primary tumour, distant metastases are very frequent (Jereb et al., 1980), the reason for which is not yet clear. The high sensitivity of the tumour to radiation and the high rate of recurrence point towards defects in the DNA damage repair mechanisms of the host. Nasopharyngeal carcinoma has been generally associated with EBV (Macsween and Crawford, 2003). Several EBV proteins and also DNA have been detected in the NPC tissues and serum of patients (Wolf et al., 1973, Krishna et al., 2004, Yap et al., 2007) which point towards the strong association between NPC and EBV infection. Majority of the undifferentiated type of NPCs are reported positive for EBV, while a number of reports show some of the lesions to be positive for Human papillomavirus (HPV) (Lo et al., 2010). Other than EBV infection, an increased susceptibility for NPC is also reported to be brought about by multiple factors such as ethnicity and environmental factors (Bailey et al., 2006).

Table 1.1: Global incidence rate of nasopharyngeal carcinoma (Chang and Adami, 2006)

Region and population (if applicable)	Years	Incidence rate (per 100,000 person-years)*	
		Males	Females
China and East Asia			
China, Hong Kong	1993-1997	21.4	8.3
China, Taiwan	1997	8.9	3.4
China, Shanghai	1993-1997	4.2	1.5
China, Tianjin	1993-1997	1.7	0.5
China, Beijing	1993-1997	1.0	0.6
Japan, Osaka Prefecture	1993-1997	0.5	0.1
Korea, Seoul	1993-1997	1.0	0.3
Southeast Asia			
Singapore, Chinese†	1998-2002	12.5	4.2
Singapore, Malay†	1998-2002	5.7	2.0
Singapore, Indian†	1998-2002	1.5	0.1
Malaysia, Sarawak Bidayuh (native)‡	1996-1998	31.5	11.8
Malaysia, Sarawak Chinese‡	1996-1998	12.0	4.1
Malaysia, Sarawak Malay‡	1996-1998	7.8	1.9
Viet Nam, Hanoi	1993-1997	10.4	4.6
Viet Nam, Ho Chi Minh City	1995-1998	4.8	1.7
Thailand, Bangkok	1995-1997	4.5	1.6
Philippines, Manila	1993-1997	7.2	2.5
Arctic			
Canada, Northwest Territories	1983-1997	9.2	6.0
Greenland, native§	1992-2002	12.7	9.2
United States, Alaska native‖	1992-2002	7.8	2.4
Middle East/North Africa			
Algeria, Algiers	1993-1997	2.7	1.3
Israel, Jews born in Africa or Asia	1993-1997	1.4	1.9
Israel, non-Jews	1993-1997	1.0	0.5
Kuwait, Kuwaitis	1994-1997	2.6	0.9
Kuwait, non-Kuwaitis	1994-1997	0.5	0.4
North America			
Canada	1993-1997	0.8	0.3
United States, White‖	1998-2002	0.4	0.2
United States, Black‖	1998-2002	0.8	0.3
United States, Hawaii Chinese	1993-1997	10.7	3.8
United States, Hawaii Filipino	1993-1997	3.5	1.5
United States, Hawaii native	1993-1997	3.6	0.9
United States, Los Angeles Chinese	1993-1997	7.6	2.4
United States, Los Angeles Filipino	1993-1997	3.7	1.6

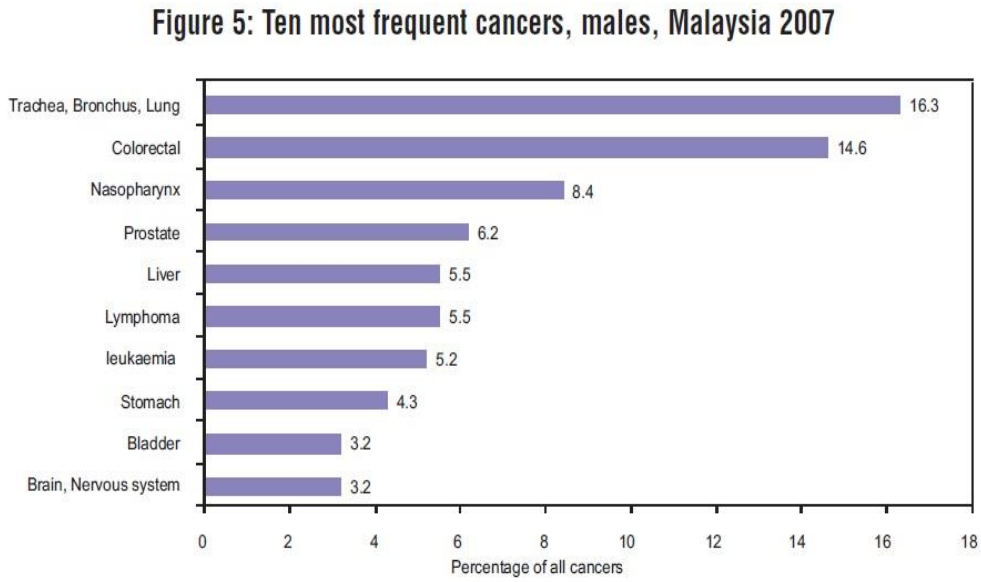

Figure 1.1: Ten most frequent cancers in males, peninsular Malaysia 2007 (Omar and Tamin, 2011)

1.2.1 Histopathology

Histologically, NPC has been classified into three types.

- Keratinizing squamous cell carcinoma (KSCC)
- Non-keratinizing squamous cell carcinoma (NKSCC)
- Undifferentiated carcinoma (UDC)

Of the three varieties, UDC is the most common one. It consists of uniform cells with a poorly defined cytoplasmic border that gives the tumour a syncytial appearance. The nuclei are pale and rounded in shape, with a distinct nuclear edge and multiple prominent eosinophilic nucleoli. Tumour cells generally form trabeculae or solid sheets, and mitosis is common. Small lymphocytes which are usually seen between tumour cells, resulted in the name lymphoepithelioma or ‘schminke tumours’ as a synonym for NPC in the past. Epithelial origin of the tumour has been confirmed by the observation of desmosomes by electron microscopy and cytokeratin positivity by immunohistochemistry, enabling to distinguish NPC from malignant lymphoma. Keratinizing and non-keratinizing forms of NPC have a more conventional appearance, with intercellular bridges/prickles and a squamous epithelial pattern with flattened cells superficially or keratin pearls (Ramsay, 1992).

1.2.2 WHO classification

NPC has been classified by World Health Organization (WHO) into three categories based on the histopathological classification.

- WHO type I – Keratinizing squamous cell carcinoma with various degrees of differentiation and similar to carcinoma that derived from other sites of the head and neck.
- WHO type II – Non Keratinizing epidermoid carcinoma
- WHO type III – Undifferentiated carcinoma or lymphoepithelioma. It has tumour infiltration with T lymphocytes which themselves are not malignant. It is also the most common form.

WHO type II and type III are frequently grouped as Undifferentiated Carcinoma Type (UNCT) (Wenig, 1999). The histological type may be of prognostic significance with UNCT having a higher local control rate after treatment with radiotherapy (RT) than KSCC. UNCT is also shown to fail more distally than locally.

1.2.3 Spread of tumour

NPC can remain asymptomatic and undiagnosed for a long time due to its inaccessibility. It has a marked potential for invasion and metastasis, and hence most patients present with locally or regionally advanced disease. Major adverse prognostic factors of NPC are age older than 40 years at the time of diagnosis, advanced TNM stage, skull based invasion and facial bone invasion (Farias et al., 2003). NPC is often submucosal. It can spread submucosally without marked projection into the nasal cavity. NPC has a predictable and orderly development of cervical metastasis. Bilateral lymphatic spread is usually very common as

nasopharynx is a midline structure. Systemic spreading occurs by blood stream. The most common site of spread is lung, followed by bone. Bulky primary tumour is more prone to metastasis and nodal status is used as a predictive marker of eventual development of distant metastasis.

1.2.4 Staging

Clinical staging of NPC begins with physical examination such as evaluation by nasopharyngoscopy. Radiographic assessment is also carried out to recognize the extent of primary and lymph node involvement. Magnetic resonance imaging (MRI) is used to find out invasion of muscles, nerves or intracranial extension. The International Union Against Cancer/American Joint Committee on Cancer Nasopharynx Staging System uses the primary tumour (T1-T4), lymph nodes (N0-N3), and systemic metastasis (M0-M1) classification which is widely used in most countries (Table 1.2) (Liu et al., 2008).

Table 1.2: Staging grouping (UICC – 1997)

Stage	Primary tumour	Lymph node	Systemic metastasis
0	TIS	N0	M0
I	T1	N0	M0
IIA	T2a	N0	M0
IIB	T1	N1	M0
	T2a	N1	M0
	T2b	N0,N1	M0
III	T1	N2	M0
	T2a, T2b	N2	M0
	T3	N0-2	M0
	T3	N0-2	M0
IVA	T4	N0-2	M0
IVB	Any T	N3	M0
IVC	Any T	Any N	M1

1.2.5 Metastasis

Metastasis is one of the primary features of malignant tumours. It is governed by a group of proteinases that degrade the extra cellular component. In metastasis, tumour cells first leave the primary site and invade the local host tissue followed by entry into the circulation and arrest at a distant vascular bed. Then, extravasation of the tumour cells into the target organ and proliferation as a secondary colony takes place. Invasive potential of tumour has important prognostic significance.

It has been suggested that EBV induces metastatic factors in NPC via the LMP-1. LMP-1 was shown to directly contribute to metastasis (Horikawa et al., 2007). Studies have reported that the presence of EBV DNA in the peripheral blood of NPC patients indicate higher risk of developing distant metastasis as well as a lower survival rate (Lin et al., 2001). Other viruses have also been reported to induce metastatic potential. An example in this context is that of HPV. Besides this, HPV was also identified in tonsillar squamous cell carcinoma with regional metastases (Strome et al., 2002).

1.2.6 Recurrence

Early stage NPC is highly curable with radiotherapy. However, most of the patients with NPC have late stage disease at presentation (Abdullah et al., 2009). A high incidence of loco-regional recurrences has been reported in NPC. Majority of the loco-regional recurrences develop within 3 years after radiation therapy outside or at the margin of the treatment portal. However, recurrences have also been observed after a long latent period (Li et al., 2012). Distant metastases also frequently occur within 3 years after treatment. The most common site for distant metastases is the

bones, liver or the lungs (Lu et al., 2009). Frequent follow up examination is important because some of the loco-regional and distance recurrences can also appear between 6 and 10 years (Nozaki et al., 1998). It is hypothesized that the high incidences of late recurrence in NPCs could be due to a defective DNA repair mechanism in the host. High levels of abnormal DNA repair function in the cells could increase the resistance of the cells to treatment and also DNA repair-mediated genomic and chromosomal instability. Studies have shown close associations between members of DNA repair proteins and recurrence both in NPCs and other cancers (Wang et al., 2010, Silva et al., 2010, Goode et al., 2002).

1.2.7 Treatment

Radiotherapy (RT) is the mainstay of treatment and it may or may not be followed with Chemotherapy (CT). Five years overall survival rates following radiotherapy are stage dependent and it can range from 70% to 80% for stage I, and 20% to 40% for stage IV. Most of the NPC recurrences are observed in the first 2 years after therapy and it can manifest loco-regionally or systemically or both (Yamazaki et al., 2011).

1.2.8 Influence of environmental factors in nasopharyngeal carcinoma

NPC presents with interesting epidemiologic and biologic characteristics. NPC is endemic in particular populations in specific geographic regions. The endemic areas include southern parts of China and South-East Asia. NPCs that occur in endemic areas are usually of WHO Type II or III. In United States of America and Western Europe, it often occurs as WHO Type I and is mostly related to exposure to the

typical head and neck cancer risk factors, such as the exposure to alcohol and tobacco. Epidemiological studies have implicated the nasopharynx as a tobacco susceptible cancer site. It is also thought that NPC can be influenced by diet. Cantonese-style salted fish which is a common food in local diet is thought to be an etiological factor for NPC. Several case control studies conducted in diverse populations in different parts of Asia and Europe have confirmed the link between diet and NPC (Polesel et al., 2012, Yu et al., 1989). A direct link between the carcinogenic substances such as nitrosamines, genotoxic and EBV activating substances in preserved food such as salted fish and the occurrence of NPC has also been reported in a number of studies in humans and cell cultures (Shao et al., 1988, Ward et al., 2000, Yu et al., 1986).

1.3 Epstein-Barr virus

Epstein-Barr virus (EBV), also known as Human herpesvirus 4 belongs to Herpesviridae family of virus which is the primary cause of infectious mononucleosis. It is the first human cancer-causing virus discovered by Anthony Epstein, Bert Achong and Yvonne Barr from Burkitt's lymphoma cells nearly 50 years ago (Epstein et al., 1964). EBV is one of the most common viruses which infects human, often after initial asymptomatic subclinical infection (Pathmanathan, 1993). Ninety five percent of adults between 35 and 40 years of age world-wide are infected by EBV (Chang and Adami, 2006). Primary EBV infections in third world countries typically occur in early childhood and are sub-clinical. In Hong Kong, nearly 80% of the children are EBV positive by age 10 and 100% by adulthood (Humans, 2004). The virus remains latent for a long time and is associated with development of several cancers including NPCs later in life. Transmission is mainly

through saliva in the developing countries where there is crowding and lack of hygiene. On the contrary, in more developed countries, primary infections occur at a later age and may present as acute infectious mononucleosis (CDC, 2006). The infected B cells remain in the circulatory system avoiding detection by both cellular and humoral immune system (Kurth et al., 2000). The infected B cells do not differentiate further: it remains in an intermediate (proliferative) stage without differentiating into antibody producing plasma cells. However, it is believed that infected B cells are responsible for spreading EBV infection to tissues and organs of the body. IgG and IgM antibodies, appear early in response to the viral capsid antigen (VCA) followed by antibodies to early antigen (EA) that appear three or four weeks after the onset of the illness and finally against the Epstein-Barr Nuclear Antigen (EBNA) component of the virus (Henie et al., 1974).

EBV was thought to be evolved from non-human primate origin. It has similar sequences and genetic organization with EBV-like viruses of chimpanzees and rhesus monkeys, and they infect only B cells and epithelial cells of primate origin (Cohen, 2000). The route of entry of EBV into epithelial cells still remains vague. EBV first binds the viral envelop glycoprotein gp350/220 to the cellular complement receptor CD21 or CR2. The viral protein gp42 binds to a HLA class II co-receptor on the host cell. Cell to cell contact is thought to be the mechanism for the infection of epithelial cells by EBV (Knox and Young, 1995). EBV is believed to initiate the infection in human epithelial cells of the oropharynx which is suitable for viral replication. After the primary infection, EBV can diffuse across the basal membrane and cause latent infection in B lymphocytes. B lymphocytes migrating through the throat are latently infected and are thought to be the source of continued virus spread due to low level of spontaneous virus replication in B cells, leading to reinfection of

epithelial cells. This infection is the central cause of infection in distal normal epithelial surfaces such as nasopharynx and frequent shedding into the saliva. This persistent and active lytic infection can remain for many years (Thompson and Kurzrock, 2004).

EBV positivity has been frequently reported in normal nasal mucosa. Chen et al. detected 8.4% EBV positivity in epithelia of non-tumour nasopharyngeal biopsies (Chen et al., 1996). It was found that nasal polyps, the etiology of which is not very clear, carry EBV DNA in 69- 85% of the cases (Tao et al., 1996). EBV has also been detected by PCR in 80% of normal nasopharyngeal tissue in asymptomatic Chinese individuals, 8.3% of normal oral mucosa and 77.8% of oral premalignant lesions (Tao et al., 1995, Cheung et al., 1993). In the humans, EBV is capable of infecting B lymphocytes, squamous epithelial cells, glandular epithelial cells, myoepithelial cells, smooth muscle cells, T cells, NK cells, plasma cells, and follicular dendritic cells (Gulley and Tang, 2008). EBV can often be cultured from the pharynx of most asymptomatic individuals. The presence of replicating virus in hairy cells of oral leukoplakia, a benign disease of the oral cavity, usually found in HIV patients and in NPC, coupled with the productive infection of epithelial cells in culture, suggest that epithelial cells could have an important role to play in EBV infection (Faulkner et al., 2000). Healthy adults, however, harbour the virus in the B cells and usually do not produce the oncogenic proteins. It should be noted that PCR analysis does not discriminate between the tissue types and hence the EBV positivity remains high in normal tissues even when the EBV infection is limited to the lymphoid cells.

1.3.1 EBV genome

EBV is a double stranded DNA virus. EBV genome consists of 172 kbp linear DNA molecules which consist of 0.5 kbp terminal repeats and numerous internal repeats separated by unique areas. EBV executes two major programs which are lytic cycle or latent cycle, each with its own objectives. In lytic cycle the objective is to produce infectious virions, while in latent cycle it does not result in production of virus particles. Around 100 viral proteins are expressed during lytic cycle but only a limited and distinct set of viral proteins are expressed in the latent cycle which include Epstein-Barr nuclear antigen (EBNA)-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-leader protein (EBNA-LP) and latent membrane proteins (LMP)-1, LMP-2A and LMP-2B and the Epstein-Barr encoded RNAs (EBERs) (Kieff and Rickinson, 2007). EBV genome is also found to code for more than twenty microRNAs (Grundhoff et al., 2006). There are at least three programs present in a latent cycle; Latency I, II and III, as reported in Burkitt's lymphoma cell lines (Calderwood et al., 2007b).

Table 1.3: EBV proteins expressed in latent cycles

Programs	Viral proteins expressed
Latency I	EBER1&2 EBNA1
Latency II	EBER1&2 LMP2A LMP2B EBNA1 LMP1
Latency III	EBER1&2 LMP2A LMP2B EBNA1 LMP1 EBNA2,3,4,5,6

1.3.2 NPC and Epstein-Barr Virus

EBV has been closely associated with human cancers, including Burkitt's lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma and nasopharyngeal carcinoma.

Epstein-Barr Virus (EBV) belongs to the family herpesviridae and was the first virus identified to be associated with human cancers. Epstein-Barr Virus is consistently detected in nasopharyngeal carcinoma (Niedobitek, 2000), especially in the WHO type III non-keratinizing undifferentiated type. Elevation of serum IgA to EBV virus capsid antigen (VCA) is often detected before the development of NPC by several years, suggesting that EBV reactivation and replication occurs before the beginning of NPC development (Ji et al., 2007). EBV establishes latent infection in its host and persists lifelong and the proteins produced by these viruses affect the host protein functioning. As much as 173 interactions between EBV and human proteins have been identified (Calderwood et al., 2007a).

EBV viral genome is clonal in dysplastic cells as demonstrated by homogenous number of terminal repeat elements and cells in carcinoma *in situ*, demonstrating that the tumour originated from a single infected cell (Raab-Traub and Flynn, 1986). It also suggests that EBV infection is an early event in the multi-step progression of NPC. The consistent presence of EBV in pre-invasive and tumour cells of EBV associated cancers, especially in the case of NPC, makes it useful as a marker for NPC and also for detecting NPC risk in high risk individuals (Yu et al., 2011). An increase in EBV specific antibody titre after therapy for NPC is associated with a poor prognosis and a declining or constant level of antibody is associated with a better prognosis. Undifferentiated NPC is most consistently associated with EBV regardless of its geographical origin despite the fact that EBV infection is ubiquitous.

While EBV is believed to be involved in the development of NPC, its infection alone is inadequate to start the tumorigenesis of NPC.

EBV is known as the etiologic agent of acute infectious mononucleosis, and it is strongly associated with Burkitt's lymphoma and undifferentiated NPC. EBV is also associated with other diseases such as EBV associated hemophagocytic syndrome, chronic active EBV infection, T-cell lymphoma, natural killer cell lymphoma, lymphoproliferative disease in immune-compromised hosts, Hodgkin's disease, pyothorax-associated B-cell lymphoma and smooth muscle tumours and in other cancers such as gastric carcinoma and primary invasive ductal breast cancer. The detection of EBV viral DNA and also its nuclear antigen in tumours, especially in nasopharyngeal carcinoma, has revealed that EBV can infect epithelial cells and could contribute with the transformation to cancer (Thompson and Kurzrock, 2004). EBV clonal genome is evident by the homogenous number of terminal repeat elements, and it is found in cells in pre-invasive lesions suggesting that it is directly involved in the process of cell transformation (Neri et al., 1991).

1.4 Human Papilloma Virus

Human Papilloma Virus is a double stranded DNA virus from the family of Papillomaviridae. It has been associated with a number of diseases and conditions such as skin warts, genital warts and also cancers such as those of the uterine cervix, oral cavity, tonsil, and penis. There are more than 100 types of HPV that have been identified based on their genomic sequences (Morbini et al., 2012). HPV infect and replicate only in the basal cells of stratified epithelium (Schiller et al., 2010).

In benign lesions, HPV present as a multiple copy episome. While in the malignant lesions, the HPV DNA is integrated into the host genome. The papilloma virus life cycle differs from other virus in such a way that they are the only viruses that begin their infectious process at an extracellular site. First, the virus binds its L1 major capsid protein to heparan sulfate proteoglycans (HSPGs) on segments of the basement membrane (BM) that are exposed after epithelial trauma. The conformational change exposes the N-terminus of L2 minor capsid protein to furin cleavage. L2 proteolysis reveals a previously obstructed surface of L1. It binds to an unknown cell surface receptor on keratinocytes that have migrated over the basement membrane to close the wound (Schiller et al., 2010). The whole infectious process is slow and takes up to 12 – 24 hours before the start of transcription. The infection requires the availability of epidermal or mucosal epithelial cells that are still able to proliferate (basal layer cells) (Hoffmann et al., 2006). Specific early genes such as E5, E6 and E7 are expressed resulting in heightened proliferation and the lateral expansion of the infected cells (Boulenouar et al., 2010). Late viral gene expression is initiated following the entry into the suprabasal layers. Viral genome is then replicated followed by production of structural proteins. Complete viral particles are assembled and released in the upper layers of the epidermis or mucosa (Figure 1.2). HPV persist in the basal cells for long periods. It does not cause cells in the basal layer to lyse since the production of the virus is restricted to the suprabasal cells.

Humans are the only known reservoirs of HPV. Viral transmission occurs through direct physical contact or by contaminated surfaces. HPV only replicates in terminally differentiated epithelial cells and diagnosis of infection through tissue culture has not been possible (CDC, 2012). Both normal epithelia, epithelia in benign lesions and cancer lesions have been reported to be infected by HPV.

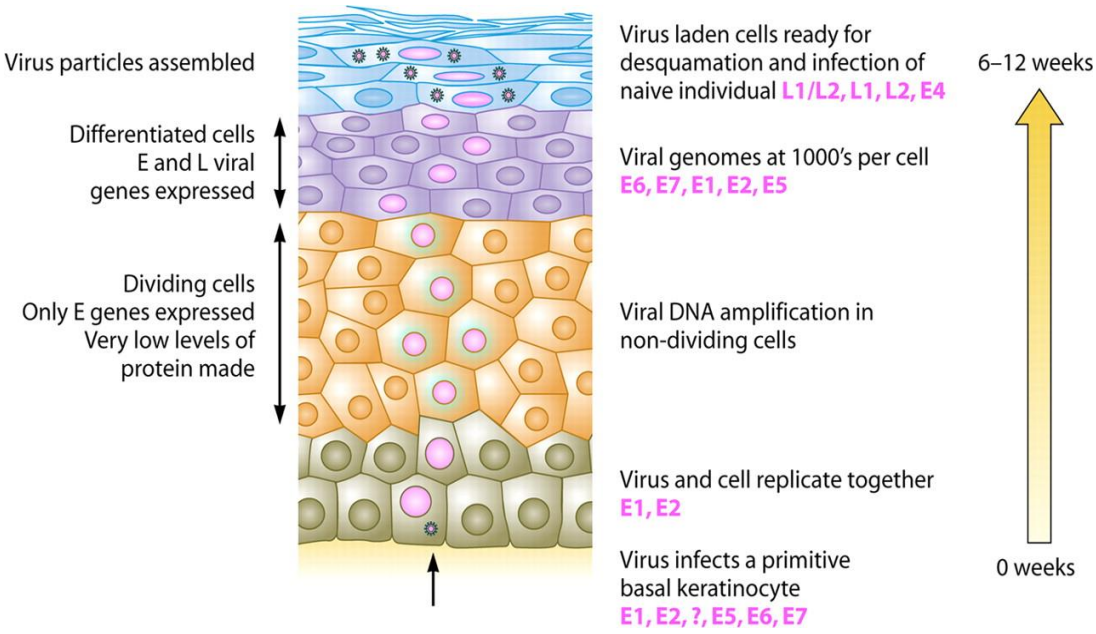

Figure 1.2: HPV life cycle (Stanley, 2012)

1.4.1 HPV genome

HPVs are double stranded, circular DNA viruses that exist as a nuclear episome in infected cells. It is classified into subgroups based on the infection sites - cutaneous or mucosal epithelia. Mucosal HPVs are further classified into high risk (HPV-16, -18, -31, -45), intermediate risk (HPV-33, -35, -39 and others) and low risk (HPV-6, -11) types. It is classified based on their epidemiologic association with cancer and benign epithelial hyper-proliferation (zur Hausen, 1996). HPV genome consists of 7900 bp of double stranded circular DNA and it is divided into eight open reading frames, i.e. E6, E7, E1, E2, E4, E5 and L2 and L1 - coding for early (E) or late (L) functions (Ault, 2006). HPV integrates its genome into host cell DNA in the course of cancer development. The ring molecules are most often opened within the E2

open reading frame, disturbing the gene continuity. Some part of open reading frame are regularly deleted after integration, i.e. part of E2 and open reading frames that are adjacent to E2 - E4, E5 and part of L2.

1.4.2 Role of HPV in cancer

HPV infections have been associated with more than 5% of cancers. HPV has been associated with predominantly the cancer of uterine cervix, carcinoma of the anogenital tract, vagina, penis, and anus and some head and neck cancers such as those of oropharynx and nasopharynx. It is estimated that HPV in HNSCC range from 25 - 70% or more with different rates for different anatomic regions of the head and neck and different geographic regions (Thibaudeau et al., 2013). For example, its incidence in laryngeal carcinomas ranges from 4-20% and 100% in verrucous laryngeal carcinomas (Gillison et al., 1999, McKaig et al., 1998). Its prevalence rate in oral cavity is estimated to range between 35-80%. HPV associated squamous cell carcinoma has been identified in 80% cases of oral leukoplakia and 46% of cases of carcinomas of tongue and floor of mouth (Maitland et al., 1987, Betiol et al., 2012). HPV positivity has also been reported in carcinomas of the sinonasal tract. Bishop et al. reports an incidence of 21% positivity in sinonasal carcinomas (Bishop et al., 2013). In Malaysia, the study carried out in state of Kelantan showed 51.4% HPV positivity among oral cancers (Saini et al., 2011).

In the case of cervical cancer, the most frequently associated are HPV 16 and 18 types. In peninsular Malaysia, cervix cancer burden is reported to be 12.2 cases/100,000 populations. The incidence of this cancer has been reported to be higher in the Malay population (49.8%) followed by the Chinese (34.2%) and

Indians (11.3%) (Aljunid et al., 2010). The HPV infection rate was reported to be 100% in cervix cancer lesions and 92% in intraepithelial lesions in women from areas serviced by the Universiti Kebangsaan Malaysia Medical Centre in Kuala Lumpur (Sharifah et al., 2009). The prevalence of HPV infection in the 10 normal smears was not reported in the study by Sharifah et al. Wong and Sam reported from Women in Selangor, Malaysia an incidence of 46.7% in non-cancerous women pointing towards a high incidence of HPV in Malaysian individuals. However, the results are not reflective of Malaysia as a country due to the lack of awareness, resources and infrastructure especially in rural areas which has led to low rates of screening. The estimated screening rate was only about 6% of women in Malaysia (Wong and Sam, 2007). Resistance towards Pap tests has been contributed by the health policies, cultural inhibitions and misperceptions regarding the disease as well as the screening procedure (Wong and Sam, 2007).

HPV infection is also associated with benign lesions and normal epithelium in the upper airway, including focal epithelial hyperplasia, inverted nasal papillomas and onset benign respiratory papillomatosis. The sharing of similar non-keratinising characteristics with the cervix epithelia is thought to be a factor for attracting HPVs to the epithelia in the head and neck region, larynx and some other organs (Franceschi et al., 2011). Gonzales et al. have reported HPV infection as high as 91% in HPV associated oral benign lesions with Duray et al. from Belgium reporting 77% of the laryngeal benign lesions to be positive for HPV (Gonzalez et al., 2007, Duray et al., 2011). The HPV incidence rate in adenoid lesions from Turkey has been reported to be about 6% (BALOĞLU et al., 2010). The two Malaysian studies reporting the HPV prevalence in 105 normal oral epithelium from the state of Kelantan (Saini et al., 2010) and in non-cancerous smears from Selangor (Chong et

al., 2010) of 24.8% and 46.7% respectively point towards the high HPV prevalence rate in the Malaysian population. The global incidence of HPV is estimated to be about 10% in the normal population (Bruni et al., 2010).

Table 1.4: Detection of HPV in normal oral lesions

Author & Year	Number of normal cases	% HPV +ve
Woods et al. (1993)	9	67.00%
Nagpal et al. (2002)	26	26.90%
Chen et al. (2002)	29	37.90%
Zhang et al. (2004)	40	55.00%
Terai et al. (1999)	37	81.10%
Sanchez-Vargas et al. (2010)	46	72.00%
Tominaga et al. (1996)	3	100.00%

1.5 Viral co-infection in cancer lesions

Viral co-infection is a state where two or more types of viral particles simultaneously infect a single cell. Viral co-infection has been a field of interest due to the complication it can bring to the patient disease state. This can be shown in the case of various viral co-infection such as HIV (Human Immunodeficiency Virus) and Hepatitis C virus which can lead to the higher risk of development of cancer in the liver (Merchante et al., 2013). There have been studies which showed EBV and HPV co-infection in various diseases. Study in anogenital warts showed that HPV DNA sequences were detected more often in HIV positive patients. EBV infection was also

observed, however no association was found with other viruses (Bernard et al., 1992). Bernard et al suggested that HPV co-infection may be associated with the neoplastic transformation of anogenital lesions. HPV and EBV co-infection was also found in HIV infected and also renal transplant oral healthy mucosa (Ammatuna et al., 2001). It was suggested that HPV and EBV co-infection may increase the risk for development of oral premalignant and malignant lesions. Besides viruses, EBV was also found to co-infect with bacteria such as *Helicobacter pylori* to cause severe gastritis in paediatric patients, which may increase the risk of the development of more serious lesions in later life (Cardenas-Mondragon et al., 2013). The importance of viral co-infection in cancer development prompted us to study the two main viruses which are thought to be involved in NPC i.e. EBV and HPV. EBV and HPV co-infection in NPC have been reported in various studies around the world (Laantri et al., 2011, Maxwell et al., 2010, Punwaney et al., 1999, Dogan et al., 2013). However, no reports are available on HPV and its co-infection in Malaysian NPC.

1.6 EBV LMP-1

EBV latent membrane protein 1 (LMP-1) is a viral protein associated with Epstein-Barr virus, abundantly expressed in EBV infected cells and functions as a key protein in B cell transformation and immortalization (Cahir McFarland et al., 1999). EBV mediated B-cell proliferation is also dependent on this particular protein by the activation of CD40 receptor. (Kilger et al., 1998). LMP-1 is a 66kDA integral membrane protein. It is comprised of a short amino acid cytoplasmic N-terminus (amino acids 1-23), six hydrophobic alpha-helical transmembrane spanning regions (amino acids 24–186) and a large 200 amino acid cytoplasmic C-terminal tail (amino acids 187–386) (Dawson et al., 2012).