Chapter 1

Introduction
1. General characteristics of nasopharyngeal carcinoma (NPC)

1.1. Anatomy and histopathology

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells covering the nasopharyngeal surface, most often within the lateral nasopharyngeal recess or fossa Rosenmüller. It differs significantly from other cancers of the head and neck area in its occurrence, causes, clinical behavior and treatment. The histological classification of NPC proposed by World Health Organization (WHO) categorized tumors into three histopathological types based on degree of differentiation. It includes keratinizing squamous cell carcinoma (WHO type I) which is highly differentiated and characterized by epithelial growth patterns and keratin filaments, non-keratinizing squamous cell carcinoma (WHO type II) with retaining epithelial cell shape and growth pattern and undifferentiated carcinoma (WHO type III) which does not produce keratin and lack of distinctive growth pattern. There is a different prevalence of histological subtypes of NPC in non-endemic and endemic regions. In the non-endemic (western) countries such as United States (US) and Europe (EU), WHO type I NPC comprised 75% of the cases and were found most often in non-Hispanic whites. However, there are increasing data at present showing that non-keratinizing tumor dominate the NPC incidence in Europe. (Wildeman et al, manuscript in preparation) In endemic areas such as Southern China (and South East Asia), WHO type III NPC accounts for more than 97% of the all NPC cases. In our center (Dr Sardjito Hospital, Yogyakarta) WHO type III is presented by 90% of NPC patients coming during year 2006-2010. (Hariwiyanto 2011, unpublished data)

1.2. Epidemiology

NPC has a remarkable geographical and racial incidence. For most countries throughout the world it represents an uncommon malignancy. In the western world its standard age-adjusted incidence for both male and female is less than 1/100,000 population. Intermediate incidences are observed in several populations including Southeast Asian countries (such as Malaysia, Thailand, Vietnam and Indonesia) and in natives of the Arctic region, Northern Africa and the Middle East, with annual incidence of 5-7/100,000 population. In contrast, it is extremely frequent in Southern China with annual incidence of around 20/100,000. Regional hot spots exist in Southeast Asia outside China with high NPC incidence rates, even exceeding 30/100,000 in a native population in Serawak. Although geographic areas have generally been categorized as high-, intermediate- and low-incidence regions, the racial distribution of NPC within regions is not identical. In the Southern China, highest rates are noted as being twice as high among the Cantonese speaking groups who inhabit the central region of Guangdong, of which Hong Kong is a part, compared to those in other regional language groups. In the US, the highest NPC rate is found among Chinese Americans, followed by Filipino Americans, then
Japanese Americans, Blacks, Hispanics and finally whites. However, the incidence rate among Chinese people born in North America is lower than those born in Southern China, but remains higher than the local inhabitants, indicating a role of exogenous factors in NPC pathogenesis. In Southeast Asia, it seems that NPC risk varies with degree of racial and social admixture with Southern Chinese.

In almost all populations, the incidence of NPC is 2-3 fold higher in males than in females. In most low-risk populations, NPC incidence increases with increased age. In China the peak incidence is reached towards the end of the fourth decade, after which there is no further rise. In high risk populations the incidence reaches the highest rate around 50-59 years and declines thereafter. This difference suggests the involvement of exposure to carcinogenic agents early in life in low risk populations. In Mediterranean populations NPC shows a bimodal age distribution with about 20% juvenile cases. In Indonesia, this bimodal distribution does not occur, but a linear increase in incidence is observed starting at 4-5 years of age and peaking around the 4th-5th decade in life. (Adham et al., Chinese J Cancer 2012, accepted) The age-related incidence curve of NPC is unusual as in most other head and neck cancers there is a continuous rise in incidence with increasing age.

1.3. Clinical presentations, diagnosis and prognosis

Patients with NPC often present with a spectrum of symptoms. Patients may present with epistaxis, nasal obstruction and discharge because of the tumor presence in the nasopharynx. The tumor may cause dysfunction of the Eustachian tube which is associated with lateroposterior extension of the tumor mass to paranasopharyngeal space. In this situation patients may present with tinnitus and deafness (middle ear obstruction), by the effects of local invasion or locoregional involvement. Headache, diplopia, facial pain and numbness may present when tumor extends superiorly, invades into skull base and cause palsy of the fifth and sixth cranial nerves. Neck mass can be the first symptom. Lee at al. (1997) summarized the symptoms at presentation as neck mass (76%) which relates to regional metastasis, nasal dysfunction (73%), aural dysfunction (62%), headache (35%), diplopia (11%), facial numbness (8%), weight loss (7%) and trismus (3%). The most common presenting symptom at diagnosis is cervical lymphadenopathy, followed by nasal, aural and neurological symptoms. Similar symptoms were found when comparing young and adults NPC subjects. However, NPC is one of the most difficult diseases to diagnose at an early stage due to few and non-specific early signs and hidden location of the nasopharynx. Early symptoms may frequently not be recognized by the patients and doctors.

Clinical investigation includes endoscopy to assess the primary tumor and provide information on mucosal involvement and tumor extension into nasal fossae and oropharynx. Computed tomography (CT)-scan and magnetic resonance imaging (MRI) are necessary to assess the extension of the primary tumor and lymph node involvement. MRI is more sensitive than CT-scan, with regards to soft
tissue extension, regional nodal metastasis and bone marrow infiltration. Although MRI can clearly assess blood vessels and base of skull erosion, CT-scan is considered a better tool for exploration through the cranial base and defining bone erosion. Examination for distant metastasis is done by chest rontgenography, total bone scan, liver ultrasonography and currently positron emission tomography (PET) scanning.\textsuperscript{21,22} The golden standard for NPC biopsy is histological examination. Alternative procedures including molecular analysis of nasopharyngeal brushings are proposed.\textsuperscript{23}

Figure 1. Clinical manifestation of NPC.
In a clockwise rotation, an anatomy picture of nasopharynx and the surrounding regions, an NPC patient showing massive neck lymph node and necrotic tissue, an endoscopy examination showing a bloody lumpy mass on nasopharyngeal region and a CT-scan result with appearance of nasopharyngeal mass. (courtesy Herdini,C.)

Prognosis of NPC is related to the disease extent as measured by UICC staging system. The prognosis of an NPC patient is strongly influenced by tumor stage at diagnosis with 90% of 5-year overall survival at stage I and 50-70% at stage III-
The American Joint Committee on Cancer (AJCC) prognostic categories help to determine appropriate treatment methods. Factors that may influence prognosis include age, gender, presence of keratinization and lymph node metastasis. Better prognosis is seen in younger patients and female gender. Epidemiologic studies have identified WHO histological type as an independent prognostic factor. Type II and III tumors are more often controlled by radiation than type I tumor associated with significantly better 5-year overall survival rate.

1.4. Risk factors of NPC

Epidemiological studies suggested a multifactorial etiology of NPC involving ethnic, genetic susceptibility and environmental factors. Chinese ethnicity is thought to be a significant susceptibility for NPC. Chinese communities, especially living in the southern part of China are known as the highest NPC incidence population in the world. In the low-incidence area of USA, NPC was highest among Chinese residents, followed distantly by other ethnicity from other countries of Asia and Africa. In South East Asia, NPC is also more common in Thai, Macaonese and Malay indigenous populations which have an intermarriage history with people from Chinese origins. Thus high NPC incidence seems retained by Chinese offsprings who migrated to other non-endemic countries. However, in Indonesia NPC presents commonly among local people without apparent Chinese prevalence. (Adham et al., Chinese J Cancer 2012, accepted; Hariwiyanto, unpublished data) The NPC family clustering in endemic areas also suggests a strong ethnic and genetic influence. Not only documented in high-incidence, familial aggregation was also observed in low-incidence populations. Furthermore, more than 10% of NPC cases have a family history of the disease, making familial risk of NPC the highest compared to other cancers. In many epidemiology studies, the excess risk was generally 4- to 10-fold among individuals with a first-degree relative with NPC, compared with those without a family history. Familial NPC has also been linked to genetic predispositions such as human leukocyte antigen (HLA) genotypes and susceptibility loci on chromosome 3, 4 and 5. However, the diversity of NPC incidence even among Chinese populations in China, and the inconsistent relationship between genetic susceptibility and NPC suggest an environmental role in NPC development. In NPC genetic factors and environmental exposures probably play a combined role. In a complex segregation analysis of familial NPC in Southern China, multiple genetic and environmental factors, rather than a single major susceptibility gene, seemed most likely to explain the pattern of inheritance.

Among the environmental factors, much attention has been focused on a well-documented association with Epstein-Barr virus (EBV) infection (detailed in section 2). The involvement of EBV in NPC was firstly postulated in 1966 as a serological study observed that NPC patients showed antibodies against EBV. Subsequent observations demonstrated that NPC patients have elevated IgG and IgA antibody titers to the EBV viral capsid antigen (VCA), IgA against early antigen
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(EA) and increased IgG against the latent Epstein-Barr nuclear antigens 1 and 2 (EBNA1 and EBNA2) and EBV specific DNase.\textsuperscript{44-46} Furthermore, these antibody titers, especially of IgA, are found to precede tumor appearance by several years\textsuperscript{47, 48} and correlate with tumor burden, remission and recurrence.\textsuperscript{49-51} Recent studies have provided insight into the molecular basis and diversity of anti EBV IgA and IgG responses which allowed the development of more defined serological tools.\textsuperscript{52-56}

The link of EBV infection with NPC development was further confirmed by demonstration of EBV DNA, RNA and/or gene products in tumor cells of nearly all undifferentiated carcinoma (WHO type III) cases regardless of geographic origin.\textsuperscript{23,57,58} The other two types of NPC are less frequently related in non-endemic regions, with EBV positivity in less than 50\% of cases. However, in endemic NPC areas all the type I and II NPC tumors were EBV-associated.\textsuperscript{59,60} Virtually all NPC cases in Indonesia and other endemic regions, irrespective of WHO type, show active EBV infection in all tumor cells by distinct molecular staining techniques, as will be detailed later in this introduction.

In the familial context, an increased risk for EBV-associated NPC and other infectious agent-related cancers was recorded among families with a history of NPC especially the multiplex cases.\textsuperscript{61} Sufficient opportunity for EBV reactivation within family was also indicated from studies on IgA detection in the sera among members of the core family of NPC cases.\textsuperscript{62,63}

Given the universal prevalence of EBV infection but the unique geographic distribution of NPC, factors other than EBV infection are considered to be important determinants for NPC risk. Many non-viral carcinogenic agents are consistently associated with food.\textsuperscript{14} Cantonese-style salted fish has been observed in a large number of case-control studies in Cantonese, other Southern Chinese, Northern Chinese and Thais populations living in different regions of Asia and North America.\textsuperscript{64-68} The relative risk of NPC associated with weekly consumption ranged from 1.4-3.2 and with daily consumption ranged from 1.8-7.5 compared with no or rare consumption.\textsuperscript{64,66,67,69-71} Exposure at weaning associated to higher risk than exposure at adulthood.\textsuperscript{64,65,69,72} Experimental data on rats have verified the evidence of Cantonese-style salted fish as a human nasopharyngeal carcinogenic in a dose-dependent manner.\textsuperscript{73,74} The presence of carcinogenic nitrosamines, butyrate, direct genotoxins and EBV-activating substances were also observed in salt-preserved fish.\textsuperscript{75,76} Moreover, genes involved in DNA repair and nitrosamine metabolism are observed to be dysregulated in NPC.\textsuperscript{77} Other local preserved food are also common among the populations of intermediate incidence of NPC. In these populations childhood exposure was also significantly associated with the risk of NPC.\textsuperscript{78} Like salted fish, these preserved diet showed the presence of nitrosamines, genotoxic and EBV-activating substances.\textsuperscript{75,79} In low incidence region (US) the role of preserved meat which contains high level of added nitrite has also been reported to increase the risk among the frequent consumers.\textsuperscript{80}
1.5. Treatment of NPC

NPC is highly radiosensitive and radiotherapy remains the standard treatment for all stages of NPC without distant metastasis.\textsuperscript{81,82} Conventional two dimensional (2D) radiotherapy alone resulted in controlling T1 and T2 tumors in between 70-90\% of cases and T3 and T4 tumors in 50-75\% cases.\textsuperscript{81,83} Nodal control is achieved in 90\% of N0 and N1 cases and 70\% of N2 and N3 cases. A booster dose using intracavity brachytherapy improved tumor control by 16\% in T1 and T2 tumors.\textsuperscript{81} Stereotactic radiosurgery has also been performed\textsuperscript{84,85} for the booster dose and is shown better for the treatment of persistent and recurrent NPC cases because of the side effect associated with hypofractionated treatment.\textsuperscript{86} For treatment of local residual disease photodynamic therapy (PDT) can be used additionally.\textsuperscript{87,88} Intensity-modulated radiotherapy (IMRT) has been lately evolved as a new standard radiotherapy technique as it can overcome the limitations of 2D radiotherapy such as protection of adjacent radiosensitive organs while giving high dose to targets. IMRT can be used as preservation for the surrounding organs. In the case of extensive tumors it can achieve good dose differential between the tumor and the dose-limiting organs and therefore ensure high dose in tumor and reduce toxicity affecting the normal organs. With IMRT local tumor control is achieved in more than 90\% NPC cases,\textsuperscript{89,90} even among patients with advanced T3-4 disease.\textsuperscript{91}

The majority (75–90\%) of newly diagnosed NPC patients have loco-regionally advanced disease, commonly with cervical nodal metastasis.\textsuperscript{7} Because NPC is also chemosensitive, currently the standard of care for these patients consists of concurrent chemo-radiotherapy. This treatment approach results in cure for the vast majority of patients, with 5-year disease-free and overall survival rates of 81-90\% and 78-85\%, respectively (using either conventional radiotherapy or IMRT).\textsuperscript{92} Cisplatin is a standard chemotherapy agent of concurrent chemo-radiotherapy and also provides a benefit in locoregional and distant control. Adjuvant chemotherapy based on cisplatin and fluorouracil may be beneficial, although there is a little evidence of improved survival. In case of locoregional recurrence of NPC, a multidisciplinary approach may lead to the salvage of a subset of patients. For metastatic NPC, a first-line doublet chemotherapy (platinum based) achieves 50-80\% response rates with a median time to progression of 5-11 months. Other drugs such as fluorouracil, paclitaxel, docetaxel, gemcitabine, capecitabine, irinotecan, vinorelbine, ifosfamide, doxorubicin and oxaliplatin may be efficacious.\textsuperscript{93}

New therapies are being developed such as the ones targeting carcinoma-related epidermal growth factor receptor (EGFR) and EBV. The approach of adding EGFR-targeted therapy to conventional treatment approaches is being actively studied in locoregionally advanced NPC using Cetuximab and more recently developed analogues like Nimotuzumab.\textsuperscript{94} EBV-based immunotherapy against NPC cells aimed at boosting the levels of cytotoxicity T lymphocytes (CTLs) that recognize the subdominant viral antigens such as EBNA1, LMP1 and LMP2.\textsuperscript{95-99} Currently
several strategies on EBV lytic induction therapy are under investigation.\textsuperscript{100,101} (Tan et al. 2011, study in preparation)

2. Epstein-Barr virus (EBV) infection

2.1. General features

EBV is a gamma herpes virus which infects more than 90% of the adult population in the world.\textsuperscript{102} Humans are the only natural host for EBV where the virus transmits via saliva.\textsuperscript{103} Primary infection normally takes place in early childhood and remains clinically unapparent in most developing countries. The high rate of childhood infection probably reaches 100% in the first years of life in endemic populations due to pre-chewed food consumption upon weaning. Infection of older children and adolescents or adults, as occurs in majority cases in developed countries, may lead to infectious mononucleosis (IM), a self-limiting lymphoproliferative disease.\textsuperscript{104}

EBV is orally transmitted and infectious virus can be observed in oropharyngeal secretions from IM patients, from immunosuppressed patients and at lower levels from healthy EBV seropositive individuals.\textsuperscript{105} Once infection occurs it is generally life long with viral episomes persisting in circulating memory B cells.\textsuperscript{106,107} Epithelial coinfection is common leading to persistent virus secretion in saliva. EBV co-exists long term with most human hosts without inducing serious disorders.

Even though serologic studies suggest that most of the adult population in the world is EBV-infected,\textsuperscript{108} the virus is implicated in the development of many malignancies only in particular individuals. Those malignancies include post-transplant lymphoma, acquired immune deficiency syndrome (AIDS)-associated lymphomas, Burkitt’s lymphoma, Hodgkin’s disease, T-cell lymphoma, NPC and gastric cancer.\textsuperscript{109}

2.2. Structure of EBV

The EBV virion consists of toroid-shaped protein core, containing a linear-double stranded DNA molecule of 172 kilobase pairs (kb) surrounded by an icosahedral capsid, amorphous tegument and lipid envelope with viral glycoprotein (gp) spikes on its surface.\textsuperscript{103} The diameter of the viral particle is 120-200 nm and consists of 25-35 proteins and host specific phospholipids derived from nuclear membrane.\textsuperscript{110} The most abundant EBV envelope protein is gp350/220 (BLLF1) which interacts with CD21 on the host cells to initiate infection. The minor envelope protein gp42 (BZLF2) mediates membrane fusion and virion entry by binding to host MHC-II molecules.\textsuperscript{111} The tegument consists of BLRF2, BRRF2, BDLF2 and BKRF4, and host actin, coflin, tubulin, heat shock protein (hsp)90 and hsp70.\textsuperscript{110} Capsid formation initiates by major capsid protein (VCA-p160; BcLF1) assembly
around a scaffold-core (VCA-p40; BdRF1) which is degraded by a viral protease (BVRF1) upon DNA entry into the mature capsid. The small capsid protein VCA-p18 (BFFR3) closes the outer shell of the capsid linking the tegument. Following a passage through the nuclear membrane, the tegumented capsid becomes enveloped at the cytoplasmic membranes of the Golgi-region, from which it is released into the extracellular space by exocytosis.

2.3. The genome of EBV and open reading frame

The EBV genome is characterized by a series of 0.5 kb terminal direct repeats (TRs) at both ends and internal repeat sequences (IR1-4) that divide the genome into short and long, largely unique sequence domains. Upon infection the linear viral genome will circularize by joining at the terminal repeats. There are about 80-100 open reading frames (ORFs) only 50-60 of which are characterized in some extent (Figure 2). Some regions are characterized by extensive splicing and some ORFs span the genome terminus, and are therefore only expressed in latent infection. The ORFs are systematically named according to the size of the BamHI restriction fragment containing the RNA site start with either rightward or leftward transcriptional orientation, e.g. BARF1 means BamHI-A rightward frame 1.

The EBV genetic sequence was resolved as early as 1984 and is assembled based on B95.8 and Raji (EBV type 1) sequences, and annotated as EBV wild type (wt) sequence. However, both B95.8 and Raji show some genomic deletions and mutations that are likely derived during extensive in vitro propagation. EBV isolates can be divided into 2 major types, type A (prototype, B95.8) and B (now called type 1 and 2) based on allelic polymorphism in latent genes of Epstein Barr nuclear antigen 2 (EBNA2) and EBNA3 proteins. Between EBV type 1 and 2 the sequence variation for EBNA2A, 3A, 3B and 3C is 36%, 10%, 12% and 19%, respectively. In term of the efficiency of growth transformation of the host cell, EBV type 1 is more efficient than EBV type 2. These type differences have been used to trace virus transmission within families and from transplant donors to recipients. In many Western and Asian countries EBV type 1 is more prevalent than EBV type 2, whereas both types are widespread in central Africa, New Guinea and certain other countries. Mixed type is frequently detected in HIV patients even though it is also occasionally found in normal individuals.

More recently the complete genome for the Chinese NPC-linked prototype GD-1 strain and the EBV type 2 (AG876) was published, advancing the knowledge on genome functions in different EBV isolates around the world. The new strain of GD1 consists of 171,656 bp with various deletions (including a 34-amino acid deletion in EBNA3C and a 35-amino acid deletion in EBNA2), insertions and point mutations compared to the prototype B95.8 virus. Sequence variation was also found in LMP1, LMP2, EBNA1 and BZLF1. Other genes appeared rather conserved including vIL-10, EBER and BARF1.
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Figure 2. EBV genome (A) and open reading frame (ORF) (B) showing all latent genes. (adapted from\textsuperscript{131,132})

2.4. EBV infection

2.4.1. Natural history of EBV infection (primary infection and persistence)

Following this initial infection, EBV persists in a latent state in memory B lymphocytes where it can remain indefinitely.\textsuperscript{133,134} The main route of EBV entry is the upper aerodigestive tract. EBV virions are transmitted through the saliva into epithelial cells of the oral pharynx, and subsequently to resting B cells (Figure 3). The nasopharynx and oropharynx have been assumed to be the site for EBV primary
infection as well as viral replication which contributes to its persistence.\textsuperscript{131,135} The nasal-associated lymphoid tissue, Waldeyer’s ring, is believed to be the site for EBV primary and persistent EBV infection.\textsuperscript{106,136} Saliva containing EBV virions is sampled by the tonsil, where infection occurs not at the apical surface of epithelial cells, but at the crypt (invagination) of the tonsil. EBV will cross the epithelial barrier to reach submucosal naive B cells residing in the mantle zone facing the surface. Thus, incoming virus infects epithelial cells or infiltrating B lymphocytes in the epithelium of the naso- or oro-pharyngeal mucosa where it establishes a primary focus of latent infection (transformation) and lytic replication. Virus released from EBV-infected epithelial cells or B cells with lytic infection can be transmitted from host to host via saliva to infect other mucosal cells.\textsuperscript{137}

The mucosal lymphocytes of the upper aerodigestive tract can migrate between the surface epithelium and the stromal tissues or to the other lymphoid tissues or even back to the site again. Soon after primary infection, EBV-infected infiltrating B cells will migrate back into the stroma in the mucosal lymphoid tissue, express the latency III program and proliferate.\textsuperscript{138-140} Many of these proliferating cells are eliminated by virus-specific cytotoxic T cells (CTLs). However some cells subsequently pass through a germinal center (GC) reaction, in which LMP1 and LMP2 expression is observed (latency II)\textsuperscript{141,142} in turn generating resting memory B cells with the latency 0 pattern of viral gene expression. In latency 0 B cells downregulate antigen expression and establish a stable reservoir of resting memory B cells containing viral genome, in which viral antigen expression is mostly suppressed. Some of these memory B cells pass through mucosal lymphoid tissue and eventually differentiate into plasma cells. EBV-infected plasma cells arising from the GC reaction may enter the lytic cycle and support viral replication close to the mucosal epithelium and provide a source of infectious virions for other B cells or epithelial cells.\textsuperscript{143} EBV-specific T cells will respond and control the proliferation of EBV-infected B cells, but not infected memory B cells since they lack immunogenic EBV antigens. EBV-infected memory B cells persist at a frequency of ~1-50/10\textsuperscript{6} B cells in the peripheral blood and thus serve as long term latent reservoir for the virus.\textsuperscript{144}
Figure 3. Interaction between Epstein-Barr virus and host cells.¹³¹
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The reservoir of EBV-infected memory B cells becomes subject to the physiological controls directing memory B cell migration and differentiation.\textsuperscript{131} Infrequently, these EBV-infected cells might be recruited into GC reactions, involving the activation of different latency programmes, after which they might either re-enter their reservoir as memory cells or enact to plasma cell differentiation. These cells possibly move to mucosal sites in the oropharynx and initiate viral replication. Virions released at these sites might introduce foci of lytic replication in permissive epithelial cells, allowing low level shedding of infectious virus in the oropharynx, and might also initiate new growth-transforming latency III infections of naive and/or memory B cells.\textsuperscript{143} These new infections might possibly reload the B cell reservoir, but are more likely to be completely removed by memory T cell response.

EBV-infected small lymphoid cells have been consistently detected in normal nasopharyngeal mucosa, tonsils and other mucosa-associated lymphoid tissue.\textsuperscript{136,138,145} Low levels of persistent lytic infection can occur occasionally in EBV-infected lymphocytes in this normal tissue indicated by the detection of rare lytic cycles in the non-neoplastic nasopharyngeal or oropharyngeal mucosa and tonsils,\textsuperscript{136,138,139,141,142,146} even among normal PBMCs.\textsuperscript{147} Due to the migration of lymphocytes, EBV-infected cells will spread the viral infection to other mucosa and lymph nodes and to the peripheral blood.\textsuperscript{142} Similarly, sporadic EBV-positive lymphoid cells have been detected in normal gastric mucosa and other mucosal and lymphoid tissues in normal individuals.\textsuperscript{145,148,149} These EBV-infected mucosal lymphoid cells could serve as a reservoir for the virus.

2.4.2. B cell infection and latency program

In general the entry of herpesviruses requires the concerted efforts of multiple glycoproteins in a multi step process that results in binding of the virus to the cell surface, interaction with cellular entry receptors, membrane virion fusion, and internalization of the virion.\textsuperscript{150} In the primary phase of infection, B cell-specific complement receptor type 2 (CR2), or CD21, is recognized by EBV major membrane gp-350/220 (gp350). The interactions between EBV gp350 and CR2 are responsible for attachment of the virus with high affinity to the cell surface.\textsuperscript{151,152} High density of gp350 ensures crosslinking of the CR2 signaling complex which drives the resting B cell from G0 to G1 phase of the cell cycle. By interacting with the cellular C3d, a bioactive fragment of complement protein-3 (C3) that is deposited on the surface of foreign antigens, CR2 can generate downstream signals to stimulate the humoral response. Binding of gp 350/220 also triggers capping of CR2 and endocytosis of the virus.\textsuperscript{152,153}

Entry into the B cell involves fusion of the virus envelope with a cell membrane. The fusion of the EBV envelope requires four envelope glycoproteins, gB and a complex of gHgLgp42.\textsuperscript{154-156} Within the complex of glycoproteins in the
virus, an interaction between gp42 and a secondary receptor, MHC class II (HLA-DR), provides the trigger for B cell fusion and virus entry into the cell.\textsuperscript{155,157}

Infection with EBV causes naive B cells to transform into proliferating blasts, which can then differentiate in vivo into long-lived resting memory B cells through the process of the GC reaction. Different patterns of latent EBV gene expression are associated with various types of infected lymphocytes. These can be observed in cell culture either as a result of infecting primary resting human B cells (latency III, the growth program) or in cell lines derived from EBV associated cancers (latency I or II).\textsuperscript{108} The different latency programmes were discovered in such cell lines but have now been related to the biology of EBV infection \textit{in vivo}.

Upon viral entry into the resting B cell, EBV nucleocapsid is transported to host nucleus and releases its linear DNA content. Gradually, all nine latent viral proteins and two EBV encoded small RNAs (the EBERs) as well as rightward transcripts from the BamHI-A region (BARTS) are expressed, establishing the latency III or growth programme.\textsuperscript{109,158,159} The latent proteins include EBV nuclear antigens EBNA1, EBNA2, EBNA3A, 3B, 3C and leader protein, EBNA-LP, and the latent membrane proteins LMP1, LMP2A and LMP2B. The expression of latent genes are stimulated by transcription factor EBNA2. The gene expression is initiated from a promoter in the BamW region (W promoter/Wp). Expression of EBNA3C will then activate G1 progression within the cell cycle and activate naive B cells to become proliferating blasts. Activated B cells will migrate to the follicle and subsequently downregulate Cp-driven EBNA2 and EBNA3A-C, switching to Qp-driven EBNA1. The virus protein expression later becomes restricted to the latency II or default programme\textsuperscript{141,159} in which only EBNA1, LMP1, LMP2A and the EBER RNAs are expressed. These two latent membrane proteins produce the signal for the latently infected B cell blasts to form germinal centres,\textsuperscript{160} which causes the infected B cell to undergo GC reaction, proliferation and differentiation into latently infected memory B cells. The virus enters latent persistence and downregulates the expression of viral proteins,\textsuperscript{161} when only LMP2A is possibly expressed.\textsuperscript{162} Latently infected memory cells circulate between Waldeyer’s ring and the peripheral blood without being detected by the immune system.\textsuperscript{142} Only when memory B cells divide, a natural process to maintain memory cell numbers, is EBNA1 expressed,\textsuperscript{161} allowing the viral genome to replicate alongside the host chromosomes. This is known as latency I or true latency program. In the circulation, EBV infected memory B cell will have all latent genes silenced by promoter methylation, except Qp-driven EBNA1 and the non-coding transcript EBER1,2 and BARTs. In latently infected memory B cells, the virus remains silent. Function of EBV latent genes in viral and cellular transcriptions are summarized in Table 1.

\begin{table}
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Gene} & \textbf{Function} \\
\hline
EBNA1 & Promote cell proliferation \\
EBNA2 & Transform cell to blast \\
EBNA3 & Activate cell cycle \\
LMP1 & Produce signals for GC \\
LMP2A & Maintain latency \\
EBERs & Regulate viral expression \\
BARTs & Silencing viral genes \\
\hline
\end{tabular}
\caption{Function of EBV latent genes}
\end{table}
Table 1. Function of EBV latent genes

<table>
<thead>
<tr>
<th>Gene products</th>
<th>Function</th>
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<tbody>
<tr>
<td>EBNA1</td>
<td>Maintenance of EBV episome through sequence-specific binding at OriP and chromosome.\textsuperscript{63} Prevents the virus from proteosomal degradation and presentation to MHC class I via gly-ala repeat.\textsuperscript{164} Destabilizes p53 via interaction with cellular ubiquitin-specific protease (USP7).\textsuperscript{165,166}</td>
</tr>
<tr>
<td>EBNA2</td>
<td>EBV transformation together with EBNA-LP.\textsuperscript{167} Interact with RBP-Jκ to transcriptionally activate CD23 and other cellular genes and viral LMP1 and LMP2.\textsuperscript{168-170}</td>
</tr>
<tr>
<td>EBNA-LP</td>
<td>Upregulation of transcription factors needed for B cell growth.\textsuperscript{171}</td>
</tr>
<tr>
<td>EBNA-3A</td>
<td>Like other EBNAs balances EBNA2 effect on RBP-Jκ transcription factor.\textsuperscript{172}</td>
</tr>
<tr>
<td>EBNA-3B</td>
<td>Transcriptional regulator.\textsuperscript{173}</td>
</tr>
<tr>
<td>EBNA-3C</td>
<td>Essential for EBV-mediated transformation of primary B lymphocytes and interacts with RBP-Jκ. Promotes LMP1 expression in the presence of EBNA2.\textsuperscript{174}</td>
</tr>
<tr>
<td>LMP1</td>
<td>Essential for EBV transformation of B cell in vitro and drives proliferation through NF-κB, AP-1 and JAK/STAT activation.\textsuperscript{175,176} Mimics CD40 activity by providing growth and differentiation signals to B cell.\textsuperscript{177} Upregulation of anti-apoptotic proteins (bcl-2, A20).\textsuperscript{178,179} Induces malignant transformation.\textsuperscript{180} Activates transcription of epidermal growth factor receptor (EGFR) in epithelial cells.\textsuperscript{181}</td>
</tr>
<tr>
<td>LMP2A</td>
<td>Inhibits signaling through BCR and promotes the proliferation and survival of B cells.\textsuperscript{182}</td>
</tr>
<tr>
<td>LMP2B</td>
<td>Function unclear.</td>
</tr>
<tr>
<td>EBER1,2</td>
<td>Counteracts the antiviral effects of interferon and PKR activation in infected cells.\textsuperscript{183}</td>
</tr>
<tr>
<td>BARF1</td>
<td>Induce tumorigenicity and malignant transformation in primary epithelial cells.\textsuperscript{184,185} Induces apoptosis-resistance via upregulating bcl-2.\textsuperscript{186} Shares some homolog with CSF1 and modulates monocytes activation.\textsuperscript{187,188}</td>
</tr>
<tr>
<td>BARTs</td>
<td>Encoding miRNA that regulate EBV latent infection and modulate host immune responses by targeting a variety of viral and cellular genes.\textsuperscript{189-193}</td>
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The process of establishing a latent EBV infection by transforming naive B cells into resting memory cells in germinal centers mimics the natural process of B cell activation in response to a foreign antigen.\textsuperscript{194,195} In immune-activated B cells, antigen-binding leads to the transition becoming a memory B cell but in an EBV-driven B cell, the viral proteins provide the signals necessary for this process. Lymphoblasts resulting from antigen activation show a similar cell surface phenotype and morphology to those produced by EBV infection in cell culture.\textsuperscript{196,197}
EBV latent gene expression in various EBV-associated malignancies and EBV-derived cell lines has led to the identification of three different and distinct latency programmes. These latency programmes are the result of differential promoter activity and are influenced by host cell factors. In peripheral blood of healthy person there is no detectable latent mRNA or proteins (only EBERs, BARTs and occasionally LMP2 are expressed), except when these cells divide when EBNA1 is also temporarily expressed. This latency is called latency 0 or I, respectively (true latency). In Burkitt lymphoma, expression of EBERs and the BARTs are found in addition to Qp promoter-induced EBNA1, characterizing the latency I. Upon cytokine triggering or lymph node entry, latency-0/I B-cells may re-express the LMP1 and LMP2 genes, representing the default or latency-II program. In HD, EBV-associated gastric cancer and NPC, LMP1 and LMP2 (A and B) are also expressed besides EBERs and EBNA1 (latency II). The latency type associated with expression of all latent genes is referred to as latency III and is observed in immunosuppression-associated lymphoproliferations and IM. Three promoters are responsible for the transcription of EBV nuclear antigens. In establishing latency III, EBNA transcription after viral infection is initiated from Wp, followed by a switch to Cp with concomitant downregulation of Wp activity. In latency I and II, EBNA2, 3 and LP are not expressed. EBNA1 expression is initiated neither from Wp or Cp. Instead, another promoter Qp is used, which is autoregulated by EBNA1 levels. LMP1 and LMP2B transcription is controlled by a bidirectional promoter close to terminal repeats, with LMP1 is transcribed in the left direction and LMP2B in the right direction across the closed terminal repeats in the latent episomal genome. LMP2A is transcribed from a promoter about 3000 bp upstream of the promoter of LMP1 and LMP2B.

2.4.3. Epithelial cell infection

EBV is frequently found in certain epithelial pathologies such as NPC, gastric adenocarcinoma and oral hairy leukoplakia, indicating its epithelial tropism in vivo. Most of the EBV-positive epithelial cell lines have negative or extremely low CD21 expression suggesting that the mechanism is CD21 independent. A study on polarized human tongues and oropharyngeal epithelial cells shows that EBV enters these cells through multiple mechanisms depending on the membrane domain that are distinct from those of lymphocyte infection. The apical membrane of polarized cells is the site of highly efficient cell-to-cell contact of EBV-infected salivary B cells with epithelial apical membranes suggests as the primary mode of EBV entry into oropharyngeal epithelium, since the apical site is resistant to cell-free EBV attachment and entry. Cell-free virions also infect oropharyngeal cells at the basolateral membrane when the epithelium is disrupted or injured. After initial infection virus may spread directly across lateral membrane to adjacent epithelial cells. Virions are released from epithelial cells at both the apical and basolateral membranes.
Some studies showed the possibility of CR2 usage for EBV infection in epithelial cell that were engineered to express high levels of CR2. \(^{204, 205}\) EBV-mediated cross-linking of CR2 in epithelial cells, with lack other components of the CR2 signaling complex found on B cells, and stimulates relocalization and clustering of CR2 in the formin homolog. However, whether epithelial cells normally express CR2 \textit{in vivo} remains uncertain.

Since epithelial cells generally do not express CR2, other possible mechanisms of the viral attachment to epithelial cells have been proposed that involve neither gp350/220 nor CR2. The first was a demonstration that virus coated with immunoglobulin A specific to gp 350/220 can bind productively to the polymeric immunoglobulin-A receptor (pIgR). \(^{206}\) The epithelial pIgR can mediate internalization of infectious virus-linked immunoglobulin A (IgA-EBV) complex. Anti-EBV-gp350 IgA were used to prove the ability of EBV to infect epithelial cells. \(^{207}\) The phenomena suggest that antibody-enhanced infection by pIgR-mediated transcytosis of pIgA-EBV through epithelium facilitates endogenous spread of EBV in long term virus carriers. \(^{207, 208}\) This may be particularly relevant to infection via the basolateral surface of an epithelial cell in an immune host. However, since in polarized cells EBV was transported intact from the basolateral to the apical surface, it maybe more relevant to \textit{trans} epithelial transport than direct infection. \(^{207}\) The second mechanism revealed that in the absence of CR2 a complex of two additional glycoproteins, gH and gL, can serve as epithelial ligands. Both proteins, which associate noncovalently, are usually referred to as a unit, the gHgL complex. \(^{155, 209}\) A soluble form of gHgL made in baculovirus can bind specifically to epithelial cells, but not B cells, and its binding can be reduced by a monoclonal antibody specific for the gHgL complex. \(^{205}\) The same antibody can also reduce virus binding. \(^{155}\) These observation indicates that there is an epithelial cell receptor for gHgL that can serve in attachment. The identity of the molecule is not yet known, but operationally it has been referred to as gHgLR. \(^{210}\) It was also recently demonstrated that integrins \(\alpha\nu\beta6\) and \(\alpha\nu\beta8\) can serve as specific receptors for gHgL and that binding to gHgL can provide the trigger for direct virus fusion with the epithelial cell plasma membrane. \(^{211}\)

Another possible mechanism was demonstrated by an interaction between a multispan EBV membrane protein containing an extracellular RGD-motif encoded by BMRF2 and exoskeletal fibronectins and integrins on polarized epithelial cells. Antibodies to integrins and to a BMRF2 fusion protein only partially block binding to polarized epithelial cells but have a significant impact on infection via the basolateral surface of the polarized monolayer. \(^{203, 212}\)

Fusion of the EBV envelope with an epithelial cell requires three glycoproteins, gHgL and gB. The requirement of gB level in epithelial penetration was higher than B cell penetration. \(^{213}\) The fusion is triggered by an interaction between gp42 and MHC class II. \(^{214}\) Fusion with epithelial cell also requires a trigger that is transmitted directly to gHgL, and not via gp42 and gB, as required in B cell fusion. \(^{210}\)
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2.4.4. Non-coding transcripts

Of all latency types of EBV infection, two products are always expressed, the EBERs (EBER1 and EBER2) and transcripts from the BamHI-A region, referred to as BamHI-rightwards transcripts (BARTs). Transcripts of EBERs and BARTs are only found in RNA format, with as yet unclear functions. The persistent and abundant expression suggest that EBER and BARTs play an important biological role. More recently, there has been increasing interest in the presence of different virus-encoded and cellular micro-RNAs (miRNAs) in EBV-infected B cells and epithelial cells.\(^{215}\) EBV encodes for two families of miRNA, one deriving from the BHRF1 and one from the BART regions. Roles of EBV-encoded miRNAs in the transcriptional regulation of both the viral and cellular genome have been described, but much work is required to characterize the function of these miRNAs.

EBERs are the most abundant viral transcript in latently infected cells. These genes are highly expressed, up to 1-10 million copies per cell.\(^{216}\) As a consequence, EBER (especially EBER1, as it showed higher expression levels and better stability compared to EBER2) has been extensively used as the golden standard for identification of EBV latent infection in tissue isolates.\(^{145}\) However, it is also shown that EBER may present at variable levels from negative to abundant, even in one tissue specimen,\(^{217}\) suggesting a possibility of EBER negative status of some EBV latently positive cells. This may also be an artifact of tissue preparation. A recent study demonstrated that EBER contributes to efficient growth transformation of B cells. EBERs may protect the infected cell from natural antiviral effects mediated via pattern recognition sensors (TLR3, 7 and 9) and interferons, by binding to signaling intermediates such as RIG1 and PKR. Overall, EBERs are non-essential for B lymphocyte transformation, apoptosis resistance and cytokine productions, as these effects maybe covered by more powerful LMP1 functions, but their expression improves the transformation efficiency and may contribute to long-term survival of the infected cell.\(^{183}\)

There is abundant expression of BARTs or complementary-strand transcripts, in most EBV driven tumors, but especially in NPC.\(^{163,218}\) These transcripts were first identified in NPC tissues, and subsequently in other EBV malignancies, such as Burkitt’s lymphoma,\(^{219}\) Hodgkin disease,\(^{220}\) nasal lymphoma\(^{221}\) and gastric carcinoma,\(^{222}\) as well as in PBMCs from healthy individuals.\(^{223}\) The function of BARTs is largely unknown but their detection in B cells from normal donors and in all EBV-associated tumors suggests that they possibly have important roles in virus persistence. The proteins coded by BARTs have not been fully characterized, but several ORFs have been identified, including BARF0 (RK-BARF0), RPMS1 and A73.\(^{224}\) The RK-BARF0 encodes an endoplasmic reticulum-targeting signal peptide, interacting with the Notch4 ligand binding domain, indicating that it can modulate Notch signaling. RK-BARF0 can also activate the expression of LMP1.\(^{225,226}\) The RPMS1 product is a nuclear protein binding CBF1 and is thus involved in Notch signal transduction, and can inhibit the transcription activation induced through CBF1 by NotchIC or EBNA2.\(^{220,224}\) The A73 protein is a cytoplasmic protein which
interacts with RACK1, a cell cycle regulatory protein also involved in signaling of protein kinase C and Src tyrosine kinases, suggesting a role for A73 in cell growth control. Because gene-products of ORFs located in the BARTs are not detectable in tumor material, nor are (RK-)BARF0 ORF-specific transcripts, the in vivo relevance of the above findings in transfected cell systems is questioned, and alternative functions for BARTs are suggested, including the recently discovered BART encoded miRNA.

miRNAs are small non-protein-coding RNA molecules that regulate gene expression at the translational level. miRNAs are members of a family of small RNAs, typically 19-30 nucleotides, which includes small nuclear RNA (snRNA) involved in mRNA splicing, small nucleolar RNA which direct modification of ribosomal RNA and short interfering RNA (siRNA) produced from long double stranded RNA precursors. EBV is the first human virus reported to encode miRNAs. To date, a total of 25 EBV miRNA precursors with 44 mature miRNAs have been reported, increasing the number and complexity of potentially biologically active molecules encoded by EBV during latent infection. These EBV miRNA precursors are clustered in two regions of the EBV genome. Three precursors (miR-BHRF1-1 to miRBHRF1-3) with four mature miRNAs are located within the mRNA of the BHRF1 gene. An additional 22 precursors (miR-BART1 to miR-BART22) with 40 mature miRNAs are located in intronic regions of the BART transcripts, derived into two clusters of multispliced BART, cluster one and cluster two. There is an exception of miR-BART2 which is considered an individual miRNA and suggested to play a role in cleavage of viral RNA encoding virus DNA polymerase (BALF5). Studies using Northern blotting and cloning methods have clearly documented two distinct expression patterns of EBV miRNAs in EBV-infected B lymphoma cells and EBV-positive NPC cell lines. The expression of BHRF1 miRNAs can be detected only in lytically infected cells, whereas expression of BART miRNAs can be detected in all forms of latency. Like most miRNAs discovered to date, the functions of the EBV miRNAs are still poorly understood. Functional studies revealed that these viral miRNAs regulate EBV latent infection and modulate host immune responses by targeting a variety of viral and cellular genes. More recently it is also demonstrated that mature miRNAs are secreted by EBV-infected B cells through exosomes. These EBV-miRNAs are functional because internalization of exosomes by (monocyte) dendritic cell/DC results in a dose-dependent, miRNA mediated repression of confirmed EBV target genes.

### 2.4.5. EBV latency proteins

#### 2.4.6. 2.4.5.1. The EBNA proteins

EBNA1 is critical for maintenance and replication of the viral episome by the host DNA polymerase during latent infection. EBNA1 binds to the origin of replication for the plasmid form of the viral genome and is essential for plasmid replication. EBNA1 also binds to cellular chromosomes and mediates equivalent
partitioning of the viral genomes to the daughter cells. Although EBNA1 is expressed in all dividing EBV infected cells, the protein is poorly recognized by CD8 CTLs and the latently infected cells can be invisible to the host immune system.\textsuperscript{236} The lack of CTL recognition of EBNA1 is due to the presence of a large repeated element of glycine and alanine. This sequence prevents the virus from proteosomal degradation and presentation by MHC class I molecules.\textsuperscript{164} EBNA1 activates the expression of other EBV latency genes important for cell immortalization and can autoregulate its own expression.\textsuperscript{237} EBNA1 expression was shown to protect cells from apoptosis by reducing the accumulation of p53 in response to DNA damage.\textsuperscript{238} The anti-apoptotic effect of EBNA1 required amino acids 395-450 which mediates EBNA1 binding to the cellular ubiquitin-specific protease, USP7.\textsuperscript{165} USP7 binds and stabilizes p53\textsuperscript{239} and EBNA1 was observed to block the interaction of USP7 with p53 by competing for the same binding pocket on USP7.\textsuperscript{166,238} More recently, EBNA1 was shown to affect function of PML nuclear bodies involved in DNA repair,\textsuperscript{240} leading to increased susceptibility for DNA damage in EBV infected cells and to induce survivin, providing apoptosis resistance to the EBV infected cell.\textsuperscript{241}

EBNA2 is essential for B cell growth transformation and is also a transcriptional transactivator.\textsuperscript{242} To activate gene expression, the interaction of EBNA2 with its responsive elements occurs via the RBP-J\textsubscript{k} protein,\textsuperscript{168} resembling the Notch signaling pathway. Activated Notch has been shown to partially substitute for EBNA2 in transformation.\textsuperscript{170} EBNA2 also interacts with multiple cellular transcriptional proteins through which it regulates the viral promoters for the latent membrane proteins, LMP1 and LMP2, for the B cell activation marker, CD23, and the EBV receptor, CD21.\textsuperscript{169} Together with EBNA leader protein (EBNA-LP or EBNA5), EBNA2 is involved in G0-G1 transition.\textsuperscript{167}

EBNA3A-C interact with the cellular RBP-J\textsubscript{k} transcription factors\textsuperscript{172} and may modulate transactivation of the LMP1 promoter and other genes by the EBNA2 protein. EBNA3A and EBNA3C are essential for B cell transformation in vitro whereas EBNA3B is dispensable.\textsuperscript{243} EBNA3C upregulates CD21 expression in vitro, augments the EBNA2-driven upregulation of LMP1 expression\textsuperscript{174} and repress the Cp promoter by interaction with human histone deacetylase.\textsuperscript{244}

2.4.5.2. Latent membrane protein 1

LMP1 is the major transforming protein of EBV behaving as an oncogene in rodent fibroblast and being essential for B cell transformation in vitro and causing lymphoma in LMP1 transgenic mice.\textsuperscript{245} LMP1 has important effects on cellular gene expression and induces expression of multiple genes including adhesion molecules, anti-apoptotic functions, growth factors and growth factor receptors.\textsuperscript{178,179} LMP1 interacts with the cellular molecules that mediate signals from the tumor necrosis factor family of receptors (TNFR).\textsuperscript{246} TRAFs form heterotrimeric complexes that transduce signals to activate the NF-κB transcription factors, induce cellular growth or induce apoptosis. LMP1 resembles CD40, a member of the TNFR family, and
can partially substitute for CD40 in vivo providing both growth and differentiation responses in B cells. The LMP1 protein has a short cytoplasmic amino terminus, a six membrane-spanning domain that is responsible for spontaneous aggregation in the plasma membrane, and signaling domains in the carboxyl terminus. Two distinct functional domains, referred to as cytoplasmic carboxyl terminus activation regions 1 and 2 (CTAR1 and CTAR2), have been identified on the basis of their ability to activate the NF-κB transcription factor pathway. CTAR1 binds TRAFs 1, 2, 3 and 5 while CTAR2 binds the TNF receptor-associated death domain protein (TRADD) and its partner, the TNF receptor-interacting protein (RIP). Signaling from these two domains leads to NF-κB activation as well as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) activation. The region between CTAR1 and CTAR2 (so-called CTAR3) has been suggested to be responsible for the JAK/STAT pathway. LMP1 can activate phosphatidylinositol 3-kinase, a lipid kinase responsible for activating a diverse range of cellular processes in response to extracellular stimuli and this effect contributes to the oncogenicity of LMP1. Expression of LMP1 affects different cellular genes in lymphocytes and epithelial cells. In both cell types, NF-κB transcription factors are activated, although different forms of NF-κB are activated in the two cell types. Activation of NF-κB increases expression of B-cell activation markers in lymphoid cells and expression of the A20 gene in both lymphoid and epithelial cells. In epithelial cells, LMP1 activates transcription of the epidermal growth factor receptor (EGFR), which is also detected at high levels in NPC. The LMP1 protein was shown to associate with exosomes, regulating its transforming activity and modulating the cellular microenvironment for immune evasion.

2.4.5.3. Latent membrane protein 2 (LMP2)

The LMP2 proteins, LMP2A and LMP2B, are not required for EBV-induced B cell transformation in vitro. However, expression of LMP2A in B cells is an essential factor in maintaining EBV in a non-replicative state. LMP2A can promote the proliferation and survival of B cells in the absence of signaling through the B cell receptor (BCR). LMP2A can transform epithelial cells and enhance their adhesion and motility, effects that might be mediated by the activation of the PI3-kinase–AKT pathway. LMP2 interferes with expression of specific transcription factors that regulate B cell development resulting in enhancement of cellular survival. LMP2A was also observed to induce expression of a range of genes that are involved in cell-cycle induction, inhibition of apoptosis and suppression of cell-mediated immunity. In epithelial cells expression of LMP2 increases the levels of β-catenin and induces its nuclear translocation. This property requires activation of Akt and phosphorylation of GSK3. Activation of β-catenin occurs frequently in the development of carcinoma generally through genetic mutations and it is likely that activation of this pathway is an important factor in EBV effects on epithelial cell growth. Powerful effects of LMP2 on epithelial cell growth are also suggested by a
study demonstrating that EBV infected epithelial cell clones emerged more rapidly from primary infected cultures. This growth advantage was linked to those clones that had fewer numbers of terminal repeats, a property that resulted in increased expression of LMP2.\textsuperscript{263}

### 2.4.7. BamHI-A righward frame 1 (BARF1)

The EBV-encoded BARF1 gene is located in the BamH1-A fragment of the EBV genome.\textsuperscript{184} The BARFI ORF gene is transcribed just before the onset of EBV DNA synthesis in EBV producer cells and translated into a 33 kd early protein (p33) which is recognized by some high EA titer NPC sera.\textsuperscript{264} The subsequent study showed that BARF1 has oncogenic activity by inducing morphological change, anchorage-independent growth and tumorigenic transformation of rodent fibroblast.\textsuperscript{184} A 54-amino acid region of the NH2 terminus is capable to induce the expression of the anti-apoptotic protein bcl-2 and suppress PARP cleavage, thus is considered essential for the transforming activity.\textsuperscript{186,265} Although initially described an an EBV-encoded early gene in B cells, BARF1 is expressed in EBV positive gastric cancers and NPC, independent of lytic gene expression, defining it as a latency associated carcinoma-specific EBV gene product.\textsuperscript{109,266-268} Transfection with the BARF1 gene to EBV-negative Burkitt’s lymphoma-derived cell lines was also shown to enhance the tumorigenicity.\textsuperscript{269,270} BARF1 also can immortalize primary monkey kidney epithelial cells,\textsuperscript{271} but the immortalized cells were not tumorigenic in nude mice.\textsuperscript{272} A more recent study confirmed the role of BARF1 as EBV encoded carcinogenic factor.\textsuperscript{185} However, because BARF1 seems to be rapidly and completely secreted \textit{in vivo}, its equivocal detection in tumor tissue has proven difficult.\textsuperscript{273} Secreted BARF1 forms a hexameric molecule which binds m-CSF1 with high affinity.\textsuperscript{274} BARF1 shares some homology with the c-fms, the receptor for colony-stimulating factor 1 (CSF1)\textsuperscript{187} and the secreted form of BARF1 can activate cell cycle as growth factor.\textsuperscript{275} Several reports showed that the EBV-encoded BARF1 gene is expressed in a high proportion of NPC cases, suggesting that BARF1 plays an important role in the pathogenesis of NPC.\textsuperscript{23,266 267,276,277}

### 2.4.8. EBV lytic replication

Reactivation from latency to lytic replication will lead to a cascade of viral gene activation and expression with function to produce encapsidated, enveloped virions. This process will release infectious virions, that can be transmitted to uninfected cells and new hosts and host cell death. The transmission of the virus from host to host is an essential aspect of viral pathogenesis. The saliva from immunocompetent hosts often contains infectious EBV,\textsuperscript{278} indicating that lytically infected cells in or near the oral cavity must exist, even if they are difficult to detect in the presence of a vigorous cytotoxic T-cell response.\textsuperscript{138} A recent study confirmed and extended these findings showing that most healthy EBV carriers produce about one million virus particles per hour in saliva.\textsuperscript{107} Therefore the great majority (90\% or more) of the
human population becomes infected with this virus. Antigen-mediated activation of B cells which lead to memory B cell differentiation towards the plasma cell stage is considered as a physiologic stimulus for lytic reactivation of EBV.\textsuperscript{143} Reactivation can be induced by B cell receptor (BCR) stimulation through crosslinking surface immunoglobulin.\textsuperscript{279}

Viral gene expression in the lytic cycle follows a temporal and sequential order, immediate early, early and late genes. The immediate early gene products are transcriptional transactivators that initiate a cascade of lytic viral gene expression. The early viral genes encode viral proteins that initiate replication of the viral genome from the origin of lytic cycle replication (oriLyt) site. Late viral gene transcription occurs following viral replication allowing expression of structural proteins.\textsuperscript{173}

Viral immediate early genes are induced directly by signal transduction from the BCR, independent of the expression of other proteins.\textsuperscript{279} This process leads to the expression of immediate early mRNAs encoding the proteins BZLF1 (variably named ZEBRA, Zta, or EB1) and BRLF1 (Rta).\textsuperscript{280-282} However, BZLF1 is the major immediate early protein controlling the lytic cycle. When expressed in latently infected cells, BZLF1 protein alone is sufficient to activate the entire EBV lytic cycle cascade, leading to production of infectious virus.\textsuperscript{282,283} BZLF1 further activates its expression by binding to its own promoter and activates the adjacent gene BLRF1.\textsuperscript{284} BZLF1 and BLRF1 then act in synergy to activate transcription of a subset of early lytic cycle genes many of which encode proteins required for lytic viral DNA replication, such as DNA polymerase (viral pol).\textsuperscript{285,286} BZLF1 plays a distinct role in activating viral lytic DNA replication by binding to the oriLyt and by interacting with and recruiting viral proteins that are essential for lytic replication.\textsuperscript{287-291} Lytic replication initiates at one of two origins of oriLyt and then proceeds repeatedly around the circular viral episome, producing long linear concatemers of the viral genome. The linear concatemers are clipped within the terminal repeat sequences (TR) to produce complete linear double-stranded viral genomes.\textsuperscript{289,292,293} Late genes which encode capsid proteins, viral tegument proteins and viral membrane proteins are transcribed following lytic viral replication. The viral genome is packaged into a capsid in the nucleus and secreted by budding through the nuclear membrane into the cytoplasm as tegument-coated capsids to generate transmissable virions. A second process of budding occurs at the Golgi membrane providing the outer viral envelope and yielding the final viral product which is released from post-Golgi vesicles via exocytosis.\textsuperscript{294}

Several chemicals have been observed to induce the EBV lytic cycle in latently infected cells. The mechanism of all chemicals appear to activate the expression of the virally encoded lytic cycle activator genes. Certain cytokines, particularly TGF-β and glucocorticoid hormones, can also induce lytic viral infection in a subset of Burkitt’s lymphoma lines \textit{in vitro} and could potentially reactivate EBV \textit{in vivo}.\textsuperscript{295,296} Nitrosamines, a compound found in preserved food, is observed to induce EBV reactivation.\textsuperscript{75,297} These defined chemicals have suspected oncogenic/carcinogenic activity and are commonly found in environment and food constituents in developing
countries. Sodium butyrate, an inhibitor of histone deacetylase (HDAC), is a potent lytic cycle activator, even though not all populations of B cell tumors respond. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C agonist, is also known to have capability in inducing viral replication in many cases, even though the result is not consistent. In some B cell lines, treatment with the DNA methyltransferase inhibitor 5-azacytidine very rapidly and potently causes induction of EBV BZLF1 and BRLF1 mRNAs. Many of the cytotoxic regimens used for cancer treatment, including gamma irradiation and some chemotherapy agents, can activate the signal transduction pathways, including p38 kinase, MAP kinase, PI3-kinase, and EGR pathways and result in lytic infection. The interaction between CD4 T cells and EBV-infected B cells has also been reported to induce lytic infection. Finally, there is increasing evidence that severe host and host cell stress in response to many different toxic stimuli (including anxiety, chemotherapy and irradiation) can induce lytic EBV infection as well.

2.5. EBV and oncogenesis

Inactive latent EBV generally has no serious consequences, allowing a vast majority of healthy adults asymptptomatically carry the virus for life. However once it becomes active it can cause a wide spectrum of malignancies. The EBV-associated malignancies include epithelial tumors such as nasopharyngeal and gastric carcinomas; mesenchymal tumors such as follicular dendritic cell tumor/sarcoma; and lymphoid malignancies such as Burkitt’s lymphoma, AIDS-associated lymphoma, immunodeficiency-associated lymphoproliferative disorders, extranodal natural killer (NK) cell/T-cell lymphoma and Hodgkin’s lymphoma.

Figure 4 showed putative check points in the EBV life cycle that might give rise to EBV-associated malignancies. The events that occur normally in healthy carriers are denoted on the left panel as discussed in section 2.4. EBV generally infects naive B cells in Waldeyer’s ring and these cells can differentiate into memory cells and out of the cell cycle so that the virus is not pathogenic. A primary EBV infection during the first or second decade of life can result in IM. Most patients recover from IM without any sequelae, although a variety of complications can occur such as splenic infarction and rupture, upper airway obstruction and neurological complications. EBV can cause chronic infections in individuals without apparent immunodeficiency. This condition is called chronic active EBV (CAEBV) infection. CAEBV infection, characterized by chronic or recurrent infectious mononucleosis-like symptoms, basically affects children and young adults.

EBV-infected B cells enter either the lytic cycle or the latent cycle. In the lytic cycle, viral particles are reproduced and shed into saliva, again infecting other mucosal cells and lymphocytes. The B cells entering the latent cycle migrate back into the lymphoid tissue. There, lymphocytes enter the growth program
which is characterized with rapid polyclonal expansion of the infected B cells as lymphoblasts. A considerable ratio of the proliferated lymphocytes is eliminated by CTLs before and through a germinal center reaction. Thereafter, the infected B cells express the latency program. In this program, expression of antigen molecules that induce cytotoxic response by infected-B cell specific T cells is ceased and the infected B cells become resting memory B cells. As a result, EBV escapes surveillance by the immune system, accomplishing lifelong infection in resting memory B cells. Burkitt’s lymphoma is thought to arise from a germinal center generating lymphoblasts that are stucked at the proliferative stage because of the activated c-myc oncogene.\textsuperscript{313} As a consequence the cells only express EBNA1 (latency program). Hodgkin’s lymphoma is considered to arise from EBV-infected B cells blocked at the germinal center cell stage. This results in constitutive expression of the default program expressing EBNA1, LMP1 and LMP2. Burkitt’s lymphoma and Hodgkin’s lymphoma development can be induced by cellular mutation that occur during the immunological disturbance associated with acute EBV infection. Because the levels of infected cells are so high at this time there is a reasonable possibility that the cell undergoing mutation will have EBV in it by chance. Post-transplant lymphoproliferative disorder (PTLD) may arise if another cell other than the naïve B cell in Waldeyer’s ring becomes infected and expresses the growth program. This cell continues to proliferate because it cannot differentiate out of the cell cycle. This may occur in immunosuppressive state when these bystander B cell blasts that should be destroyed in the germinal center are rescued in the suppressed CTL response. Other lymphoid malignancies such as B-NHL and AIDS-NHL may arise in this stage expressing the growth program with full spectrum of latent gene products (EBNA1-6 and all three LMPs). Occasionally, EBV-infected memory B cells replicate the virus and release infectious viruses into saliva. Some EBV-infected memory B cells differentiate into plasma cells. The virus is also released from plasma cells, entering the lytic cycle as they migrate into peripheral tissues. EBV released from B cells in the lytic cycle are considered to be the viral source in EBV-related epithelial malignancies such as NPC and gastric carcinomas. NPC is characterized by default program expressing EBERs, Qp promoter-driven EBNA1, LMP1, LMP2A, LMP2B and BARF1. Gastric carcinoma expressed EBNA1, LMP2A, LMP2B and BARF1. In these malignancies all other EBNAs are absent.\textsuperscript{109, 314, 315}
Figure 4. EBV life cycle and oncogenesis

On the left side is the “normal” life cycle of EBV and on the right side are the different diseases and malignancies arising from different stages of acute and persistent infection. The cancer stages are considered to arise because of a combination of (internal or external) events that co-operate with EBV transforming genes/functions to immortalize/freeze the particular phase of normal EBV infection in the affected cell type.\textsuperscript{109, 314}

2.6. EBV in NPC pathogenesis

2.6.1. Characteristics of EBV infection in NPC development

The development of NPC is an important consequence of epithelial infection with EBV which leads to malignant transformation. The EBV-associated, undifferentiated form of NPC, WHO type III, shows the most consistent worldwide association with EBV. NPC tumors are characterized by the presence of undifferentiated carcinoma cells and a prominent lymphocytic infiltrate. This interaction between tumor cells and lymphocytes seems to be crucial for the continued propagation of the malignant component.\textsuperscript{109}

EBV infection has been shown to be an early event in the development of NPC. It was actually difficult to find early premalignant lesion of NPC such as dysplasia and carcinoma in situ (CIS).\textsuperscript{58} Extreme rarity of lesions without concomitant carcinoma (3%) and a rapid development of invasive carcinoma (within one year) indicated a rapid progression of the initiated cell from dysplasia to CIS and invasive NPC, being contrast to many other cancers that show CIS for years. Despite the infrequency of such premalignant lesions, EBV has been identified in high grade
dysplasia and isolated CIS. All cases of CIS expressed EBERs and LMP1 proving that the lesions were homogeneously infected with EBV. The presence of a single clonal form of EBV implies that the hyperplasia or dysplasia represents the outgrowth of a single EBV-infected progenitor cell prior to clonal expansion, confirming previous observation using southern-blot hybridization. This also emphasizes the presence of EBV at the beginning of tumor development rather than a secondary infection. However, a large scale study in high risk Chinese populations demonstrated that in early dysplasia EBV was present in a subset of cells while EBV infection in high grade dysplasia was homogeneous and uniform. These studies suggest that an early genetic change precedes EBV infection and induces dysplastic growth and that the EBV infected cells apparently overtake the population and the lesion rapidly progress to CIS. A genetic factor may also influence the ability of EBV to establish a latent infection in epithelial cells rather than replicate as is believed to occur in normal oropharyngeal cells. Finally, chronic inflammation of the oro-nasopharynx may provide a stimulatory environment for EBV transfer from lymphocytes to epithelial cells, correlating with increased viral shedding as encountered in NPC.

Based on the viral detection in early NPC, EBV is believed to play an important role in disease pathogenesis, with latent and clonal viral genomes. Besides, EBV infection is not present in normal nasopharyngeal epithelium nor in low-grade dysplasia. Among the latent genes expressed in NPC is the viral oncoproteins LMP1. The expression of EBV LMP1 would facilitate tumour cell growth and survival advantages, maintaining the malignant phenotype. LMP1 has been suggested to activate STAT signaling and activated nuclear STAT was detected in NPC. This may indicate that LMP1 in NPC is auto regulated and that perhaps constitutive STAT activation is a factor that leads to tumor development. However, the rate of LMP1 detection in NPC tumor tissues is highly variable (20-90% of cases), in contrast to Hodgkin lymphoma and nasal NK/T-cell lymphoma, which almost always express LMP1.

2.6.2. Latent EBV gene expression in NPC

EBV infection in NPC is primarily latent, indicated by the presence of EBV episomes using electron microscopy, which was confirmed using restriction enzyme analysis. EBV latent gene expression is predominantly restricted to the EBNA1 from the Qp promoter, transcription of LMP1 and LMP2 (LMP2A and LMP2B) and the non-coding EBERs and BamHIA transcripts, classified as type II latency.

LMP1 and LMP2 appear to be heterogeneously expressed between different NPC cases, with juveniles having more abundant LMP1 expression. In LMP1 positive NPC tumors the LMP1 expression occasionally is heterogeneous throughout the tumor. No relation was found between LMP1 expression and hypoxia, as defined by HIF1α expression, not to local (neo)angiogenesis as defined by CD36 expression. However, it has been reported that all early precancerous cells are positive for
LMP1.\textsuperscript{316} How LMP1 (and LMP2) expression are regulated in NPC tumors remains illusive.

The expression of BARTs and BARF1, a transforming EBV oncogene,\textsuperscript{270} was detected in a large proportion of NPC tumours,\textsuperscript{23,26,27,26,332} suggesting a crucial pathogenic role for these RNAs/proteins in NPC. Oncogenic role of BARF1 in epithelial cells was also indicated by its capacity of tumorigenicity.\textsuperscript{269} It has also been suggested that BARF1 expression may compensate for the reduced expression of LMP1 in NPC, for instance by inducing apoptosis resistance via upregulation of bcl-2.\textsuperscript{265} Secreted BARF1 protein formed a complex with purified colony stimulating factor 1 (CSF1) molecule, so that the activation of macrophage in vitro was inhibited by the formation of this complex.\textsuperscript{187} In combination with poly (IC), CSF1 is known to induce alpha interferon in monocytes.\textsuperscript{188} This suggests that BARF1 protein could intervene in immunomodulation.

Recently, multiple EBV-encoded microRNAs (up to 44) have also been detected in EBV-infected B cells and carcinomas including NPC and gastric carcinoma, although their pathobiological functions are still not very clear.\textsuperscript{215,230,231} Recent studies imply that EBV miRNAs may be linked to the pathogenesis of NPC. It has been shown that miR-BART2 (BART2-5p) targets the viral DNA polymerase BALF5 for degradation, effectively inhibiting EBV lytic replication.\textsuperscript{190} Cluster one BART miRNAs (BART1-5p, BART16-5p and BART17-5p) have been reported to suppress the expression of the viral LMP1.\textsuperscript{189} On the other hand miRNAs from the BHRF1 region (BHRF1-3) have been associated with replication of the virus and modulate host immune responses by targeting the interferon-induced chemokine CXCL11.\textsuperscript{191} BART2-5p targets the host stress-induced natural killer (NK) cell ligand, MICB, to escape recognition and escape from NK cell killing.\textsuperscript{193} BART5-5p has been shown to promote host cell survival by targeting a proapoptotic molecule, p53 up-regulated modulator of apoptosis (PUMA).\textsuperscript{192} Together these observations indicate that EBV virus actively utilizes its miRNAs to manipulate various viral and cellular functions. Recent studies using real-time PCR technology confirmed the expression patterns observed in cell lines and provided further insights into the wide expression range of BART miRNAs in NPC clinical samples.\textsuperscript{333,334}

### 2.6.3. Lytic EBV in NPC

In some scattered tumor samples linear genomes were also detected suggesting that in an occasional cell within the tumor, the virus may reactivate from latency and produce virus. In fact, it has been proposed that keratinization of small tumor fields within the NPC tumor may be responsible for the switch to lytic replication.\textsuperscript{335} This sporadic replication can be a source of the antigenic stimulus that stimulates the elevated antibody response to viral replicative antigens that are indicative of tumor development or relapse.\textsuperscript{317,336} Moreover, oropharyngeal epithelial cells with evidence of viral replication have been detected during primary infection and reactivated infection and are considered as the source for
viral shedding. The high IgA titers to EBV replicative antigens that precede the development of NPC patients to VCA indicate increased viral replication and antigenic stimulation. The elevated IgA titers may play role in NPC development by enhancing epithelial infection as it has been shown that secretory IgA facilitates entry into epithelial cells. In the development of NPC, increased viral replication, perhaps at lymphoid or mucosal epithelial interfaces, increases the possibility of establishing a latent, transforming infection of an epithelial cell that may have already had an initiating genetic change. This establishment of latent infection and expression of viral oncogene in an epithelial cell are likely to be critical events that lead to the development of NPC. Genetic changes may participate and facilitate latent infection or are synergistic with EBV transforming proteins. This process could also be influenced by the genetic or environmental factors during the malignant outgrowth.

2.6.4. EBV immune responses and evasion in NPC

EBV infection is normally well-controlled by both natural and adaptive immune responses, the latter including molecularly diverse antibody (humoral) and T-cell based (cellular) reactivities against an array of EBV antigens. Both aberrant humoral immunity and cellular immunity are observed in NPC patients. In general, EBV-specific antibody responses in NPC patients are more strong and more diverse as compared to regional healthy EBV carriers, and can be utilized as diagnostic marker. In contrast the EBV-specific cellular responses are normal or reduced compared to those in healthy EBV carriers. In particular, at the level of tumor-infiltrating lymphocytes a reduced T-cell reactivity is noticed, suggesting of local tumor-induced immune suppression (tumor immune evasion). EBV-associated NPC occurs in immune competent individuals and would be considered capable of evoking cellular immune response to the virus. The tumor however, develops immune evasion through mechanisms that are not completely understood, but include resistance to apoptosis and local T cell silencing. NPC cells (in vitro) may secrete small vesicles, called exosomes, that may silence local T cells through LMP1 or cell regulatory molecules such as galectin-9. Furthermore, NPC cells secrete BARF1 protein, which has immunomodulating properties via scavenging of M-CSF (CSF-1), leading to altered behavior of tumor-infiltrating myeloid cells. Both BARF1 and LMP1 containing exosomes can be detected in the circulation of NPC patients, and may have general immune suppressive effects. Originating from the nasopharyngeal mucosal epithelium, NPC is characterized by infiltrating lymphocytes that secrete cytokines, such as TGF-β, IL5, IL6 and IL10. These cytokines induce class switching and as a consequence IgA antibody will be generated. The elevated IgG and IgA responses to EBV antigens reflect B cell responseto antigens expressed (early) during the tumor process, probably triggered by the release of viral components from the tumor site. Interestingly, IgG and IgA antibodies in NPC frequently have different antigen-recognition
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repertoire, suggesting distinct B cell triggering events. The viral lytic activation underlying NPC development triggers a strong humoral immune response, but tumor-associated membrane proteins, such as LMP1, LMP2, and BARF1 hardly induce a significant humoral immune response. The nuclear protein EBNA1 induces a strong antibody response, reflecting its release from tumor cells. This suggests that lytic virus reactivation accompanies the process of tumor development. However, a direct demonstration of this lytic process has not been achieved at the level of the tumor, except in the study of Zhang et al. which showed very limited viral replication in only some keratinizing tumor cells of NPC.

3. Epigenetic aspect in NPC

Epigenetics is defined as molecular factors and processes around DNA that regulate genome activity, independent of DNA sequence, and that are mitotically or meiotically stable. Different mechanisms are involved in epigenetic regulation and the development of cancer including DNA methylation, histone modifications and RNA interference. Among these epigenetic alterations, DNA methylation is probably the most widely explored.

3.1. DNA methylation and transcriptional repression

DNA methylation is a post replication modification in which a methyl group is covalently added to the 5-carbon of cytosine bases that are located in cytosine-guanosine dinucleotides (CpGs). The “p” in CpG represents the fact that they are phosphodiester-linked. In general, the CpG dinucleotide is greatly under-represented throughout mammalian genomes due to the spontaneous mutational deamination of 5-methylcytosine to thymine in the germline during evolution. However, there are regions called CpG islands which can be found in small genomic regions of about one kb to several kb. About 10-15% of total genomic CpG sites in mammalia occur in this CpG-rich regions. A CpG island is mostly located in the 5’ regions of approximately half of the human gene-promoter regions, thus interfering with the transcription initiation of the nearby gene. CpG islands are usually unmethylated in normal tissues. Exceptions are those of imprinted genes and X-chromosome inactivation which are methylated in the normal situation.

DNA methyltransferases (DNMTs) are enzymes responsible for maintenance of cellular DNA methylation pattern during cell division. All the DNMTs can catalyze the transfer of a methyl group to DNA and use S-adenosyl methionine as the methyl donor. Mammalian DNA methyltransferase can be classified into two general groups based on their preferred DNA substrate. DNMT1 is the most abundant DNA methyltransferase in mammalian somatic cells. It has a high affinity for hemimethylated DNA double strands and copies the methylation pattern after DNA replication. With the presence of DNMT1 it is therefore possible for replicating cells to maintain their pattern of gene expression from one cell generation to the
next. DNMT3a and DNMT3b have been characterized as de novo methyltransferases for their equal preference for hemimethylated and unmethylated DNA substrates. These enzymes are responsible for introducing cytosine methylation at previously unmethylated CpG sites.\textsuperscript{365}

DNA methylation functions as a host defense mechanism. Approximately 80% of the CpGs in the human genome are located in the non-clustered CpGs in repetitive sequences within parasitic DNA elements or retrotransposons (e.g. endogenous retrovirus, L1 elements or ALU elements). These non-clustered CpGs are usually hypermethylated and therefore DNA methylation occurs to silence the expression of these elements and limits their spread through the genome. Otherwise, these parasitic DNA elements will mediate recombination between non-allelic repeats which may result in chromosome rearrangements or translocations. In addition, transcription activation of retrotransposons within the introns of coding genes may interfere with the expression of host genes.\textsuperscript{366,367}

Another function of DNA methylation is transcriptional repression. DNA methylation can affect gene expression by two different mechanisms. First, DNA methylation can prevent binding of specific transcription factors to CpG-containing DNA-binding elements.\textsuperscript{368} In the second mechanism, certain methyl-CpG-binding proteins, such as MBD1, MBD2, MBD4, methyl cytosine binding protein (MeCP) especially MeCP2, and Kaiso, specifically recognize mammalian methylation marks.\textsuperscript{369,370} These proteins can either directly repress transcription or recruit enzymes that catalyze posttranscriptional modifications of histones. They can also recruit chromatin-remodeling complexes that affect the chromatin structure. Most genes silenced by methylation are mediated through this way.\textsuperscript{371}

Genomic DNA is highly folded and compacted by histone and non-histone proteins in the form of chromatin. The basic unit of chromatin is called nucleosome, which is typically composed of an octamer of the four core histones H\textsubscript{A}, H\textsubscript{2B}, H\textsubscript{3} and H\textsubscript{4} with 146 base pairs of DNA wrapped around the histones. Each core histone has amino acids at the N-terminal end of about 25-40 residues long.\textsuperscript{372} This histone tail can be subjected to various post-translational modifications, including methylation, acetylation, phosphorylation of serines and ubiquitination. The histone modifications are considered to form a histone code that interacts with chromatin-associated proteins and translated into a transcriptionally active or repressed genetic state.\textsuperscript{373} The functional consequences of the methylation of histones depends on the type of residue, lysine (K) or arginine and the specific site that the methylation modifies (e.g., K\textsubscript{4}, K\textsubscript{9}, or K\textsubscript{27}).\textsuperscript{374,375} Methylation of H\textsubscript{3} at K\textsubscript{4} is closely linked to transcriptional activation, whereas methylation of H\textsubscript{3} at K\textsubscript{9} or K\textsubscript{27} and of H\textsubscript{4} at K\textsubscript{20} are associated with transcriptional repression.\textsuperscript{376}

Acetylated histones are usually associated with transcriptionally active chromatin which is permissive for transcription factor binding to the cognate sites. Deacetylated histones can reserve the process of chromatin activation and thereby are associated with inactive chromatin. The mechanism underlying this chromatin switch is the fact that acetylation can neutralize the basic charge of histone tails.
which may reduce the affinity between histone-histone, histone-DNA and histone-other regulatory proteins. The binding of methyl-CpG-binding proteins (MeCPs) to the methylated CpG islands could recruit histone deacetylase complexes, which remove the acetyl groups from the N termini of the histones, and causing a more condensed conformation so that transcription factors cannot reach the target DNA. Thus, CpG island hypermethylation causes a change in chromatin structure and in histone modification collectively resulting in a transcriptionally silenced state (Figure 5). The enzymes responsible for bringing about the steady-state balance of histone acetylation/deacetylation are histone acetyltransferase (HATs) and histone deacetylase (HDACs).

Figure 5. Chromatin structure of active and inactive promoter. A. Transcriptionally active chromatin is characterized by unmethylated cytosines and hyperacetylation of histones H3 and H4 at multiple sites and trimethylation of histone H3 at lysine 4. B. When cytosines become methylated they bind MBDs that attract HDACs. HDAC remove acetyl groups (Ac) from the histone tails (H3 and H4). Complexes containing one or both of the histone methyltransferases G9a and SUV39H1 are responsible for trimethylation of H3 at lysine 9 (H3K9me3), establishing chromatin in a ‘closed’ repressive state. Methyl cytosines established by the DNMTs serve as docking sites for methyl-CpG binding domain (MBD) proteins such as MeCP2.

2.2. Epigenetic regulation of EBV gene expression

EBV is a typical virus using the host DNA methylation program for silencing its viral genes, either in normal or neoplastic tissue. By epigenetic mechanism EBV regulates its promoters and limits viral gene expression thus suppressing a
family of immunodominant antigens. A transcriptional regulation is important for viral persistence in the host eluding from potent immune response generated against EBV proteins. In tight latency there is no virus production and only a limited set of viral promoters is activated. By this trick EBV can escape the host immune surveillance and contribute to the carcinogenesis of EBV related malignancies (see Figure 4).

2.2.1. Methylation and EBV latent genes

Latent EBV promoters control the expression of viral genes that are associated to growth and transformation. Latency type gene expression is regulated largely through differential promoter utilization. Upon initial infection of B cells, Wp is the first and only promoter activated. Initial transcription from Wp promoter allows the expression of EBNA2, which is then recruited to Cp indirectly by binding to a DNA-binding protein and transcriptional repressor RBP-Jκ/CFB1. Later Wp transcription is shut down due to de novo promoter methylation and Cp becomes active and remains the dominant latent promoter in established LCLs. Wp is heavily methylated in PBMCs from normal individuals and constantly hypermethylated in all EBV-associated carcinomas and lymphomas.

Methylation of the EBNA2 response element and its downstream region completely silences Cp through the direct inhibition of RBP-Jκ/CFB1 binding and the binding of transcriptional repressors (MeCP2, MBD1-4) to these regions. This also increases H4 and H3 acetylation and may be associated with a chromatin structure permissive for transcription in latency III. Cp is variably methylated in normal PBMCs, unmethylated in latency III cell lines and tumors (PTLD), but hypermethylated and silenced in all the EBV-positive B cells and all the EBV positive cell lines and tumors with a latency I or II infection (BL, NPC, NL, HL and gastric carcinoma).

EBNA1 expression can be driven by four promoters, Cp, Wp, Qp and Fp. During the earliest stages of infection transcription is initiated from Wp. Once host cell transformation is established there is a switch towards the Cp. They also encode EBNA 2-6 and BHRF1-linked miRNAs, which are expressed upon differential splicing events. In latency I, where EBNA1 is only expressed, Wp and Cp are silenced and EBNA1 expression is driven largely through the Qp. EBNA1 mRNA expression is maintained in latency III by Cp promoter utilization and mRNA processing. Qp is considered as the major latent promoter for EBNA1 transcription in vivo. EBNA1 protein restricts the usage of Qp transcription in type III cells where EBNA1 proteins levels are elevated. In the absence of EBNA1 protein in the cell Qp is most active. Thus, EBNA1 can auto regulate its own expression levels through promoter selection and help to coordinate the switch between latency types. By using Qp EBV expresses the indispensable viral protein EBNA1 when all other EBV proteins are switched off. Qp is unmethylated in normal PBMCs, although its expression is variable. Moreover, Qp remains as hypomethylated in all situations.
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(EBV-associated tumors and cell lines)\textsuperscript{219,407} and therefore remains highly active. Fp is an early lytic promoter, being activated when EBV enters into lytic cycle,\textsuperscript{408,409} but virtually silenced in latency I and II cell lines or tumors.\textsuperscript{410} Fp is also densely methylated in PBMCs from normal individuals,\textsuperscript{391} but demethylated and active in early IM, PTLD and AIDS-associated oral hairy leukoplakia tissues.\textsuperscript{411}

LMP1 is present in the majority of EBV-driven tumors but appears absent in some neoplasms, in particular in Burkitt’s lymphoma (where \textit{c-myc} drives the cell cycle progression) and EBV positive gastric cancer. In NPC, the variability of LMP1 expression between different tumors and within a tumor can be related to the methylation status or regulatory sequences (LMP1 regulatory sequence/LRS). LMP1 is expressed in NPC isolates with unmethylated LRS but is absent from the ones with highly methylated LRS. A relationship between LMP1 expression and hypoxia-related host (de)methylation and HIF1α expression was not found for NPC.\textsuperscript{331} Latency type I cells do not express LMP1 and are highly methylated at LRS whereas LRS is unmethylated in latency type III cells and latency type II, which express LMP1. EBNA2 also activates LMP1 (and LMP2) transcription to maintain type III gene expression.\textsuperscript{412}

\subsection*{2.2.2. Methylation and EBV lytic genes}

The EBV lytic cycle is triggered by expression of two immediate early genes, Zta and Rta, that are driven from the early gene promoters, Zp and Rp. The tight regulation of Zp and Rp is equally crucial for viral persistence as for Wp and Cp. Activity of the Zp is regulated by AP-1, CREB, YY1 and Zta itself. Rp is regulated by Sp1, YY1 and Zif268. Zp and Rp are found hypermethylated in nearly all latency I and II EBV Burkitt’s lymphoma cell lines. In EBV-associated tumors, including BL, NPC and nasal NK/T lymphomas, both promoters are heavily hypermethylated and Zta and Rta transcripts are not detectable. DNA methylation inhibitor 5-azacytidine induces activation of the lytic cycle in LCLs. DNMT3A knockout mice was used and this revealed that Zp was more dependent on proper methylation than Rp. This indicates that DNA methylation may not be a major regulator of Zp activity in LCLs but for Rp it is.\textsuperscript{394} The reliance of EBV on DNA methylation to regulate its early lytic gene transcription may vary with cell type or with the transcription factor milieu. More recently it is observed that DNA methylation of Rp inhibits Rta activation by cellular transcription factors. Surprisingly, it enhances the ability of Zta to activate Rp and therefore is considered to be a unique feature of Zta.\textsuperscript{413} Rp is the only EBV promoter known to be regulated in this unusual manner. It has also been demonstrated that methylation-dependent Zta binding to critical viral promoters may enhance lytic reactivation in latently infected cells, where the viral genome is heavily methylated. The methylation-dependent Zta activation may also help the virus to establish latency following infection.\textsuperscript{414}
2.3. Epigenetic role in NPC development

NPC is distinguished from other malignancies by the number of genes targeted for silencing by promoter methylation. The key TSGs such as p53 and Rb which are found to be mutated in 50% of all tumors are not frequently mutated in NPC. Extensive observations for activated oncogenes or tumor suppressor loss did not show characteristic translocations, mutations in p53 or Rb alterations, or activating Ras mutations.\textsuperscript{415-417} This suggests that EBV genes may affect these pathways, directly or indirectly, such that there is no selection for mutation in these genes.\textsuperscript{418} However, additional genetic changes could develop during tumor growth and contribute to tumor progression and metastasis.\textsuperscript{419} In contrast, epigenetic events of known or candidate TSGs has been reported in NPC. Alternative to genetic changes, aberrant epigenetic mechanisms disrupt multiple genes involved in cell signaling pathways through DNA methylation of promoter CpG islands and/or histone modifications. These epigenetic alterations grant cell growth advantage and contribute to the initiation and progression of NPC by alterations in the multistep oncogenesis such as apoptosis, cell cycle and mitotic checkpoint regulation, intracellular adhesion, DNA damage repair, cytoskeleton organization, Wnt-signaling pathway, tumor invasion and metastasis.\textsuperscript{419,420} Moreover, p53 regulation in NPC is also due to methylation-mediated silencing which promotes p53 signaling by deubiquitinating p53 and p14 and ubiquinating MDM2 for degradation and p53 stabilization.\textsuperscript{421}

Rass association family 1 gene (RASSF1A), a TSG involved in Ras signaling, cell cycle arrest, apoptosis and DNA repair, and p16, a cell cycle regulation gene, were first TSGs detected as hypermethylated in NPC. By PCR screening in NPC samples it was detected that the p16 cyclin dependent kinase inhibitor at 9p21 and the RASSF1A gene at 3p14 are frequently lost.\textsuperscript{422} In vitro studies have shown that reintroduction of the RASSF1A gene into an NPC cell line inhibited growth.\textsuperscript{423} This finding suggests that inactivation of RASSF1A is an important contributor to NPC development. Through more extensive epigenetic studies, many more putative TSGs were demonstrated to be silenced by epigenetic alterations in NPC than previously thought, indicating that the molecular pathogenesis of NPC is complex. High levels of CpG methylation spread throughout the cellular genome especially in EBV-associated NPC and many candidate TSGs were aberrantly methylated in their 5' CpG islands (Table 2).

2.4. Interaction between EBV and epigenetic modulation in NPC

Comparison between EBV-positive and EBV-negative head and neck cancers showed higher frequency of epigenetic changes in EBV-associated NPC. This suggests a relationship between EBV latent infection and aberrant methylation in this cancer. Based on our observations and other existing reports, a pathogenesis model for NPC was proposed as a consequence of combined exposure to specific carcinogens, genetic changes and EBV infection (Figure 6).
Table 2. List of methylated candidate TSGs involved in cell signaling in NPC.

<table>
<thead>
<tr>
<th>Classification</th>
<th>TSG</th>
<th>Full name</th>
<th>Other name</th>
<th>Location</th>
<th>Functions</th>
<th>Alterations in NPC</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rass GATPase</td>
<td>DAB2</td>
<td>Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)</td>
<td>DOC2</td>
<td>5p13</td>
<td>mitogen-responsive phosphoprotein, suppresses the mitogenic signaling via Ras pathway, cell differentiation, cell proliferation</td>
<td>methylated</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>RASAL1</td>
<td>Ras protein activator like 1 (GAP1 like)</td>
<td>RASAL</td>
<td>12q23-24</td>
<td>Ras GTPase-activating protein, negatively regulate Ras signaling</td>
<td>methylated</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>RASSF1</td>
<td>Ras association (Ra/GDS/AF-6) domain family 1</td>
<td>RASSF1A</td>
<td>3p21.3</td>
<td>Ras effector protein, Ras signaling, cell cycle arrest, apoptosis, DNA repair, inhibits type accumulation of cyclin D1</td>
<td>methylated, mutated</td>
<td>10-14</td>
</tr>
<tr>
<td></td>
<td>SCGB3A1</td>
<td>Secretoglobin, family 3A, member 1</td>
<td>HIN1</td>
<td>5q35-qter</td>
<td>AKT signaling pathway, cell communication</td>
<td>methylated</td>
<td>16</td>
</tr>
<tr>
<td>Rho GTPase</td>
<td>DLC1</td>
<td>Deleted in liver cancer 1</td>
<td>ARHGAP7, STARD12, p122 RhoGAP</td>
<td>8p22.3</td>
<td>Cell cytoskeleton organization, GTPase activator, signal transduction, cell adhesion, invasion</td>
<td>methylated, deleted (LOH)</td>
<td>18</td>
</tr>
<tr>
<td>p53</td>
<td>UCHL1</td>
<td>Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thio/esterase)</td>
<td>PARK5, PGP9.5</td>
<td>4p14</td>
<td>Apoptosis, binds p53/MDM2 complex and activates p53 signaling</td>
<td>methylated</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>TP73</td>
<td>Tumor protein p73</td>
<td>p73</td>
<td>1p36.3</td>
<td>Cell cycle, DNA damage response, apoptosis, transcription factor</td>
<td>methylated</td>
<td>10</td>
</tr>
<tr>
<td>Wnt/β-catenin</td>
<td>WIF1</td>
<td>Wnt inhibitory factor 1</td>
<td>WIF-1</td>
<td>12q14.3</td>
<td>Wnt signaling pathway, binds and inhibits WNT proteins, protein tyrosine kinase activity</td>
<td>methylated</td>
<td>26</td>
</tr>
<tr>
<td>Gene</td>
<td>Chromosome</td>
<td>Function</td>
<td>Abnormalities</td>
<td>Other Remarks</td>
<td></td>
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</tr>
<tr>
<td>BRD7</td>
<td>9p21</td>
<td>Transcriptional regulation, cell cycle regulation</td>
<td>methylated</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>CDKN2A</td>
<td>9p21</td>
<td>Cell cycle regulation, inhibits CDK4 kinase</td>
<td>methylated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16, INK4A</td>
<td>9p21</td>
<td>Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)</td>
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<td></td>
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<td>PTEN</td>
<td>10q23</td>
<td>Cell cycle regulation, upregulates p21 and p27 protein</td>
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<td>MGMT</td>
<td>10q26</td>
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<td>methylated</td>
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<td></td>
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<tr>
<td>MLH1</td>
<td>3p21.3</td>
<td>DNA mismatch repair protein, cell cycle G2/M arrest</td>
<td>methylated</td>
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</tr>
<tr>
<td>FHIT</td>
<td>3p14.2</td>
<td>Cell cycle regulation, G1-S phase regulation, upregulates p21 and p27 protein</td>
<td>methylated</td>
<td></td>
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</tr>
<tr>
<td>MIPOL1</td>
<td>14q13.3</td>
<td>DNA damage response</td>
<td>methylated</td>
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<td>DNA damage response</td>
<td>methylated</td>
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<tr>
<td>MGMT</td>
<td>3p21.3</td>
<td>DNA repair, senses and integrates DNA damage/repair-related signals with replication, cell cycle and genomic instability</td>
<td>methylated</td>
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<td>AIF</td>
<td>16q12</td>
<td>Alternate open reading frame</td>
<td>methylated</td>
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<td>BRD7</td>
<td>16q12</td>
<td>Transcriptional regulation, cell cycle regulation</td>
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<tr>
<td>CDKN2B</td>
<td>16q12</td>
<td>Cell cycle regulation, inhibits CDK4 kinase</td>
<td>methylated</td>
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<td>ARF</td>
<td>14q13.3</td>
<td>Cell cycle regulation, G1-S phase regulation, upregulates p21 and p27 protein</td>
<td>methylated</td>
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<td>CHFR</td>
<td>12q24.33</td>
<td>Mitotic checkpoint protein early in G2-M transition, cell cycle regulation</td>
<td>methylated</td>
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<td>FHIT</td>
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<td>MPO1L</td>
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<td>Cell cycle regulation, G1-S phase regulation, upregulates p21 and p27 protein</td>
<td>methylated</td>
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<td>DNA damage response</td>
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<tr>
<td>GADD45G</td>
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<td>DNA damage response</td>
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<td>MGMT</td>
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<td>BRD7</td>
<td>9p21</td>
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<td>CDKN2A</td>
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<td>p16, INK4A</td>
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<td>Cell cycle regulation, upregulates p21 and p27 protein</td>
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<td>CDHE, ECAD, LCAM, CD324</td>
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<td>OPCML</td>
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<td>OPCM, OBCAM</td>
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<td>CASP8</td>
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In Indonesia EBV infection occurs at a young age and children are exposed to frequent infection/inflammation of the head and neck region, triggering frequent EBV reactivation. Increased EBV reactivation, evidenced by EBV DNA load in saliva and elevated EA and VCA IgG as well as IgA serology frequently precedes NPC. Due to poor food quality, frequent smoking and other environmental factors Indonesian people are exposed to (co-)carcinogenic substances from a young age onwards. These combined factors may be predisposed to NPC development, facilitated by EBNA1 and LMP1 driven enhanced survival of “damaged” cells. EBV-encoded proteins (such as LMP1, LMP2A and other viral proteins) are key players in disrupting cell signaling in NPC through aberrant promoter methylation. Furthermore, the methylation of these TSGs could serve as epigenetic biomarkers. The overview of the role of epigenetic disruption of cell signaling regulators mediated by EBV infection is summarized in Figure 7.
Figure 7. Overview of the role of epigenetic disruption of cell signaling regulators mediated by EBV infection during NPC tumorigenesis.\textsuperscript{426}

DAB2, disabled-2; DLC1, deleted in liver cancer 1; DNMT, DNA methyltransferase; EBV, Epstein-Barr virus; HIN1, high-in-normal 1; LMP1, latent membrane protein 1; LMP2A, latent membrane protein 2A; NPC, nasopharyngeal carcinoma; PcG, Polycomb protein; RASAL1, Ras GAP-activating-like protein 1; RASSF1A, Ras association domain family 1A; TSG, tumor suppressor gene; UCHL1, ubiquitin carboxyl-terminal hydrolase L1; WIF1, Wnt inhibitory factor-1. Ras GTPase signaling negative regulators, Rho GTPase signaling negative regulators, p53 signaling positive regulators, Wnt/catenin signaling negative regulators, cell cycle control-DNA damage signaling regulators, cell adhesion regulators and apoptosis regulators play important roles in the initiation and progression of NPC. Epigenetic silencing of these antagonists or activators through promoter CpG methylation or histone modifications, initiated or mediated by EBV-encoded viral proteins, disrupts multiple cell signaling pathways during NPC tumorigenesis.

Next to silencing the viral genes, EBV has capabilities in altering the host genome methylation profile. Such modified methylation profiles in cellular genes may play roles in viral pathogenesis and tumorigenesis. Epithelial cells expressing LMP1 have higher invasive ability in correlation with reduced E-cadherin expression.\textsuperscript{425} A reduction of E-cadherin is observed as a result of LMP1-induced hypermethylation of E-cadherin gene promoter through activation of DNMT3A and 3B and triggering the JNK-AP-1 signaling pathway.\textsuperscript{426} It was also demonstrated that DNMT1, 3A, 3B,
MeCP2 and HDAC1 form a protein complex on the E-cadherin promoter in LMP1 positive cells. Addition of demethylating drugs in LMP1 expressing epithelial cells restored the expression of E-cadherin, which suggests that LMP1 regulates the expression of specific cellular genes via epigenetic mechanisms. The E-cadherin promoter was methylated in about 50% of primary tumor which increased with advanced and invasive tumor stages. Three of the miR-BART target the 3’-untranslated regions of the LMP1 mRNA and block the translation of the LMP1 protein in NPC cells. The high level expression of those miRNAs in NPC tumors may explain the low level LMP1 expression in some NPC tumors.\(^\text{109}\) p53 protein has also been shown to be regulated by various post-translational modifications such as phosphorylation and ubiquitination, modulated by LMP1.\(^\text{427,428}\) More importantly, it was recently demonstrated that the universal EBV protein EBNA1 subverts the regulation of p53, by binding to USP7/HAUSP and inhibiting p53-deubiquitination, leading to increased p53 turnover. Hereby EBV may overcome p53 mediated cell cycle control, a key event in the control of normal cell proliferation.\(^\text{238}\)

The role of EBV in DNA methylation was also observed for EBNA3A and EBNA3C, which contribute to enhanced B cell survival by inhibiting the expression of the death-inducing protein Bim.\(^\text{429}\) This repression involves remodeling of the Bim gene promoter by polycomb proteins and DNA methylation within an unusually large CpG-island that flanks the transcription initiation site.\(^\text{430}\) EBNA3A and EBNA3C functionally cooperate in the polycomb-mediated chromatin remodeling of p16. Both EBV proteins can bind the highly conserved co-repressor of transcription CtBP, and these interactions appear to be required for the efficient repression of p16. Thus by inducing the heritable repression of two major TSGs, one that induces cell death (Bim) and one that induces growth arrest (p16), EBV profoundly alters latently infected B cells and their progeny.\(^\text{431}\) Generally it seems that EBV has established epigenetic strategies to alter host gene expression to facilitate its life cycle. In B cells EBNA3 proteins enhance cell survival and proliferation whereas in epithelial cells the LMPs appears to be more important.\(^\text{432}\) In each case the infected cells become more prone to malignant transformation.\(^\text{378}\) The disruption of DNA repair by EBNA1 acting on PML bodies, together with EBNA1-driven loss of p53 control and EBNA1 inducing oxygen radical formation to induce genetic damage, may increase the oncogenic risk in EBV carrying cells, whereas EBNA1-driven upregulation of survival may stabilize genetically damaged cells, thus evolutionary contributing to increased survival of the infected cell.\(^\text{240,433}\)

### 3. Early identification of nasophayngeal carcinoma in Indonesia

NPC is the most common head and neck malignancy in Indonesia, with an annual incidence rate of 6.2/100,000 population recorded in our local region.\(^\text{434}\) In year 2006-2010, 90% of NPC patients coming to Dr Sardjito Hospital in Yogyakarta represented WHO type III, being closely linked to EBV infection.\(^\text{52}\) (Hariwiyanto
Early stage NPC has much better treatment response rate and improved 5-year survival rate with radiotherapy alone. Late stages NPC has more severe morbidity and the standard treatment is chemo-radiotherapy which has increased side effects. In our local hospital, during the last five years, 86% of patients came with late stage NPC, mainly due to the vague and unidentified symptoms of NPC in early stage. As people mostly are affected at the prime of their life (peak age-incidence at 35-65 years), the impact of NPC is generally devastating and has great socio-economic impact. These situations negatively affect the treatment success and increases the mortality rate. Accordingly, the NPC treatment results in our local center are poor.

Like many other cancers, three fundamental approaches could help to reduce the poor outcome of NPC. These include primary prevention, early detection of disease (secondary prevention) and improved treatment of established cancer. Indeed, with rapid technological advances in imaging methods, improving radiotherapy techniques, and integration with chemotherapy, much better treatment results have been achieved in the modern era. In our local situation in Indonesia there has been no decrease of NPC incidence or death rate. Importantly, NPC screening as a means of secondary prevention was limited by the general lack of community and clinician knowledge of the symptoms of NPC whereas recently developed and available screening methods are relatively affordable and available. Well-organized screening has never been conducted in Indonesia despite the disease’s high incidence (the same problem holds for other types of cancer as well). The importance of introducing a screening program for early detection of NPC is clinically relevant. Primary prevention should aim at risk factor education of the community, introducing food-quality programs at the government level for decreasing NPC risk factors in our local population and educating regional primary health care workers for identification of NPC at early stage.

Regarding NPC screening, NPC-associated biological markers are not yet available in Indonesia due to cost and lack of available standardized laboratories and trained staffs. Commercial test currently available in the market have disputable diagnostic value and are not well optimized for the ethnically diverse target population in Indonesia. While histological examination of nasopharyngeal biopsy is the classical standard for NPC diagnosis in clinical setting, the invasiveness of the screening procedure makes it not suitable as an early screening approach in high risk populations of NPC. Therefore less or non-invasive methods will need to accompany a good screening program performed on a large scale. To perform an NPC downgrading program, the population at risk should be defined, NPC-related risk factors specific to Indonesia need to be determined, awareness of the disease and its early stage symptoms should be increased and a screening program needs to be prepared.
3.1. Population at risk of NPC

For a screening program to be cost-effective, the target population should have a relatively high risk of getting the disease.\textsuperscript{339,440} Multiple studies in China and Taiwan showed that individuals presenting with high IgA anti-EBV levels, with a similar antigen reactivity pattern to NPC cases, have higher risk to develop the diseases compared to others who did not have the elevated antibodies.\textsuperscript{47,48,441} The presence of elevated EBV IgA antibody may precede the clinical appearance, even by as long as ten years.\textsuperscript{48,442} Based on these studies, the EBV IgA positive population currently serves as the best group to undergo serology follow-up and long-term monitoring. As a consequence, a framework for a major screening program needs to be implemented in a general population. Such a mass screening is not easy to conduct in our local population which is resource-limited and suitable tests are not yet available. A case finding program on a selected population presenting with suspicious symptoms of NPC will be a more feasible approach, as presented in this thesis. A history of chronic ear and nose disease was thought to double the risk for having NPC.\textsuperscript{443-445} Therefore, individuals with suspicious early symptoms of NPC should be subjected to preliminary risk-assessment before more extensive clinical evaluation. However, NPC is one of the most difficult diseases to recognize at an early stage due to few early non-specific symptoms and its hidden location. More attention should be paid to the general chronic problems in the head and neck area to facilitate the awareness of insidious symptoms. Moreover, more careful examination by nasoendoscopy and consultation to ENT department should be part of good clinical management. Very few data are available on this selected population of individuals with chronic complaints regarding to the presence of IgA anti-EBV.\textsuperscript{446}

In addition to populations having elevated IgA against EBV, individuals with a family history of NPC were also demonstrated to be at higher risk of developing the disease, thereby serving as another target population for screening. Family aggregation has been well documented by many epidemiology studies, either in high- or low-risk regions of NPC.\textsuperscript{29-31,447} First-degree relatives of NPC patients of multiplex cases (more than one NPC affected in the family) have relative risk (RR) 3.3 compared to the ones with only one NPC affected.\textsuperscript{448} It has also been demonstrated that this population has a higher frequency of elevated IgA anti-EBV compared to the general community,\textsuperscript{62} and having elevated EBV IgA poses a significant risk (RR of developing NPC in later years).\textsuperscript{448} However, since the familial risk factors for NPC may also associate with shared genetic susceptibility, environmental factors and social habits between family members, a family history of scattered case of NPC may also serve as a target for NPC screening. Since there are many risk factors predisposing to NPC development, the non-viral environmental factors or (co) carcinogenic substances, such as dietary and non-dietary exposure should also be investigated.
3.2. EBV-based markers for NPC diagnosis and screening

The vast majority of NPC cases are associated with EBV expression, making EBV-based assays of utmost importance for NPC identification. The method first developed for this purpose was Southern blot hybridization which then was replaced by polymerase-chain reaction (PCR) amplification techniques. Compared to PCR, Southern blot is less sensitive, more complicated and requires more than 1% of target DNA to represent EBV. Real-time quantitative PCR (Q-PCR), which can quantify EBV copy number, has replaced the conventional PCR. Elevated numbers of EBV DNA may reflect the increase of EBV activity at present, either from body fluids, lymphocytes or epithelial cells. Q-PCR is currently the preferred method for diagnosis and follow-up as well as prognosis of NPC treatment, but the methodology is not well-standardized. Aberrant viral activity may also be reflected in the pattern of transcription of EBV DNA into RNA, which can be detected by reverse transcriptase PCR (RT-PCR) and NASBA (nucleic acid sequence based amplification). RT-PCR allows detection of small amounts of RNA, by first transcript RNA to cDNA which is then amplified by using conventional PCR. NASBA is an isothermal RNA amplification method to detect unspliced transcript even in the presence of background DNA. NASBA provides better sensitivity and rapidity compared to RT-PCR. Identification of EBV-infected cells by the expression of viral gene product can be applied by morphological technique. EBER-RISH (RNA in situ hybridization) is widely used for this purpose based on the abundant expression of EBER1 and EBER2 in most EBV-positive cells. This staining is currently the golden standard for EBV detection in tissue. EBER-RISH can be complemented by monoclonal antibody-based immunohistochemistry for detection of latent and lytic viral proteins. Serology is a method to determine the status of EBV infection by measuring antibody reactivity against EBV antigens and is the most widely used at present. Generally low IgG and virtually no IgA response to EBV proteins are found in healthy EBV carriers. The opposite situation is found among NPC patients showing increased anti-EBV IgG and IgA responses, both in level (titer) and diversity (spectrum of antigens recognized). The increase in IgG or IgA antibody response is directed towards more diverse EBV proteins, with elevated IgA to EBV proteins characterizing NPC and reflecting the disease’ mucosal origin. Nevertheless, no direct correlation between molecular markers and immune responses are observed because a rise in EBV (DNA or RNA) activity does not always evoke a parallel level of antibody responses. Differences observed for these parameters can be used to discriminate healthy virus carriers from diseased ones and are relevant for the development of diagnostic tests.

IFA serology was developed early upon EBV discovery and used for detection of IgG or IgA response to antibody response to EBV complex, such as EBNA, EA(D) and VCA. The staining uses glass-immobilized, intact EBV-positive cells expressing different sets of viral antigens to determine the antibodies. IFA technique has provided information about the dynamics of antibody responses during primary EBV infection, asymptomatic carriers and in various disease states.
IFA detection of IgA response to EA(D) and VCA complexes have been widely used in NPC diagnostic work up.\textsuperscript{458} IgA to VCA shows a high sensitivity (up to 97%),\textsuperscript{459} while IgA to EA(D) displays high specificity.\textsuperscript{459,460} Therefore, IgA VCA is used largely as a tool for primary diagnosis\textsuperscript{461} and screening\textsuperscript{48,442,448,462} of NPC, with IgA EA(D) used for confirmation.\textsuperscript{463} Both IgA VCA, IgAEBNA1 and IgA EA(D) have also been proposed as a marker for treatment monitoring and may predict disease relapse.\textsuperscript{51,464}

Even though IFA is still the gold standard for NPC serodiagnosis, it is labor intensive and requires well-trained personnel to produce the cell slides and read the resulting staining patterns.\textsuperscript{465} Application of IFA method as well as EBER (the golden standard method for EBV presence) for routine use is still complicated. Both are limited by technical difficulties, time-consuming, unsuitability for automation and subjective interpretation due to lack of standardization.\textsuperscript{466} Therefore, they are not considered convenient for screening purpose. Simpler technique that can be applied for mass sampling and with possible automation is sorely needed. Specific ELISA tests can overcome some difficulties associated with IFA technique,\textsuperscript{53,355} and can be an alternative method that replace IFA. In some studies IFA was found to be less sensitive compared to ELISA when using a single EBV antigen. Single EBV antigen ELISA formats using immunodominant markers for detection of IgA VCA or IgA EA may provide adequate alternatives. Currently EBV immunoblotting and ELISA developed by our group confirmed the IFA results to a molecular feature.\textsuperscript{52,53,463} Furthermore, multiplex bead technique is a novel approach allowing simultaneous assessment of antibodies to multiple EBV antigens making EBV serology less labor intensive and providing standardization, assuming well-defined protein-antigens are used.\textsuperscript{467} However, this still needs further development.

The exploration on EBV EBNA1 and individual lytic antigens separately in various formats to IgA EBV ELISA (EA and VCA-derived antigens) had an unsatisfactory outcome. The results obtained with some purified components were in conflict with those of other studies. Overall, no consensus exists on the use of a single protein marker to diagnose NPC. This underlies the variation in antibody reactivity to multiple EBV antigens, differences in antigenic sources, selective epitope use, and populations studied, making attempts to use one-type ELISA universally difficult. An ELISA system based on use of two chemically well-defined and cheap synthetic peptides representing immunodominant epitopes of EBNA1 and VCA-p18 antigens showed relevance for NPC screening. The sensitivity and specificity of using this method were 90.1\% and 85.4\%,\textsuperscript{53} A dried blood sampling on filter paper derived from finger prick blood taking was further optimized. This method offered simpler sampling, easy transportation and handling. This also retained IgA antibody stability, efficient storage and comparable IgA and IgG against [EBNA1+VCA-p18] reactivity compared to fresh plasma taken from the arm.\textsuperscript{55}

The molecular diversity of IgG/IgA responses was used to identify the immunodominant EBV marker for discriminating NPC from healthy and non-NPC subjects. The study defined which antigens among the 80 proteins of EBV lytic antigens are useful candidates to formulate simpler yet sensitive and specific
methods for NPC screening.\textsuperscript{52} It was found that healthy individuals and NPC patients from different endemic areas shared a characteristic IgG and IgA response pattern to EBV. Healthy EBV carriers showed a restricted IgG response to EBNA1 and VCA-p18, and occasionally to VCA-p40 and ZEBRA, whereas they have a negative or weak IgA response to EBV antigens. NPC subjects showed stronger and more diverse IgG and IgA responses toward different types of EBV antigens, indicating different antigenic trigger.\textsuperscript{52,460} This EBV immunoblot diversity (scoring) provided significant diagnostic value discriminating between NPC and non-NPC tumors. The individual antigens identified by immunoblot were further analyzed to identify the (conformational or linear) epitopes involved in antibody-antigen interaction, yielding several sets of immunodominant EBV peptides for diagnostic use.\textsuperscript{53,463} Using immunoblot as a confirmation test for the above mentioned IgA/[EBNA1+VCA-p18] ELISA, the sensitivity and specificity for NPC diagnosis increased to 98.0% and 99.2% in the Indonesian NPC samples.

Nasopharyngeal biopsy is a golden standard for NPC diagnosis requiring an invasive approach which is difficult to perform in patients without obvious sign. Moreover, nasopharyngeal biopsy is not a convenient method for repeated procedure during long term clinical monitoring. The use of the nasopharyngeal brushing method in the nasopharyngeal region, resembling cervical scraping method used for cervical smears, offers a less invasive sampling method. From brushings information can be obtained through different approaches including cytology staining, EBV DNA quantification and EBV RNA profiling. EBV DNA quantification has been developed by EBV group VU University medical center based on amplification of a highly conserved region of the EBNA1 gene using standardized LightCycler PCR. The EBNA1 gene segment was chosen because of its highly conserved region among EBV isolates worldwide. Sensitivity and specificity of using this method for NPC diagnosis were 98% and 90%, respectively. EBV DNA further has been observed as a good marker for prognosis during NPC treatment.\textsuperscript{453,468} For setting of NPC clinical screening, EBV DNA has been proposed for complementary test to EBV-based serology.

In nasopharyngeal brushing, EBV mRNA has also been detected using NASBA assay for sensitive and specific detection in high EBV DNA backgrounds. This approach was able to detect BARF1 mRNA, the expression of an EBV-encoded gene which is greatly considered to be crucial for NPC development. Detection of BARF1 mRNA in NPC brushing were found in 85% isolates\textsuperscript{23} confirming previous reports of the high expression of BARF1 in NPC.\textsuperscript{276}

Regarding that another key success for NPC downgrading is the availability of tests with good diagnostic value, EBV IgA serology testing appears to fulfill the criteria. Like other EBV IgA test, ELISA IgA/[EBNA1+VCA-p18] may serve as a good screening test in our local population as it has been in-house well standardized, relatively cheap, yet reliable and less invasive. Immunobloting was proposed for
confirmation regarding its good diagnostic value confirming the positiveness derived from the ELISA assay. Since multiple markers may provide a better NPC detection, EBV DNA load in nasopharyngeal brushings can be a complementary method to the EBV-based serology tests. These assays may be applied for a screening program in a general community or in a case finding program conducted on a selected population with known risk factors. Determination of BART or BARF1 mRNA using NASBA is not a feasible method to be conducted in our center with regards of its relative high cost and the limited equipment available in our local center. Further exploration to define whether other assay can be developed and optimized to detect the presence of BARF1 (such as BARF1 antigen or antibody against BARF1) are needed.

3.3. Epigenetic (methylation) markers in NPC

Besides EBV infection, host gene may serve as a marker in carcinogenesis of NPC. Epigenetic marker is one among other proposed molecular markers readily applicable for diagnostic work. The DNA methylation and histone modification patterns associated with the development and progression of cancer have potential clinical use. DNA methylation of TSGs is widely known to be an early event in carcinogenesis and this marker is under exploration by many studies as complementary diagnostic tools. In addition, extensive exploration is also performed on epigenetic markers for prognostic factors and predictors of responses to treatment using its detection in various biological fluids and serum.\(^{469}\)

Gene promoter DNA methylation of many TSGs has been widely documented in NPC. Detection of DNA methylation has been reported in tumor biopsy, paraffin tissue and plasma. Moreover, the detection in nasopharyngeal local region has been reported with similar detection to fresh biopsy,\(^{470}\) proposing its role in a less-invasive approach of screening program in individuals with vague symptoms. DNA methylation can be detected by using methylation specific PCR (MSP), quantitative MSP, methylation-specific multiplex ligation dependent probe amplification (MS-MLPA) assay and bisulfite genomic sequencing. From all methylation-based assays MSP may be the simplest technique with relatively low cost that can be applied in developing countries. Since DNA methylation is also considered as an early event in NPC oncogenesis, its use in (early-stage) NPC identification needs further exploration.

4. Aim of the study

This thesis focuses on determining clinical, virological and host epigenetic markers for early identification of nasopharyngeal carcinoma in high risk populations in Indonesia. Several questions are raised as follows:
Chapter 2: Do the first degree-relatives of sporadic NPC patients present with elevated EBV IgA? Are they suitable candidates for a screening program?

EBV infection and family NPC history are widely known as strong risk factors associated with undifferentiated NPC. Screening of family members of multiplex NPC families has been done in China and Taiwan using IFA-based EBV IgA serology assays. Very few studies addressed the question whether family members of scattered NPC cases also have higher risk of getting NPC and thus showed aberrant reaction of IgA against EBV. This chapter determines whether first degree-relatives of sporadic NPC cases showed elevated IgA anti EBV compared to the community at large. The elevated EBV IgA antibody found in first-degree relatives will put them at risk and makes them suitable candidates for enrolling in a screening program and long-term monitoring. The use of the well-standardized ELISA IgA/[EBNA1+VCA-p18] will give information of its utility in setting up a more general screening program in our local population. The application of a simpler blood sampling method, finger prick blood collection (dried blood sample), was also studied for particular participants refusing venous blood collection. Moreover, the influence of non-viral factors as defining factor for EBV IgA seropositivity was also determined among first-degree relatives of NPC.

Chapter 3: What is the value of screening using EBV-based markers among individuals presenting with chronic problems in head and neck?

Seroepidemiology studies has been rarely conducted in a case finding approach among subjects presenting with clinical symptoms suspicious of early NPC. A 3-step EBV-based screening approach was designed and applied to selected patients with defined chronic head and neck complaints. The 3-step procedure consists of serology testing for EBV IgA, serologic confirmation using IgG diversity immunoblot score and EBV DNA measurement in nasopharyngeal brushings. These assays have revealed good diagnostic value in discriminating NPC patients from non-cancer populations at primary diagnosis. Yet their utility for identifying (early-stage) NPC cases in a screening program still needs to be investigated. This study analyzed serologic responses against EBV and viral load in prospectively examined individuals presenting with chronic problems in head and neck aiming to find NPC cases among such individuals.

Chapter 4. Is there sequence diversity in the BARF1 gene in NPC compared to healthy carriers and other EBV-associated diseases?

EBV BARF1-encoded protein was found to be expressed in most of NPC tumors and considered as an important EBV oncogene. Unlike LMP1, study of BARF1 sequence variation is limited. This study described BARF1 sequence variations among EBV isolates derived from NPC cases, healthy EBV-carriers and other EBV associated
disorders. The relationship of BARF1 genetic diversity to the EBV genotype, viral load and serology markers were investigated. Immunogenic properties of NPC derived BARF1 as a purified hexameric protein was also determined.

Chapter 5. Can antibody against BARF1 be used as a new diagnostic tool to identify NPC?

Aberrant EBV serology is generally used to support NPC diagnosis and provides an affordable approach for population screening. Antibodies targeting the BARF1 protein could be used as a new diagnostic tool to identify NPC patients. This study describes the expression and purification of Indonesian NPC-derived BARF1 having two conservative mutations, V29A and H130R (chapter 4), which are not considered to affect the three-dimensional (3D) structure significantly. The humoral immune responses against prototype BARF1 (B95-8) and purified native hexameric BARF1 were then analyzed in sera of Indonesian NPC patients compared to healthy EBV-positive and EBV-negative individuals.

Chapter 6. Can we improve NPC diagnosis by EBV-based assays using a complementary test for DNA methylation analysis?

Antibody levels against EBV may fluctuate and were shown to be elevated a long time before tumor presentation. It is therefore essential to define additional NPC progression markers for better selection of high risk patients. DNA methylation is widely considered to be an important epigenetic mechanism in carcinogenesis. In this study the frequency of promoter DNA methylation of multiple genes in Indonesian NPC cases was determined. To analyze the potential value for detection of early-stage NPC, the methylation pattern of NPC patients, healthy EBV carriers and a high risk population were compared. An additional TSG, the MAL gene, recently identified as epigenetic marker in human papilloma virus-associated cervical cancer but which has never been tested in NPC, was also analyzed.

Chapter 7. Can DNA methylation analysis be designed as multiple methylation specific PCR for easier implementation?

Multiple TSGs are known to be hypermethylated in NPC. Testing many TSGs is somewhat laborious. In this study an assay based on multiplex methylation specific-PCR (MMSP) was designed. The detection of multiplex information simultaneously in a single PCR reaction will allow detection of a panel of markers. These markers included two EBV genes and two TSGs which have been proven to be frequently methylated in NPC isolates. This study also tested nasopharyngeal sampling by swab to optimize a non-invasive approach for NPC identification.
Chapter 1

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