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Epstein–Barr virus infection and nasopharyngeal carcinoma

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Epstein–Barr virus (EBV) is associated with multiple types of human cancer, including lymphoid and epithelial cancers. The closest association with EBV infection is seen in undifferentiated nasopharyngeal carcinoma (NPC), which is endemic in the southern Chinese population. A strong association between NPC risk and the HLA locus at chromosome 6p has been identified, indicating a link between the presentation of EBV antigens to host immune cells and NPC risk. EBV infection in NPC is clonal in origin, strongly suggesting that NPC develops from the clonal expansion of a single EBV-infected cell. In epithelial cells, the default program of EBV infection is lytic replication. However, latent infection is the predominant mode of EBV infection in NPC. The establishment of latent EBV infection in pre-invasive nasopharyngeal epithelium is believed to be an early stage of NPC pathogenesis. Recent genomic study of NPC has identified multiple somatic mutations in the upstream negative regulators of NF-κB signalling. Dysregulated NF-κB signalling may contribute to the establishment of latent EBV infection in NPC. Stable EBV infection and the expression of latent EBV genes are postulated to drive the transformation of pre-invasive nasopharyngeal epithelial cells to cancer cells through multiple pathways.

This article is part of the themed issue 'Human oncogenic viruses'.

1. Overview of EBV as an oncogenic virus in human cancers

The Epstein–Barr virus (EBV) was the first human tumour virus to be discovered [1]. Also known as human herpes virus type 4 (HHV4), EBV is grouped together with other human pathogenic herpes viruses including herpes simplex virus (HSV) 1 and 2, varicella zoster virus (VZV), cytomegalovirus (CMV) and human herpes viruses (HHVs) 6, 7 and 8. It was first identified in Burkitt's lymphoma cell lines using electron microscopy [1]. The incidence of endemic Burkitt's lymphoma overlaps with malaria and HIV infection, suggesting that compromised host immunity is likely to play an essential role in the pathogenesis of EBV-associated malignancies [2,3]. EBV infection is detected in 30–40% of classical Hodgkin's lymphoma worldwide, a subset (approx. 10%) of gastric carcinomas (EBV-associated gastric cancer, EBVaGC) and 100% of non-keratinizing nasopharyngeal carcinomas (NPCs), the common histological subtype of NPC in endemic regions [2–4]. EBV infection is also detected in T- and natural killer (NK)-cell lymphomas and leiomyosarcomas and is responsible for post transplantation lymphoproliferative diseases [2]. As a lymphocryptovirus, EBV can undergo both latent and lytic phases of infection [5]. During latent infection, the EBV genome is replicated only once with each cell cycle. When induced by lytic reactivation, the EBV genome is amplified to generate a high number of viral genomes, which are packaged into infectious particles for transmission [5]. Lytic infection is the default program of EBV infection in normal epithelium, in contrast to latent infection, which is the predominant mode of EBV infection in epithelial cancers (NPC and EBVaGC) [2–4]. Hence, the establishment of latent EBV infection may represent a critical step in the pathogenesis of EBV-associated epithelial

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cancers. Presumably, somatic mutations and alterations of cell signalling in premalignant epithelial cells support the switching of the otherwise default lytic infection to latent infection, facilitating the persistent infection of EBV in epithelial cells. The activities of multiple latent EBV genes expressed in infected premalignant epithelial cells then drive their malignant transformation into cancer cells. Moreover, the expression of lytic EBV genes, which is often detected in a small subset of NPC cells, has been postulated to induce the secretion of immune evasive factors to support the growth of EBV-infected NPC *in vivo* [6–8].

2. Close association of EBV infection and NPC

The closest association of EBV infection with human tumours is with the undifferentiated histological type of NPC that is endemic to southern China and Southeast Asia [9,10]. The association of EBV infection and NPC was first discovered when high titres of serum antibodies against EBV antigens including viral capsid antigen (VCA) and early antigen diffuse (EAd/BMRF1) were detected in patients [11]. The presence of the EBV genome in NPC cells was later demonstrated by *in situ* hybridization [12]. A high incidence of NPC is also seen in northern African populations and the Inuit populations of Alaska and Canada. The risk factors for NPC include genetic predisposition, dietary factors and EBV infection [9,10]. The World Health Organization classifies NPC as (a) keratinizing and (b) non-keratinizing squamous carcinomas. NPC in endemic areas such as Hong Kong and the southern provinces of China is mostly non-keratinizing and closely associated with EBV infection. Although EBV infection is present in almost all undifferentiated NPCs and almost every NPC cell, the virus is not detected in other head and neck cancers, apart from salivary gland tumours [2,13,14]. Lytic replication is believed to be the default infection program of EBV in pharyngeal epithelial cells, which are predominantly stratified squamous epithelium with differentiating properties. Lytic replication of EBV has been detected in hairy leukoplakia, which is a type of epithelial hyperplasia that may present at the lateral surfaces of the tongue in immune-compromised patients [4]. As latent EBV infection is the predominant mode in undifferentiated NPC, the undifferentiated properties of NPC cells presumably provide a cellular environment for latent EBV infection. Heavy infiltration of lymphocytes and inflammatory stroma is another common histopathological feature of undifferentiated NPC, which may modulate the switch from lytic to latent mode of EBV infection in NPC cells. The inflammatory stroma and the rich cytokine milieu may also be essential to the growth of EBV-infected NPC cells in patients, which may explain why it is difficult to establish NPC cell lines both *in vivo* (in immune-deficient mice) and *in vitro*. Only a limited number of NPC xenografts (x666, 2117, C15, C17 and C18) and one EBV-positive NPC cell line (c666-1) are currently available for investigations, which greatly hampers progress in NPC research [15]. The lack of a representative EBV infection cell system also hindered our investigation into the functions of EBV in nasopharyngeal epithelial cells.

3. Clonal origin of EBV infection in NPC

During lytic infection, the EBV episome replicates by a rolling circle method to generate multiple copies of linearized EBV genome packaged into an infectious virus [16]. Upon infection of new host cells, the linearized EBV genomes undergo circularization to form episomes, thereby maintaining latency. The joining of the two ends of the EBV genome during circularization occurs randomly at terminal repeat (TR) regions [16]. As a result, each circularized EBV episome will have a unique but variable number of TR repeats. Homogeneous lengths of TR repeats in EBV genomes have been observed in NPC and pre-invasive lesions, indicating that EBV infection in NPC is a clonal event likely to occur at an early stage of NPC development [17]. This observation also strongly suggests that NPC develops from the clonal expansion of a single EBV-infected cell.

4. Cell types giving rise to NPC

The exact cell type in the nasopharynx that gives rise to NPC cannot be clearly defined because of the mixed populations of epithelium present in the nasopharyngeal region. The nasopharynx is behind the nasal cavity and including its anterior, lateral and posterior walls has approximately 50 cm^2 of epithelial lining in adults [18]. A detailed histological examination of 55 Chinese individuals, 23 Indians, 11 Malays and 11 Europeans revealed that around 60% of the nasopharyngeal mucosa is covered by stratified squamous epithelium (largely non-keratinizing with patches of keratinization in older subjects) and the remainder comprises a variable percentage of pseudostratified ciliated columnar epithelium with goblet cells [18]. Patches of transitional epithelium are present near the Eustachian orifices where stratified squamous epithelium and ciliated epithelium meet. Immediately above and behind the elevation of the Eustachian tube is the pharyngeal recess (fossa of Rosenmüller), which is covered by mixed stratified epithelium and ciliated epithelium with patches of transitional epithelium. The fossa of Rosenmüller is the common incidence site of nasopharyngeal carcinoma. It remains to be determined whether squamous metaplasia present at or near the fossa of Rosenmüller is susceptible to EBV infection and neoplastic transformation. Interestingly, genetic alterations have been reported in adult nasopharyngeal epithelium in a high-risk NPC population (notably with allelic loss of chromosomes 3p and 9p) before EBV infection, suggestive of a supportive role of these genetic alterations in latent EBV infection [19,20]. These genetic alterations may arise from the early exposure of populations at high risk of NPC to carcinogenic agents such as nitrosamines in salted fish, which was previously common in the Cantonese diet. A close interplay of genetic susceptibility, somatic genetic alterations and inflammatory stromal cell signalling is believed to be involved in the stabilization of latent EBV infection in premalignant nasopharyngeal epithelium and its subsequent transformation to NPC.

5. EBV entry into nasopharyngeal epithelial cells

EBV infects both B cells and epithelial cells [14,21]. Interestingly, the virus shuttles between B cells and epithelial cells during its infection cycle, which facilitates its persistence and transmission in humans [2,22]. EBV readily infects and transforms resting B lymphocytes into proliferative lymphoblastoid cells, eventually immortalizing them. In healthy individuals with competent immune systems, the proliferation

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of EBV-infected B cells will eventually subside with EBV establishing life-long infection in the circulatory memory B-cell compartment. By contrast, EBV infection of primary epithelial cells does not induce their proliferation or immortalization [23]. Human pharyngeal epithelial cells are believed to be reservoirs of lytic EBV infection in which EBV genomes are amplified with the release of infectious viral particles into saliva for transmission.

The infection routes of EBV into B cells and epithelial cells differ. During infection of B cells, the gp350 protein on the viral envelope binds to the CD21 (also called CR2) present on the B-cell surfaces, eventually leading to the internalization of EBV in the cells. The entry route of EBV into epithelial cells is much less defined. The expression status of the CD21 receptor in pharyngeal epithelium has been controversial in earlier days because of the specificity of the antibody used to detect CD21 [24]. The consensus is that CD21 expression is low or absent in normal pharyngeal epithelium. Nevertheless, mRNA transcripts of CD21 have been detected by RT-PCR in micro-dissected histological sections of tonsillar and adenoid epithelia [25]. Interestingly, the expression of CD21 transcripts increases in dysplastic pharyngeal epithelia [26]. It remains unclear whether premalignant changes in nasopharyngeal epithelium may upregulate the expression of CD21 to facilitate EBV infection. The dynamic expression of CD21 in dysplastic pharyngeal epithelium requires further validation. Nonetheless, EBV infection has been detected in high-grade dysplastic premalignant lesions of NPC [17] with elevation of host antibodies against lytic EBV proteins (including VCA and EAd) detected before clinical presentation of NPC. The early antibody response may reflect the host's immune response to the lytic viral antigen expressed during EBV infection in dysplastic nasopharyngeal epithelium at an early stage of NPC development.

The efficiency of EBV infection of B cells and epithelial cells varies greatly. In B cells, high-efficiency EBV infection can be achieved by incubating the cells directly with supernatant harvested from virus-producing B cells induced to undergo lytic infection. By contrast, infection of epithelial cells by EBV requires cell-to-cell contact for transfer of the virus from producer B cells to epithelial cells [22,27]. Highly efficient EBV infection (20–50%) of primary or immortalized nasopharyngeal epithelial cells has been achieved by a co-culture method [28]. Recent studies have identified the novel receptors neuropilin 1 (NRP1) and non-muscle myosin heavy chain IIA (NMHC-IIA), which mediate the entry of EBV into nasopharyngeal epithelial cells [29,30]. NRP1 interacts with the EBV glycoprotein, gB, to facilitate the internalization and membrane fusion of EBV, while NMHC-IIA may facilitate EBV entry into epithelial cells via binding to the EBV glycoprotein gHgL [29,30].

6. Establishment of latent EBV infection in NPC

The ability of EBV to induce the proliferation and immortalization of B cells strongly supports its role in the pathogenesis of EBV-associated lymphoid tumours [31]. Counterintuitively, EBV infection of primary nasopharyngeal epithelial cells induces growth arrest [23]. Expression of lytic EBV genes has been detected alongside the latent membrane protein LMP1. EBV infection was observed to slow down the proliferation rate of telomerase-immortalized nasopharyngeal epithelial cells [32]. Overexpression of *cyclin D1* or a p16-resistant-*Cdk4* mutant can override the growth arrest induced by EBV infection and facilitate stable EBV infection in immortalized nasopharyngeal epithelial cells [32]. Examination of viral gene expression in immortalized nasopharyngeal epithelial cells stably infected with EBV revealed representative type II latent EBV infection with suppressed lytic gene expression [32]. These observations support the postulation that genetic alterations in pre-invasive nasopharyngeal epithelium support latent EBV infection.

As noted above, the default infection program of EBV in pharyngeal epithelial cells is lytic. Hence, the switching and establishment of latent EBV infection represents an important step in the pathogenesis of NPC. The profiles of viral genes expressed during latent infection are cell-context dependent. At least three types of latency program of EBV infection are recognized, involving different diseases and infected cell types [2,3,14,21]. EBER and EBNA1 are expressed in all three types of latency program. The type of EBV infection program in Burkitt's lymphoma (B-cell origin) is referred to as a type I latency in which a minimal number of latent EBV genes (EBER and EBNA1) is detected. Type II latency is observed in epithelial cancers, including NPC and EBVaGC, and shared by several non-epithelial tumours including Hodgkin's disease and T/NK-cell tumours. In addition to EBER and EBNA1, LMP1 and LMP2A are also detected. A special type of type II latency infection is observed in EBVaGC in which LMP1 expression is low or absent. A third type of latent EBV infection program is recognized in lymphoproliferative diseases seen in immunocompromised patients such as transplant and HIV patients. It is also observed in *in vitro* EBV transformation of primary B cells when immune cells are absent [31,33]. High expression of *BART*-miRNAs is particularly striking in NPC and gastric cancer, strongly supporting the important role of *BART*-miRNAs in the pathogenesis of EBV-associated epithelial cancers [34].

7. Genetic predisposition to NPC

The significance of genetic susceptibility and environmental factors (notably the consumption of dietary nitrosamines) in NPC development have been well documented [10,35,36]. As mentioned earlier, NPC has a remarkable ethnic and geographical distribution. EBV-associated NPC is endemic to southern China and Southeast Asia, with the highest incidence seen among the southern Chinese (15–20 per 100 000 persons per year), especially those of Cantonese origin. In addition to the high incidence rate, familial aggregations of NPC and the occurrence of multiple cases of the disease in first-degree relatives have been reported in the southern Chinese population, strongly suggesting that genetic susceptibility is involved in the aetiology of the disease [35–37]. The potential implications for EBV infection and NPC are discussed below.

Among the reported host genetic factors associated with NPC, variants of major histocompatibility complex (MHC) class I genes on chromosome 6p21.3 have shown the strongest association with NPC risk in both candidate gene-based investigations and genome-wide association studies (GWAS) [35,38–41]. The MHC class I genes *HLA-A*, *HLA-B* and *HLA-C* encode proteins that play a central role in cellular host immune responses in the identification and presentation of foreign antigens, including viral antigens, to the cytotoxic T cells. The differential ability of HLA haplotypes to induce a cytotoxic immune response against EBV antigens may

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determine an individual's susceptibility to EBV infection and the development of NPC. An earlier study demonstrated that inhibited MHC class I expression in NPC is closely associated with the expression of EBV genes [7]. Multiple high-resolution genotyping studies of Chinese populations have reproducibly reported the association of specific HLA class I alleles/haplotypes with susceptibility to NPC [39,41,42]. In summary, an increased risk of NPC is found in individuals possessing the HLA-A*02:07, A*33:03 and B*38:02 alleles, whereas those with the HLA-A*11:01, HLA-A*31:01, B*13:01 and B*55:02 alleles have a decreased risk of the disease. Recent GWAS findings also demonstrated independent and strong association signals in the HLA-A, HLA-B and HLA-C loci, supporting a major role of MHC class I genes in the inherited susceptibility of NPC [38–40]. Notably, the significant GWAS signals in HLA-A and HLA-B/C loci were detected in EBV-associated cancers, but not in other virus-associated cancers such as cervical and hepatocellular cancers [41]. HLA-class I molecules may play a unique role in the regulation of the complex life cycle of EBV, including the tropism of virus infection in B cells and epithelial cells. HLA class I-mediated host immune responses may contribute to the regulation of EBV latency in B lymphocytes, preventing persistent infection in epithelial cells and the clonal expansion of virally transformed cells. Although the strong association of HLA class I genes with NPC development has been demonstrated, the challenge remains in defining the precise mechanisms involved. Our recent whole-exome/whole-genome sequencing (WES/WGS) study revealed for the first time the high frequency of somatic genetic changes in multiple MHC class I genes (*HLA-A*, *HLA-B*, *HLA-C*, *B2M*) and their key regulator, *NLRC5* [43]. Somatic mutations and rearrangements of these genes are present in 30% of NPC cases and are significantly correlated with poor patient outcomes. Somatic mutation and inactivation of MHC class I genes may disrupt the antigen presentation mechanism and allow EBV-infected NPC cells to escape from host immuno-surveillance. This discovery not only defines a major mechanism for immune evasion, but also suggests potential biomarkers for future NPC immunotherapy trials.

In addition to the MHC region, GWAS and WES studies have identified various NPC susceptibility loci with moderate risk on multiple chromosomal regions, including *MST1R* (3p21.3), *MECOM* (3q26), *CDKN2A/CDKN2B* (9p21), *CIITA* (16p13), *TNFRS19* (13p12) and *CLPTM1 L/TERT* (5p15.3) [44]. However, the functional mechanisms responsible for increased susceptibility to NPC and the relationship with EBV infection remain to be elucidated.

8. Somatic mutations in NPC

In addition to EBV infection, the accumulation of multiple somatic genetic alterations is involved in driving NPC development. Defining the somatic mutations associated with NPC and their potential involvement in regulating EBV may provide further insights into NPC pathogenesis. The acquired genetic alterations frequently identified in EBV-associated NPC are listed in table 1. Some of these genetic alterations may be involved in supporting latent EBV infection and modulating EBV gene expression in NPC. Our earlier studies identified frequent allelic loss at chromosomal loci 3p21.3 and 9p21.3 in NPC, implicating the impairment of the key tumour suppressor genes, Ras association domain family 1A (*RASSF1A*) and cyclin-dependent kinase inhibitor 2A (*p16/CDKN2A*), in the initiation of this cancer [9,53]. Because high incidences of allelic loss at 3p21.3 and 9p21 have been identified in both EBV-positive and EBV-negative pre-invasive lesions, they are likely to occur before EBV infection [19,20,53]. Inactivation of *RASSF1A* and *p16* may provide a growth advantage for the clonal expansion of EBV-infected premalignant cells. In immortalized nasopharyngeal epithelial cells, inactivation of *p16/CDKN2A* has been shown to support persistent EBV infection [32].

In the past two decades, several genome-wide studies have further deciphered common somatic changes in the NPC genome. By extensive allelotyping and comparative genome hybridization, we have defined in NPC the frequent and focal losses of alleles on chromosomes 3p, 9p, 11q, 13q, 14q and 16q and the presence of a common amplicon on chromosome 11q13 where the cyclin D1 resides [47,54,55]. These findings were further confirmed in a recent genome sequencing study of microdissected NPC [43]. In the common deletion regions, we confirmed the inactivation of multiple key tumour suppressor genes, including *p16/CDKN2A* (9p21.3), *RASSF1A* (3p21.3), NFKB inhibitor alpha (*NFKBIA*, 14q13) and TNF receptor-associated factor 3 (*TRAF3*, 14q32.3) in NPC primary tumours and tumour lines reported in our earlier studies [49,50,51,56]. Among these genes, inactivation of *p16/CDKN2A* by homozygous deletion and promoter hypermethylation has been consistently found in almost all NPC samples examined [51,56]. As a crucial driver event, *p16* inactivation disrupts the G1/S cell-cycle checkpoint and supports EBV infection to promote NPC tumorigenesis [32,57]. Promoter methylation and mutations of *RASSF1A* were also found in 83% and 9.5% of NPC cases, respectively [50]. The major biological function of *RASSF1A* is to ensure the sequential progression of mitosis and hence regulate cell-cycle progression and maintain genomic stability. Because *RASSF1A* functions as a guardian of mitosis, its inactivation may accelerate mitotic progression and lead to an increased risk for chromosomal aberrations [48,58]. In addition to the inactivation of tumour suppressors, the amplification of several oncogenes, including phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) on 3q26.3, cyclin D1 (*CCND1*) on 11q13 and lymphotoxin beta receptor (*LTBR*) on 12p13, has been reported in 8–20% of NPC tumours, providing evidence for the oncogenic activation of the PI3 K-Akt and NF-κB signalling pathways as well as cell-cycle progression in NPC [45,47,48].

Through transcriptome sequencing, we identified a novel fusion of ubiquitin protein ligase E3 component n-recognin 5 (*UBR5*) on 8q22.3 and zinc finger protein 423 (*ZNF423*) on 16q12.1 in 8.3% (12/144) of NPC primary tumours, mainly in advanced stages [59]. The oncogenic properties and transforming abilities of the *UBR5-ZNF423* fusion gene can be demonstrated in NPC cells and NIH3T3 fibroblasts. This novel transforming fusion gene may contribute to tumorigenesis by disrupting the interaction of zinc finger protein 521 (ZNF523) and early B-cell factors, thereby deregulating their downstream oncogenic targets. In addition to *UBR5–ZNF423*, a well-known transforming fusion gene, fibroblast growth factor receptor 3-transforming acidic coiled-coil-containing protein 3 (*FGFR3-TACC3*) was also detected in 4 of 159 (2.5%) primary NPC tumours [60]. The oncogene addiction of these fusion genes demonstrated in NPC models suggests that they are potential therapeutic targets for a subset of patients.

Through whole-exome and/or genome sequencing, we and others have defined the mutational landscape in more than 200

Table 1. Acquired genetic alterations frequently identified in EBV-associated NPC. SNV, single nucleotide variant.

gene	chromosomal location	mechanisms and prevalence	function	references
oncogenes				
PIK3CA	3q26.3	amplification (20%) SNV (3.6–9.6%)	activating PI3 K-AKT signalling	[43,45,46]
CCND1	11q13.3	amplification (16%)	promoting cell-cycle progression and latent EBV infection	[47]
LTBR	12p13.3	amplification (approx. 7.3–10%)	activating NF-κB signalling	[48]
ZNF423	16q12.1	translocation (8.3%)	altering the activity of early B-cell factors and inducing cell transformation	[49]
tumour suppressor genes				
RASSF1A	3p21.3	SNV (9.5%)	promoting mitotic progression and genetic instability	[50]
HLA-A, *HLA-B* and *HLA-C*	6q22.1	SNV, deletion and rearrangement (30%)	presenting critical antigen molecules for T-cell recognition of tumour antigens	[43]
CDKN2A/p16	9p21.3	homozygous deletion (approx. 35%)	promoting cell-cycle progression and EBV latent infection	[51]
NFKBIA	14q13.2	SNV (6.7%)	negative regulation of NF-κB signalling	[43]
TRAF3	14q32.3	SNV, deletion and rearrangement (17.5%)	negative regulation of NF-κB signalling	[43]
CYLD	16q12.1	SNV, deletion and rearrangement (18.6%)	negative regulation of NF-κB signalling	[43]
NLRC5	16q13	SNV (5.7%)	negative regulation of NF-κB signalling, critical transcriptional regulation of MHC class I gene expression	[43]
TP53	17p13.1	SNV (8.5–9.5%)	maintaining genome stability, inducing cell-cycle arrest, apoptosis and DNA repair	[43,52]

NPC cases [43,52,61]. In addition to the predominant C > T transition identified at NpCpG sites, Lin's and our studies also revealed that the deamination of 5-methyl-cytosine and defective DNA mismatch repair signatures is common in EBV-associated NPC but the characteristic *APOBEC* mutational signature seen in HPV-associated carcinomas was rarely found. The findings imply that fundamental differences may exist in the viral carcinogenesis of HPV- and EBV-associated cancers. In contrast to the low detection rate in previous studies, genome sequencing studies have confirmed the *TP53* mutations in 9–10% of EBV-associated NPC [43]. The enrichment of *TP53* mutations in recurrent and metastatic tumours suggests the potential role of these mutations in cancer progression. Recurrent mutations in PI3 K/MAPK pathway activators or regulators (e.g. *PIK3CA*, *PTEN*, *ERBB3*, *RAS*) and chromatin modifying enzymes (*KMT2D*, *KMT2C*, *EP300*) were also observed, but infrequently [43].

9. NF-κB signalling and EBV infection in NPC

Somatic mutations have profound effects on cell signalling that could drive NPC pathogenesis. The role of NF-κB as a pro-inflammatory stimulus in tumorigenesis and tumour progression is well established. We and others have observed a high frequency of mutations on regulators of NF-κB, which may lead to its activation [43,61]. Using micro-dissected NPC tissue and WES, we identified a high incidence of somatic genomic changes (41%) in the key negative regulators of NF-κB pathways, including *CYLD*, *TRAF3*, *NFKBIA* and NLR family CARD domain containing 5 (*NLRC5*) [43]. In addition, 26% of tumours in this NPC cohort express high levels of EBV-encoded LMP1, a well-known potent activator of NF-κB pathways in NPC [43]. Notably, integrated analysis revealed a mutually exclusive relationship among somatic NF-κB pathway aberrations and overexpression of LMP1 in NPC. These results strongly implicate the importance of dysregulated NF-κB signalling in NPC pathogenesis either by constitutive activation through somatic mutations or by the EBV-encoded latent gene product, LMP1. A proposed conceptual framework of how NF-κB is activated in NPC, involving both interplay between somatic mutations in NPC and latent EBV genes is shown in figure 1. The occurrence of somatic alterations of NF-κB regulators may supplant the need for LMP1-mediated NF-κB activation and avoid the cytotoxic effect of LMP1 overexpression in tumour cells during tumour progression [43].

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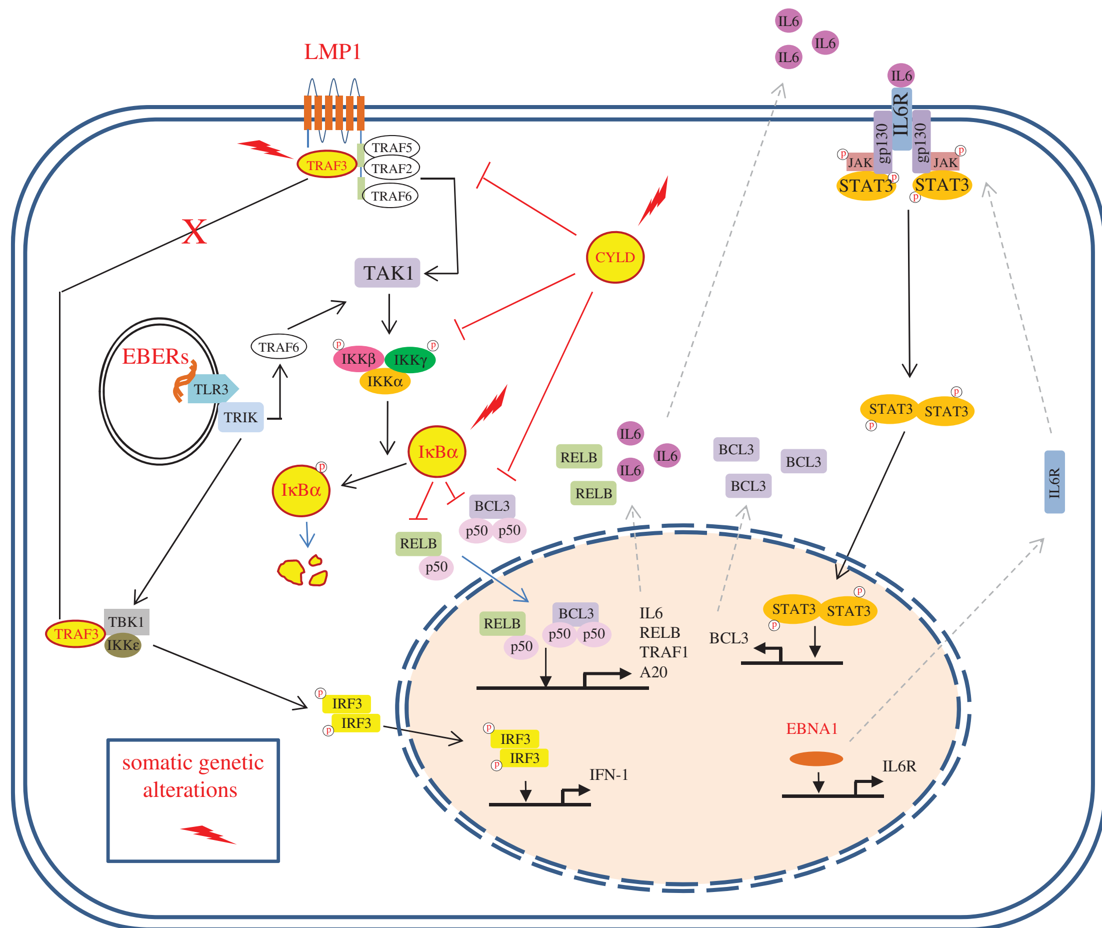

Figure 1. Activation of NF-κB and IL6/STAT3 signalling pathways in NPC cells by multiple EBV-encoded latent gene products. Through binding with TLR3, abundant EBERs could strongly activate both NF-κB and IFN3 signalling pathways. However, the inactivation of TRAF3 by either *LMP1* expression or somatic mutation will inhibit IFN3 signalling, thus protecting the cells from the EBER-induced innate immune response. LMP1 also activates the NF-kB signalling pathways through interacting with multiple TRAFs. Distinct NF-κB signals, including p50/p50/BCL3 and p50/RelB, will induce multiple survival genes (A20, survivin) and inflammatory cytokines (IL6). EBNA1 induces the ubiquitous expression of IL6R in NPC cells. The binding of highly expressed IL6 with IL6R results in constitutive activated STAT3 signalling. The activated STAT3 pathway might crosstalk with the altered NF-κB signalling by the upregulation of BCL3 in NPC cells. The common somatic mutations in NPC targeting the NF-κB pathways are also shown.

Previous studies, including ours, have revealed a distinct p50/p50/BCL3 complex as the predominant NF-κB signal in NPC [49,62]. The discovery of frequent (18.6%) somatic alterations of *CYLD*, which encodes a deubiquitinating enzyme (DUB) regulating the nuclear accumulation of BCL3, further supports the importance of constitutive activation of this atypical NF-κB signal in NPC development [63]. Nevertheless, the role of this atypical NF-κB in the establishment of latent EBV infection in NPC cells remains unclear. Current findings suggest a potential genetic explanation for the constitutive activation of NF-κB signals and the unique inflammatory character of this EBV-associated epithelial cancer. Constitutive activation of NF-κB signalling in NPC induces cell proliferation and survival, modulates the tumour microenvironment, and suppresses cellular immune responses, thereby facilitating tumour progression towards malignancy [49]. NF-κB signalling inhibits the lytic replication of EBV [64]. Aberrant NF-κB signalling pathways may contribute to the establishment of latent EBV infection and modulate latent gene expression. As shown in recent studies, activated NF-κB signalling upregulates the expression of EBV-encoded *BART*-miRNAs and long non-coding RNAs that have been found to promote the survival and tumorigenic growth properties of EBV-infected epithelial cells [34,65,66]. The frequent somatic NF-κB activating mutations identified in NPC may suggest addiction of NPC cells to activated NF-κB signals and could represent novel therapeutic targets for NPC treatment.

Recently, Hajek *et al*. [67] reported frequent mutations of *CYLD* and *TRAF3* in a subset of HPV-associated head and neck squamous cell carcinomas (HNSCC). Interestingly, these genetic alterations define distinct subsets of HPV-positive HNSCC with associated activation of transcription factor NF-κB, episomal HPV infection of tumours and improved patient survival rates. The genome sequencing data suggest that EBV-associated NPC and a distinct HNSCC subtype with episomal HPV infection may share a similar path of viral carcinogenesis.

10. Contribution of EBV infection to NPC pathogenesis

The establishment of stable infection of EBV in pre-invasive nasopharyngeal epithelium represents an early stage of NPC development. Both latent and lytic genes may be involved in the transformation of pre-invasive nasopharyngeal epithelium into NPC. Details of the potential involvement of EBV infection in NPC development are discussed below and summarized in figure 2.

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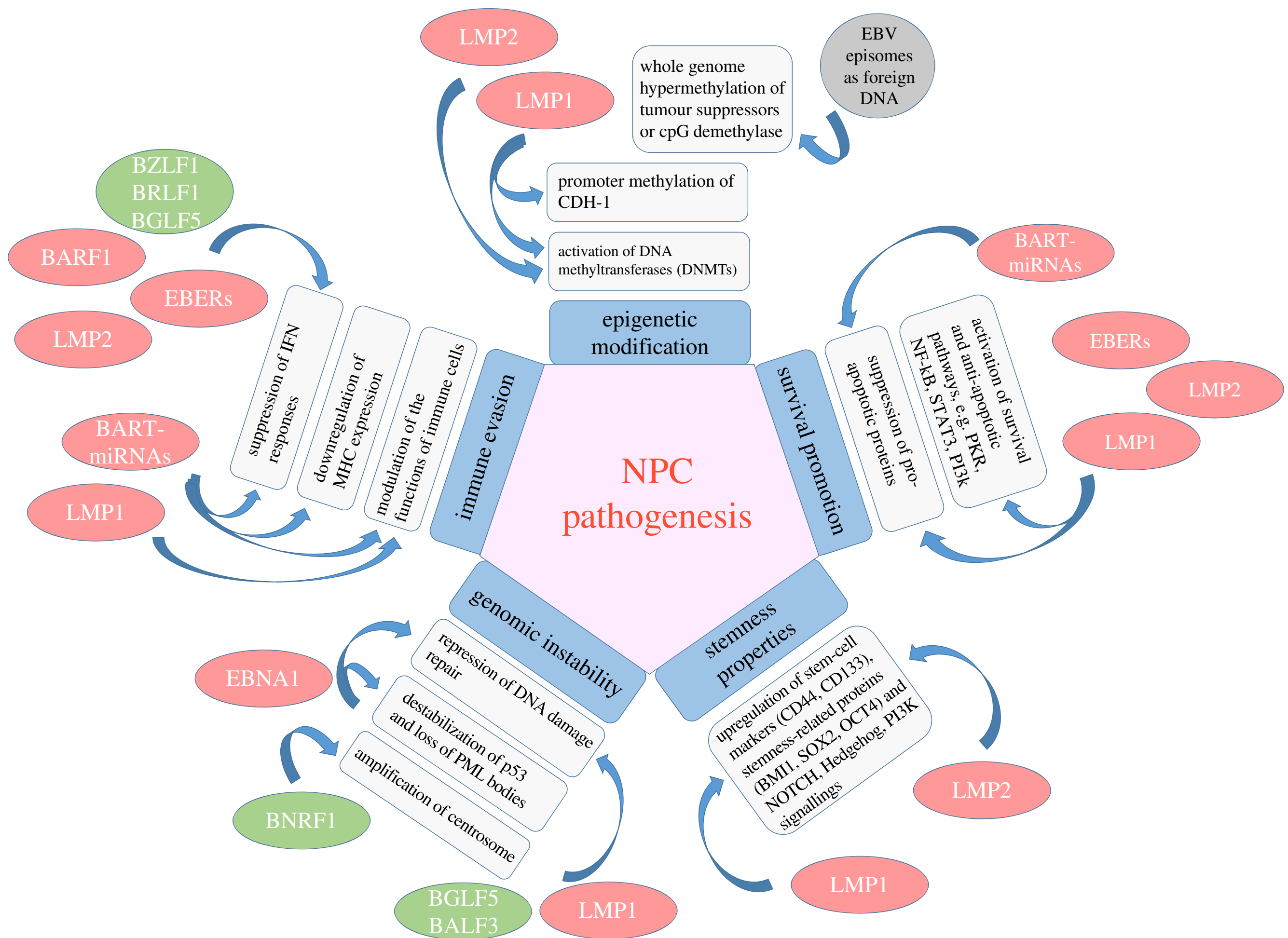

Figure 2. Contribution of EBV infection to NPC pathogenesis. EBV infection could drive NPC pathogenesis through multiple pathways. EBV infection promotes a hypermethylation phenotype in the host and induces the inactivation of tumour suppressor genes. EBV-induced hypermethylation may be a host defence response or the direct action of viral genes, such as *LMP1*, through activation of DNMTs. Both lytic and latent EBV genes may contribute to the development of NPC. The lytic EBV genes may induce genomic instability and stimulate the release of immune-suppressive cytokines for immune evasion of viral infected cells. The expression of latent genes also drives tumorigenesis and the acquisition of stemness in NPC cells. The transformation properties of *LMP1* are well documented. Many transformation properties of *LMP1* are mediated through NF-κB activation. The important role of *BART*-miRNAs in NPC pathogenesis, anti-apoptosis and immune evasion is emerging.

(a) EBV modifies epigenetic profiles of host genome

EBV infection may drive epigenetic changes during tumorigenesis [68]. CpG hypermethylation is a common mechanism involved in the inactivation of tumour suppressor genes in human cancers. Significant changes in the host cell methylomes towards CpG island hypermethylation have been observed in EBV-associated cancers, including Burkitt's lymphoma, NPC and EBVaGC [69]. A specific EBV epigenetic signature was reported in EBVaGC with a strong CpG island methylator phenotype (CIMP) compared with EBV-negative gastric cancer [70]. The epigenetic analysis of EBV-associated undifferentiated NPC is relatively less defined. Nonetheless, a comprehensive list of hypermethylated tumour suppressor genes in NPC has been reported, including promoter methylation at multiple genes at chromosome 3p21.3 including *RASSF1A* [50], chromosome 6p22.1-21.3 involving the *MHC* locus [71] and chromosome 9p21 including the *CDKN2A* (*p16* and *CDKN2B* (*p15*)) [56] and others [72]. Methylome profiling of NPC cell lines and primary tumours has revealed extensive and genome-wide methylation of cellular genes involved in Wnt, MAPK, TGF-β and hedgehog signalling.

It remains to be determined whether latent EBV infection is directly responsible for the methylation profile observed in NPC. It is known that EBV infection drives DNA methylation in the gastric cancer cell line, AGS [68]. The EBV infection of telomerase-immortalized keratinocytes also resulted in genome-wide DNA methylation [73]. Gene expression profiling using Affymetrix U133 plus 2.0 microarrays confirmed the differential gene expression in EBV-infected cells and treatment with 5-aza-cystidine to reverse methylation were able to revert the expression profiles of some of these differentially expressed genes [73]. Interestingly, such EBV-induced epigenetic changes were retained in the infected cells after the loss of EBV episomes, indicating a 'hit-and-run' effect of EBV infection [73]. DNA methylation may represent a host cell defensive response evoked to inactivate invading foreign DNA such as the EBV genome. Although methylation of the host genome induced by EBV infection may be general and non-specific, hypermethylation of CpG islands at the promoter regions of tumour suppressor genes will confer a growth advantage to EBV-infected cells. For example, methylation inactivation of specific tumour suppressor genes, such as *p16*, facilitates cell-cycle progression and supports latent EBV infection [32]. Ten-eleven translocation 1 (TET1) is a CpG demethylase which mediates DNA demethylation. A recent study reported that epigenetic inactivation of TET1 could function in a DNA methylation feedback loop to promote DNA methylation in human cancers including NPC [74]. Interestingly, another

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report showed that TET1 was recruited to activate *BZLF1* repression to reactivate EBV from latent infection [75]. Hence, in addition to enhancing CIMP in NPC, the silencing of *TET1* may also contribute to the maintenance of latent EBV infection by inhibition of *BZLF1* expression in EBV-infected NPC cells.

Expression of latent EBV genes could also be directly involved in methylation of the host genome and inactivation of tumour suppressor genes to support the growth of EBV-infected cells. E-cadherin (*CDH-1*) expression is commonly suppressed in NPC [76]. Viral LMP1 has been shown to induce methylation of the *CDH-1* promoter to downregulate the expression of cadherin [77]. DNA methylation of both the viral and host genomes is carried out by cellular DNA methyltransferases (DNMTs 1, 3A and 3B). DNMT1 is associated with the maintenance of methylation of the host genome during cell replication; DNMT3A and 3B are associated with de novo DNA methylation. *DNMT1* can be activated by LMP1 and activation of AP-1/Jun signalling is involved [78]. Moreover, EBV silencing of the *CDH-1* promoter also involves repressive chromatin modification [79]. LMP2A has also been reported to suppress the expression of *PTEN*, which is essential for the suppression of PI3 K signalling [80]. LMP2A suppression of *PTEN* expression involves phosphorylation of STAT3, which activates *DNMT1* transcription and contributes to the loss of *PTEN* expression through CpG island methylation of the *PTEN* promoter in EBVaGC [80].

(b) EBV infection induces genomic instability

Both lytic and latent EBV genes may contribute to genomic instability in EBV-infected host cells. Lytic-defective EBV has decreased ability to generate B-cell lymphoma in humanized mice models [81]. The expression of lytic EBV genes is commonly observed in small islets of NPC cells, but their roles are poorly understood [6,7]. Lytic infection and expression of viral lytic proteins may stimulate intense local inflammatory responses to promote tumorigenesis. The repeated induction of lytic infection in EBV-infected epithelial cells enhanced their tumorigenicity, suggesting the role of lytic reactivation of EBV in NPC progression [82]. Furthermore, the lytic EBV gene products may directly induce DNA damage and contribute to NPC development. Expression of the lytic EBV gene *BGLF5* increased the formation of micronuclei and induced genomic instability [83]. Another EBV lytic gene, *BALF3*, also induced micronuclei and DNA strand breaks [84]. Recurrent expression of *BALF3* in NPC cells resulted in genomic copy number aberrations and increased malignant properties including enhanced cell migration and invasion, spheroid formation and greater tumorigenicity in NOD/SCID mice [84]. A recent publication showed that B-lymphocytes infected with wild-type EBV viruses, but not BNRF1-deficient viruses, have enhanced centrosome amplification and chromosomal instability [85]. It suggests a role of BNRF1 in promoting genomic instability in the EBV-associated tumorigenesis [85].

The involvement of latent EBV genes in DNA damage and genomic instability is well documented. EBNA1 is expressed in all types of latent EBV infection [86]. EBNA1 increases tumorigenicity in EBV-negative cancer cells [87,88]. Multiple pathways may be involved through its effects on USP7, p53, PML and reactive oxygen species (ROS) [86]. USP7 can bind and stabilize multiple cellular proteins, including p53 and Mdm2. EBNA1 has a higher affinity with USP7 than p53 and Mdm2 and may outcompete them in binding to USP7. Hence, EBNA1 may destabilize p53 and/or Mdm2 in EBV-infected cells by blocking their interactions with USP7. Moreover, EBNA1 expression reduces the accumulation of p53 in response to DNA damage [89]. The expression of EBNA1 in AGS and SCM1 gastric carcinoma cells decreased their steady-state levels of p53 [90,91]. Promyelocytic leukaemia (PML) bodies are important for apoptosis, DNA repair, senescence and p53 activation. Loss of PML bodies is associated with the development and promotion of several tumours [92,93]. EBNA1 can induce the loss of PML nuclear bodies in both NPC and gastric cancer cells [89,91,94]. Induction of PML protein degradation involves the binding of EBNA1 to USP7 and the host casein kinase 2 (CK2) and their recruitment to the PML nuclear bodies. Furthermore, EBNA1 expression also decreases DNA repair efficiency, p53 acetylation and apoptosis in response to DNA damage agents [89,94]. Stable or transient expression of EBNA1 has also been shown to induce ROS, DNA damage foci and dysfunctional uncapped telomeres [95,96].

LMP1, which is commonly expressed in NPC, can repress DNA repair through c-terminal activation region 1 (CTAR1) in human epithelial cells [97]. The PI3 K/Akt pathway is involved in LMP1-mediated repression of DNA repair. DNA damage binding protein (DDB1) is involved in nucleotide excision repair. The LMP1/PI3 K/Akt pathway can inactivate FOXO3a to suppress the expression of DDB1 and contribute to the genomic instability in human epithelial cells [97]. We also recently revealed that EBV-encoded microRNAs are able to suppress ATM expression and interfere with the DNA repair mechanism (unpublished observations).

(c) EBV evades host immunity

Immune evasion is one of the hallmarks of human cancer cells. The foreign antigens expressed in virally infected cells are targets for immune detection. EBV has devised multiple strategies to evade host immune destruction. The functions of EBV lytic proteins in immune evasion during lytic reactivation of EBV in B cells are well recognized [98]. Expression of lytic EBV genes is often detected in small subsets of NPC cells [6,7]. The lytic infection of EBV in NPC is believed to be abortive in nature. Some of these lytic genes may play a role in immune evasion in EBV-infected NPC cells [8]. EBV infection and expression of multiple lytic EBV gene proteins may block the secretion of multiple antiviral cytokines. For example, the immediate early lytic proteins of EBV, including BZLF1 and BRLF1, inhibited interferon response genes and type I interferon production [99,100]. BZLF1 also induced SOC3 to inhibit JAK/STAT signalling and IFNα production by monocytes and induced a state of type I IFN-irresponsiveness [101]. The lytic EBV proteins may also influence the host's innate immunity through modulation of TLR expression on the surface of virally infected cells to interfere with intracellular NF-κB signalling. For example, BGLF5 has been reported to degrade TLR9 mRNA to reduce TLR9 expression [102].

Latent EBV gene products also play a role in the modulation of host immune responses. The EBV-encoded gene product *EBER*, which is expressed at high levels in all latency types of EBV infection, can inhibit interferon-stimulated gene activity by binding to PKR, a double-stranded RNA-dependent protein kinase [103]. Expression of LMP1 can recruit Tregs via activation of NF-κB signalling, thus upregulating expression of the chemokine CCL20 [104]. High CCL20

expression was shown to increase the migration of CD4 + FOXP3+ Tregs cells towards the tumour [49,104]. Furthermore, LMP1 can also induce Tregs to secret IL-10 [105]. The secretion of IL10 cytokine is associated with immune suppression. Colony-stimulating factor 1 (CSF-1) can stimulate macrophage differentiation and IFNα secretion. EBV encodes BARF1, a soluble form of CSF-1 receptor, which may neutralize the effects of CSF-1 leading to reduced IFNα secretion by mononuclear cells [106,107]. EBV-infected cells can secrete exosomes to modulate immune activity [108,109]. These exosomes have been shown to contain LMP1, galectin 9 and other immunomodulatory molecules that may modulate the functions of immune cells present in the NPC microenvironment [108]. A recent study reported that exosomes harvested from NPC xenografts (C15 and C17) and serum of NPC patients facilitated Treg recruitment with the involvement of CCL20 [109]. The EBV-encoded LMP2A and LMP2B dampened type I IFN responses in epithelial cells by disrupting IFN-stimulated gene transcription and by targeting IFN receptors for degradation [110].

Recent studies have revealed an important role of EBV-encoded miRNAs in immune evasion [111–113]. The EBV-encoded *BART*-miRNAs are expressed at high levels in NPC. The cellular targets of *BART*-miRNAs identified in immune evasion include major histocompatibility complex class I-related chain B (MICB), which is a target of *BART-miRNA2-5P* [114]. MICB is a stress-induced ligand that is recognized by the NKG2D receptor on NK cells and CD8 + T cells [114]. A decrease in the expression of MICB on cell surfaces will lead to a decreased cytolytic response following NKG2D activation, enabling the EBV-infected cells to evade immune detection. The TBX2/T-bet, a transcriptional activator of γIFN and regulator of the production of IL2 and Th2 cytokines, is a target of *BART-miRNA20-5p* [115]. *BART-miRNA15-3p* can inhibit the activation of NLRP3 inflammasome and the production of the pro-inflammatory cytokine IL-1 [116].

(d) EBV promotes cell survival

The balance of cell proliferation and apoptosis is crucial for the growth of cancer cells *in vivo*. The universal presence of EBV infection in NPC patients strongly suggests a selective survival advantage of EBV-infected NPC cells *in vivo*. Multiple latent EBV genes have been shown to confer a cell survival advantage by interfering with the pro-apoptotic process and may be involved in driving the clonal expansion of EBV-infected pre-invasive nasopharyngeal epithelial cells. The properties of these latent EBV genes have been extensively reviewed [14,21,117]. Their key cellular functions contributing to cell survival are highlighted below.

*EBER*s are small non-polyadenylated RNAs encoded at high level upon EBV infection of cells [118]. The role of *EBER*s to counteract the activity of PKR, which is commonly induced in host cells as an antiviral infection response, is well documented [119]. *EBER*s inhibit the phosphorylation of eIF-2α, which is the cellular substrate of PKR and is involved in signalling inhibition of protein translation. Inhibition of PKR signalling protected EBV-infected intestinal epithelial cells from Fas-mediated apoptosis induced by IFNs [119]. *EBER*s can also interact with TLR3 and induce tumour cells to produce cytokines to recruit and activate macrophages to produce a favourable microenvironment for the growth of NPC cells [120].

The expression of LMP1 is common in high-grade dysplastic lesions in the nasopharyngeal epithelium infected with EBV and may play an important role in driving the expansion of EBV-infected clones at early stages of NPC development [17]. The oncogenic properties of LMP1 and its ability to activate multiple cell signalling pathways have been extensively studied [14,21,113,121]. LMP1 is a well-known potent activator of NF-κB signalling. Many of the pathological properties of LMP1 including anti-apoptosis are mediated by its ability to activate NF-κB. The C-terminal activation domain 1 (CTAR1) activates both canonical and non-canonical NF-kB signalling [121,122], as well as the atypical NF-κB signalling involving the binding of bcl3 to p50/p50 dimers of NF-κB [62]. NF-κB signalling by LMP1 upregulates the expression of A20, c-IAP, survivin and others to enhance cell survival [121,122]. LMP1 also induces the expression of IL6 to activate IL6R and STAT3 signalling to support the growth of nasopharyngeal epithelial cells [123]. The upregulation of IL6 by LMP1 is also mediated by NF-κB signalling. The multiple roles of LMP1 in activation of NF-κB and IL6R/STAT3 are illustrated in figure 1. A positive feedback loop of LMP1/IL6R/STAT3 signalling may be involved in the activation of STAT3 to promote the latent infection of EBV in NPC cells [124]. Activation of STAT3 signalling may be involved in the upregulation of Bcl3 in NPC cells [125]. Activation of PI3 K/Akt pathways by LMP1 also phosphorylates and inactivates Bad and Foxo3α proteins, which are pro-apoptotic in cells [126,127].

The N-terminal cytoplasmic domain of LMP2A contains multiple signalling domains and contributes to the modulation of multiple signalling pathways including PI3 K/Akt, RhoA and MAPK/ERK [127]. LMP2A activation of PI3 K/Akt can inhibit cellular differentiation and promote cell survival through stabilization of ΔNp63 [128]. LMP2A activation of ERK also resists anoikis [129].

The importance of *BART*-miRNAs in promoting the survival of EBV in epithelial cancer cells is emerging [34,112,113]. *BART*-miRNAs are synthesized from the intronic regions of the BamH rightward transcript (BART) of EBV. *BART*-miRNAs are abundantly expressed in NPC, but minimally or not expressed in EBV-transformed B cells [112,113]. In the EBV-positive NPC cell line C666-1, expression of *BART*-miRNAs represents approximately 40% of all cellular miRNAs. That *BART*-miRNAs represent a significantly high proportion of microRNAs in EBV-infected NPC cells indicates their likely involvement in the growth of NPC cells. In the C17 NPC xenograft, *BART*-miRNAs represent 57% of all miRNAs [113]. Overexpression of *BART*-miRNAs promotes the growth and tumorigenesis [34,112,113] of EBV-infected AGS cells [65]. The role of *BART*-miRNAs in immune evasion has been discussed earlier. Here, the anti-apoptotic role of *BART*-miRNAs is further elaborated. Earlier studies indicated that *BART*-miRNAs target multiple pro-apoptotic cellular transcripts to enhance the survival of EBV-infected cells [111,130]. For example, the mRNA of the BH3-only protein, Bim, is a common target of multiple *BART*-miRNAs [111]. The anti-apoptotic action of bcl-2 is inhibited by Bim. Destruction of Bim transcripts by BART-miRNAs may confer a survival advantage to EBV-infected cells. PUMA, which is a downstream target of p53, is targeted by *BART-miRNA5-5p* [130]. A recent study that systematically investigated the role of EBV *BART*-miRNAs in NPC and gastric cancer cells using photoactivatable ribonucloside-enhanced cross-linking and immuno-precipitation (PAR-CLIP) revealed novel apoptotic

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targets of *BART*-miRNAs including DICE1, FEM1B, CASZ1a (targeted by *BART-miRNA3*), OCT1 (targeted by *BART-miRNA6*), ARID2 (targeted by *BART-miRNA8*), CREBBP and SH2B3 (targeted by *BART-miRNA8*), PPP3R1, PAK2 and TP53INP1 (targeted by *BART-miRNA22*) [34].

(e) EBV infection contributes to stem cell-like properties of NPC cells

The maintenance of stemness is speculated to play a crucial role in persistent latent EBV infection and malignant transformation of nasopharyngeal epithelial cells. Latent EBV genes, including LMP2A and LMP1, have been reported to induce stemness or stem cell-like properties in NPC cells. In EBV-positive precancerous lesions, EBV infection was observed in basal layers of the nasopharyngeal epithelium or immediately adjacent cells, suggesting that NPC tumour initiating cells/cancer stem cells (CSC) may arise from the EBV-infected suprabasal and basal layers of stem cells [19,20,131]. Elucidating the features of CSC in EBV-positive NPC is important in understanding the role of EBV in NPC tumorigenesis. In the EBV-positive NPC cell line C666-1, the isolated sphere-forming cells showed upregulation of multiple CSC and embryonic stem cell markers and increased tumorigenic potential [132]. By characterization of these sphere-forming cells, a $CD44^{+}SOX2^{+}$ subpopulation was defined as NPC CSC. Its occurrence was further demonstrated in multiple EBV-positive NPC patient-derived xenografts and primary tumours. Functional studies of the isolated $CD44^{+}$ NPC cells have confirmed their CSC properties including resistance to chemotherapeutic agents and greater spheroid formation efficiency [132]. Reducing the CSC population by targeting NOTCH3 and CBP/β-catenin signalling has been shown to enhance the treatment efficacy of chemotherapeutic agents in the suppression of NPC cell growth [133,134]. Interestingly, significant elevations of EBV copy number and latent gene expression (e.g. *EBER1*, *LMP1*) were found in NPC sphere-forming cells, further confirming a link between CSC and latent EBV infection [132]. In recent years, increasing evidence has supported the proposition that EBV infection could induce stem-like properties in nasopharyngeal epithelial cells. Several studies have shown that expression of LMP1 and LMP2A enhances epithelial–mesenchymal transition, upregulates stem cell marker expression and induces CSC phenotypes in nasopharyngeal epithelial cells [135,136]. Silencing LMP1 expression reduced CSCs in an EBV-positive NPC cell population [135]. These latent EBV genes may acquire the properties of CSC via altering multiple cellular mechanisms and signalling pathways in host cells. EBV was reported to activate the hedgehog signalling pathway, which is associated with stem cell maintenance via autocrine induction of the SHH ligand [137]. Furthermore, expression of LMP1 and LMP2A has been shown to induce the expression of various stemness-related gene products (BMI1 and SOX2) and stem cell surface markers (CD44v6 and CD133) via dysregulation of the hedgehog signalling pathway in nasopharyngeal epithelial cells [137]. The role of aberrant activation of NOTCH3 signalling in modulating stem cell marker expression (OCT4) and CSC properties in NPC cells was also reported in our previous study [134]. A recent study also demonstrated that activation of the PI3 K/Akt pathway by LMP1 plays a critical role in the induction of CSCs in NPC [138]. These findings support the potential roles of latent EBV proteins in the transformation of CSC by activating multiple signalling pathways and stimulating the expression of stemness properties in NPC cells.

11. Strain variations of EBV and NPC

The postulation that a specific strain of EBV may be associated with NPC development is contentious. EBV isolates worldwide can be classified as type 1 or type 2 based on the EBNA2 gene sequence [139]. Type 1 is the common EBV subtype in NPC and is the common strain in Asian and Western populations. Type 2 EBV is common in some areas of Africa. Type 1 EBV demonstrates stronger induction of lymphoblastoid cell lines from infected B cells. The tumorigenic propensity of type 1 and type 2 in human cancers is unclear [139]. Recent next-generation sequencing (NGS) examination of EBV has revealed the homogeneous clustering of EBV strains in NPC patients [140]. The specific allelotype of HLA in populations in different geographical regions and the differential ability to present epitopes of different EBV strains may be an underlying selection mechanism. While clonal EBV infection has been detected in NPC, co-infection of multiple EBVs has been detected in both immunocompromised and healthy persons [141]. It remains to be determined whether a specific EBV strain is selected for clonal expansion of EBV-infected premalignant nasopharyngeal epithelial cells into NPC. The recent cloning of M81-EBV from NPC revealed distinct infection properties [142]. In contrast to EBV isolated from infectious mononucleosis (B95-8-EBV) and Burkitt's lymphoma (Akata-EBV) and used widely in the laboratory, M81-EBV is an epitheliotropic virus strain and has a higher lytic activity upon infection of B cells. Clinically, high antibody titres against lytic EBV genes support the involvement of lytic reactivation in NPC development.

Interestingly, LMP1 variants cloned from laboratory strain B95-8 virus and LMP1 variants from NPC have differential transformation properties [143–145]. B95-8 LMP1 isolated from infectious mononucleosis was shown to be more toxic than the LMP1 isolated from NPC including the Cao-LMP1 and 2117 LMP1. LMP1 is an essential EBV-encoded oncogene [145]. The NF-kB signalling property is more potent in NPC-derived LMP1 than in LMP1 derived from the B95-8 virus isolated from infectious mononucleosis [143]. This has implications for whether a specific strain of EBV may be involved in the pathogenesis of NPC. At present, the question of whether a specific EBV strain plays a major role in the development of NPC remains to be determined and awaits larger functional studies involving the isolation of more EBV strains and their variant genes from populations at high or low risk of NPC.

12. Conclusion

A model of the clonal evolution of NPC driven by EBV infection and progressive genomic changes in precancerous nasopharyngeal epithelium is proposed based on recent findings in the genomic profiling of NPC (figure 3). Infection with EBV was detected in almost every NPC cell. EBV establishes type II latency in NPC with the expression of multiple latent EBV genes. A strong interplay between EBV infection and somatic mutations in precancerous lesions is believed to be involved in NPC development. Long-term exposure to environmental carcinogens (e.g. nitrosamines from salted fish and preserved food) of nasopharyngeal mucosa imposes DNA damage and

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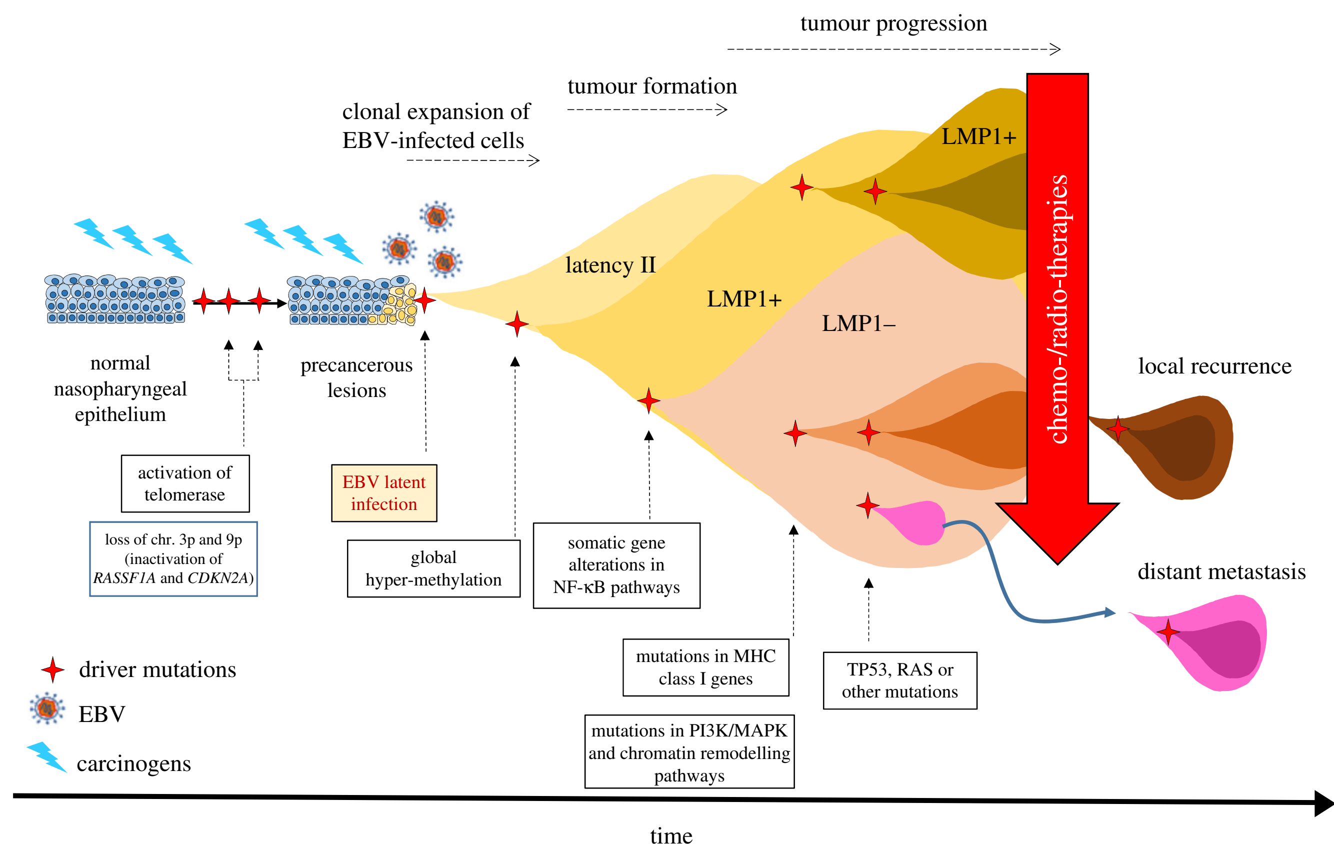

Figure 3. EBV infection and progressive genomic changes drive clonal evolution of nasopharyngeal carcinoma. In NPC, persistent EBV infection in a genetically aberrant epithelial cell and clonal expansion of infected cells are postulated to initiate tumorigenic transformation. Long-term exposure of the nasopharyngeal mucosa to environmental carcinogens (e.g. nitrosamines from salted fish and preserved food) increases DNA damage and induces various somatic genetic alterations in the epithelial cells. Accumulation of driver events including activation of telomerase activity and inactivation of tumour suppressor genes *RASSF1A* and *p16* on chromosomes 3p and 9p facilitate immortalization, genome instability and EBV infection in the histologically normal and/or dysplastic cells. Expression of latency II gene products including *EBNA1*, *LMP1*, *LMP2A*, *EBER*s and *BART*s alters multiple cellular pathways, promotes cell proliferation and modulates the host's microenvironment to drive the clonal expansion of EBV-infected pre-invasive nasopharyngeal epithelial cells. Importantly, EBV facilitates global hypermethylation, which inactivates various cancer-related genes and enhances tumour heterogeneity. During tumour development, the occurrence of acquired mutations in multiple negative regulators in NF-κB signalling alters the activities of various cancer-related genes to enhance tumour heterogeneity. The loss of LMP1 in NPC during progression may be related to acquisition of additional genetic alterations during tumour progression which compensate the functions of LMP1. Further genetic alterations are acquired during tumour progression. In advanced stages, somatic mutations of *TP53*, *RAS* and other genes may drive the growth of subclones of NPC cells as local recurrent diseases and distant metastasis after conventional cancer treatment.

induces various somatic genetic alterations in epithelial cells. Accumulation of driver events including activation of telomerase activity and inactivation of tumour suppressor genes *RASSF1A* and *p16* on chromosomes 3p and 9p may facilitate cell immortalization, genome instability and latent infection of EBV in the histologically normal and/or dysplastic nasopharyngeal epithelium. Expression of latency II EBV gene products including EBNA1, LMP1, LMP2A, *EBER*s and *BART*s alters multiple cellular pathways. Stable EBV infection and expression of latent EBV genes in a genetically aberrant nasopharyngeal epithelial cell presumably drive the clonal expansion of the EBV-infected cell. Hence, establishment of latent EBV infection in precancerous nasopharyngeal epithelium is postulated to be an early and essential step in NPC pathogenesis. The mutually exclusive relationship between somatic mutations of negative regulators upstream of NF-κB signalling and LMP1 expression in NPC indicates a special role of NF-κB activation in driving NPC pathogenesis. The activated NF-kB signalling function in driving latent EBV infection and the expression of *BART*-microRNA has been reported. Mutation of MHC class I genes in NPC cells may lower the host cell's immune response to EBV infection and facilitate the growth of EBV-infected NPC cells. Mutation of PI3 K/MAP signalling and chromatin remodelling further support the persistence of EBV infection in NPC cells. EBV infection and the expression of latent genes, notably *LMP1*, may facilitate global hypermethylation, which dysregulate various cancer-related genes and enhance NPC tumour heterogeneity. Further genetic alterations are acquired during tumour progression. In advanced NPC, somatic mutations of *TP53*, *RAS* and other genes may drive the growth of subclones of NPC cells, which emerge as local recurrent diseases and distant metastasis after conventional cancer treatment.

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