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Pathogenesis and therapeutic implications of EBV-associated epithelial cancers

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Epstein-Barr virus (EBV), one of the most common human viruses, has been associated with both lymphoid and epithelial cancers. Undifferentiated nasopharyngeal carcinoma (NPC), EBV associated gastric cancer (EBVaGC) and lymphoepithelioma-like carcinoma (LELC) are amongst the few common epithelial cancers that EBV has been associated with. The pathogenesis of EBV-associated NPC has been well described, however, the same cannot be said for primary pulmonary LELC (PPLELC) owing to the rarity of the cancer. In this review, we outline the pathogenesis of EBV-associated NPC and EBVaGCs and their recent advances. By drawing on similarities between NPC and PPLELC, we then also postulated the pathogenesis of PPLELC. A deeper understanding about the pathogenesis of EBV enables us to postulate the pathogenesis of other EBV associated cancers such as PPLELC.

KEYWORDS

Epstein-Barr virus, nasopharyngeal cancer, lymphoepithelioma-like carcinoma, primary pulmonary lymphoepithelioma-like carcinoma, pathogenesis

1 Introduction

Epstein-Barr Virus (EBV) is a ubiquitous oncovirus that affects more than 90% of adults worldwide, but is especially endemic in East Asia, South East Asia, North Africa and Polynesia (1). The oncogenic potential of EBV is well established and yet evolving still, and its role has been demonstrated in the pathogenesis of epithelial malignancies such as Epstein-Barr virus- associated gastric cancers (EBVaGCs), nasopharyngeal carcinoma (NPC), lymphoepithelial like carcinoma (LELC), as well as in hematological malignancies such as Burkitt's lymphoma, Diffuse Large B Cell Lymphoma (DLBCL), Hodgkin's disease, and natural killer T-cell lymphomas (2).

While EBV-associated epithelial cancers vary in presentation and cell origin, these malignancies have in common a viral-mediated immune-suppressed tumor immune microenvironment, mutational signatures and epigenetic hallmarks (3). Among these epithelial cancers, the pathogenesis of NPC has been the most well described, in particular

the role of EBV in transforming nasopharyngeal epithelium in a possible multi-step process from dysplasia to carcinoma (4). However, this process has not been described for other epithelial cancers, especially in lymphoepithelial like carcinoma (LELC).

We aim to discuss the pathogenesis of NPC and EBVaGC, and hypothesize the pathogenesis of LELC based on their common epigenetic, genomic and somatic mutational signatures. In particular, we examine primary pulmonary LELC (PPLELC) and consider the hypothesis of the EBV transformation of dysplastic epithelial cells in the lungs.

1.1 NPC

NPC is a head and neck cancer that is found commonly in Guangdong Province, Hong Kong, Southeast Asia, East Asia, and the Mediterranean area (5); however it is relatively uncommon in Western countries (6). A study in Singapore found that NPC has a higher incidence amongst Cantonese compared to the Teochew- or the Hokkien-dialect group (7). Similar findings were also observed in China, where the Cantonese 'boat people' in Southern China and provinces near Guangdong have a relatively higher incidence of NPC (8). This geographical clustering of NPC suggests that both genetic and environmental factors contribute to its development. Genetic factors, including HLA-A*0207 or B*4601 and disease-associated SNPs such as TLRs (which will be discussed in section 5) may increase an individual's susceptibility to NPC. Furthermore, evidence of genetic susceptibility is also shown by the increased NPC risks in those with first-degree family members with NPC (9). Environmental factors, such as the consumption of volatile nitrosamine containing preserved foods and salted fish, are also strongly linked to NPC and are commonly found in the Cantonese diet (10).

According to epidemiological studies, NPC occurs two to three times more frequently in males than females (11). It has a bimodal distribution in low-risk populations, reaching a modest peak from ages 15 to 24 years old, and reaching a higher peak from ages 65 to 79 years old (11). In high-risk populations, NPC incidence peaks at 50 to 59 years old, and subsequently decreases (12).

The World Health Organisation (WHO) classifies NPC into three major groups, namely, the keratinizing squamous subtype (Type I), nonkeratinizing squamous subtypes (Type II), and undifferentiated or poorly differentiated (Type III). EBV and its association with the non-keratinising forms of NPC has been well-established (6). This is evidenced by the presence of EBV in dysplastic nasopharyngeal epithelium, as well as its clones in NPC biopsies in a few studies (13). Anti-EBV immunoglobulin A (IgA) has also shown utility as a screening marker for NPC, especially in patients with a strong family history (14). In addition, serum EBV capsid antigen (VCA) IgA or EBV DNA titers were also found to be associated with risk of NPC, with higher levels correlating with advanced disease (9, 15).

NPC is usually found in the lateral wall of the nasopharynx, in particular, the fossa of Rosenmuller. As the disease progresses, it can either remain confined within the nasopharynx, or extend into the contralateral lateral wall, skull base, palate, nasal cavity or oropharynx (16). While NPC most commonly presents with cervical lymphadenopathy (16), it can also present as cranial nerve palsies, blood in mucus or saliva, hearing loss, serous otitis media, tinnitus, diplopia, dysphagia and dysphonia, and occasionally pain (17). In the recently published 8th edition of the TNM staging for NPC by the American Joint Committee on Cancer (AJCC) (18), NPC is classified into 5 stages with stage 0 suggesting preinvasive lesion, I to II indicating early disease, and stages III and IV broadly indicating more extensive lymph node involvement, with IVB indicating metastatic disease.

1.2 Epstein-Barr virus-associated gastric cancer

EBVaGC constitutes 10% of gastric cancer (19) and is one of the most common EBV-associated malignancies by overall incidence. It is defined by the monoclonal proliferation of latent EBV-infected carcinogenic cells (20). Like NPC, EBVaGC is more commonly found in males, younger individuals (21). However, unlike NPC and PPLELC, the incidence of EBVaGC is higher in Western countries such as Germany and the United States as compared to Asian countries (22). EBVaGC is predominantly located in the proximal stomach and remnant stomach post partial gastrectomy for gastric ulcers or cancer (20). Clinically, EBVaGC presents with loss of weight, epigastric pain, early satiety, gastrointestinal bleed, iron deficiency anemia, and nausea (3).

Similar to NPC, consumption of salty food and exposure to wood dusts increase risk of EBVaGC (23–25). *H. pylori* infection, a significant risk factor for non-EBV gastric cancer, was not found to be associated with EBVaGC.

1.3 LELC and primary pulmonary LELC

LELC is a poorly differentiated carcinoma characterized by dense lymphocytic infiltration in the stroma, histologically similar to undifferentiated NPC. It is a rare but distinct cancer that may arise from multiple organs, including the lungs, stomach, parotids, salivary gland, thymus, biliary tract, breast, prostate, and metastatic NPC has to first be excluded (26, 27).

The first recognition of the possible association of LELC of the lungs with EBV was by Begin et al (28), and since then, the presence of EBV has also been detected in LELCs of the salivary gland (29), gastric (30, 31), thymus (32), colon (33), lung (34) and the intrahepatic biliary tract (35). The identification of EBV in LELC has led to an increasing amount of research exploring the role of the oncovirus in the pathogenesis of LELC (36).

PPLELC, or LELC of the lungs, is classified as a subtype of non-small cell lung cancer (NSCLC) and is the most common type of LELC. Similar to NPC, it is found more commonly in Asians, in particular the Southern Chinese (36–38), and when found in westerners, it is typically EBV negative (39–41). It presents at a lower mean age of 51-55 years old and has a better prognosis than non-LELC lung cancers (42), with a higher incidence in non-smokers and Asian females (43). Since its first documentation

(28), current understanding of the histological resemblance, geographical clustering and molecular similarities (36) between NPC and PPLELC have prompted increasing interest in the role of EBV in PPLELC (34, 44, 45). Currently, PPLELC is the most well studied LELC subtype and will be the primary focus when discussing LELCs.

2 Pathogenesis of NPC

EBV is known to be one of the etiological agents of NPC pathogenesis (46). While lytic replication typically predominates during the acute phase of EBV infection and early dysplasia, persistent latent EBV infection, clonal expansion of infected epithelial and lymphoid cells are thought to be drivers of NPC (47), and the lytic-latent switch is an important step in malignant transformation.

EBV is potentially acquired in early childhood when the immune system is not fully developed and evolved (48). On the contrary, if acquired during teenage years, EBV potentially presents as infectious mononucleosis (49, 50). One hypothesis proposes that in early life, persistent EBV infection arises from inadequate immune clearance due to the developing immune system resulting in an infection that is likely to persist for the remainder of the carrier's lifespan. Because of this, early infection is more likely to result in latency, which when exposed to genetic and environmental factors, is particularly susceptible to malignant transformation (51). In the case of NPC, EBV expresses a type II latency program expressing specific gene products that enhances its ability to survive and spread. This will be further elaborated in the later sections.

3 Pathogenesis of EBVaGC

One hypothesis of EBV pathogenesis in EBVaGC suggests that direct ingestion of the EBV virus could result in the infection of gastric epithelial cells (52, 53). It has also been postulated that during the reactivation of the lytic phase, resident B lymphocytes present in gastric mucosa release EBV, which subsequently infects more epithelial cells, a process thought to be aided by ephrin receptor A, integrins, and non-muscle myosin heavy chain IIA (NMHCIIA) (54). This process is further facilitated by upregulation of adhesion molecule-1 through integrin β1/β2 mediated contact between B lymphocytes and gastric epithelial cells and clathrin-mediated endocytosis (3). Congruent with this hypothesis, EBV infection is found in a small number of non-neoplastic gastric mucosa, suggesting that EBV infection precedes the monoclonal expansion of EBV-infected cells in tumorigenesis (55, 56). EBV anti-VCA and anti-EBNA antibody titres are also elevated in patients with gastric dysplasia on biopsy, indicating that EBV reactivation may be related to early tumorigenesis of EBVaGC.

After establishing infection in epithelial cells or B lymphocytes, EBV maintains a type I or II latency programme. This includes the expression of latent genes like EBERs, EBNA-1, Bam-HI A rightward transcripts (BARTs), miR-BARTs, and latent membrane protein 2A (LMP2A) (57, 58). EBER1 upregulates insulin growth factor 1, promoting the growth of EBVaGC (59). It also triggers chemoresistance and cell migration through the IL-6-STAT3 pathway (60), and facilitates tumorigenesis by blunting cellular responses to DNA damage through disruption of promyelocytic leukemia nuclear bodies (61). Furthermore, EBNA1 sequesters reactive oxygen species via miR-34a and NOX2, increasing cell viability (62). LMP2A was found to activate the nuclear factor kappa-light-chain-enhancer of B cells (NF-κb) pathway to upregulate miR-155-5p, resulting in the inhibition of p-Smad2 and Smad 2, inducing epigenetic alterations in the host genome (63).

4 Proposed pathogenesis of EBV in PPLELC

PPLELC is a poorly differentiated carcinoma with a rich lymphoid infiltration in its stroma, which is histologically indistinguishable from metastatic NPC. This raises the possibility of a "converging" role of EBV transformation in these epithelial cells, leading to a poorly differentiated subtype. Whilst the majority of LELC existing in case series are of PPLELC, gastric LELC (64), intrahepatic cholangiocarcinoma (ICC)-LELC (65) and parotid LELC (29), which suggest an aerodigestive epithelial nidus of pathogenesis, not all LELCs described originate from epithelial cells directly in contact with environmental carcinogens or EBV infection.

At present, our understanding of how EBV contributes to PPLELC pathogenesis is limited, primarily relying on inferences drawn from our knowledge of EBV in the context of NPC, and similarities in the gene signatures and EBV latency in PPLELC. We propose that PPLELC adopts a similar model whereby a type II latency program in dysplastic tissue, accompanied by other predisposing factors, leads to the development of cancer (Figure 1).

Current literature has also established the presence of EBV viral load in PPLELC and its correlation to treatment response and prognostication (66–68). Here, we also posit the idea that early childhood latent infection of EBV in patients who develop PPLELC in later years possess underlying genetic predispositions such as variations in HLA-haplotypes and TLR subtypes, similar to the existing multistep model proposed in NPC (4). Subsequently, EBV oncoproteins and its downstream signaling pathways drive airway epithelium into malignant epithelial cancers (Figure 1). However, due to the rarity of this disease and its sparse literature, the genetic and environmental predispositions remain to be proven by genome-wide association studies and epidemiology studies.

5 Environmental carcinogens and genetic susceptibility of NPC, PPLELC, and EBVaGC

With the nasopharynx being located in the upper airway, and the lungs in the lower airway, both are exposed to similar viral infections and carcinogens; similarly, the nasopharynx and the upper digestive tract also share other carcinogens through inhalation and ingestion.

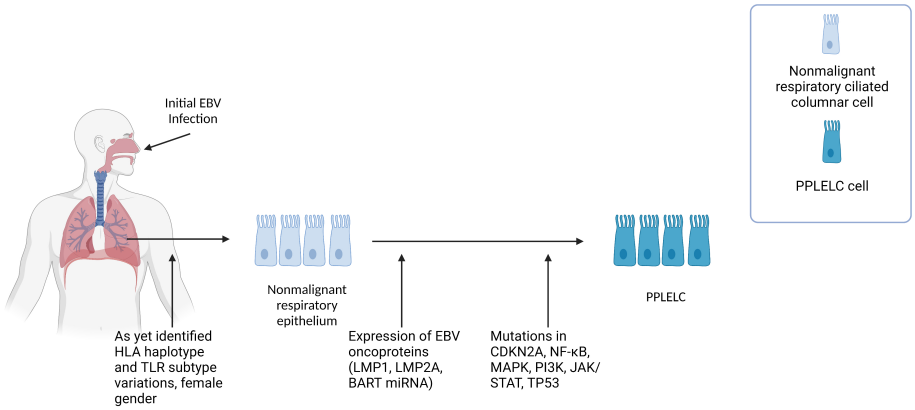

FIGURE 1
Figure 1 describes the proposed multistep pathogenesis of PPLELC. We propose that PPLELC follows a multistep pathogenesis process similar to that in NPC.

Early events

Like NPC, distinct genetic profiles (unidentified HLA haplotype, and TLR subtypes variations) and early EBV infection may result in susceptibility to EBV infection. Early infection results in a type II latency program, suggested by a molecular profile that is highly resembling that of NPC. Additional risk factors such as being of a Chinese race, female, and nonsmokers are also associated with increased incidences of PPLELC. However, our knowledge of the role of epigenetic and environmental risk factors in PPLELC is still in its infancy.

Type II latency program

In these non-malignant respiratory ciliated columnar cells, EBV can establish latency and subsequently express latent EBV oncoproteins (LMP1, LMP2A, BART miRBA). Mutations in TP53, JAK/STAT, and cell cycle genes such as CDKN2A and CCND1 further drive non-malignant respiratory epithelium to PPLELC. The molecular and mutational landscape in PPLELC bears a striking resemblance to the molecular profile of NPC, suggesting that EBV enters a type II latency program in PPLELC. While it is unclear how these type II latency oncoproteins confer oncogenic traits in PPLELC, extrapolations can be drawn from our understanding of their established roles in NPC pathogenesis. It is likely that these oncoproteins and their downstream signaling pathways help enhance cell survival and facilitate tumorigenesis, resulting in the transformation from non-malignant respiratory epithelium to PPLELC.

Several environmental risk factors have been found to increase NPC and EBVaGC risks. Dietary consumption of salt-preserved fish and preserved foods such as eggs, meat, and vegetables (12, 17, 69, 70) are highly associated with NPC. Exposure to wood dust (71–75), formaldehyde and solvents such as phenoxy acid and chlorophenol (76, 77) have also been linked to NPC and EBVaGC (23–25). Additionally, steam, flammable products, cotton dust, and smoking also increased NPC and EBVaGC risks (22, 78). A case-control study conducted among male patients in the Guangdong province demonstrated that a smoking pack-year of 40 or more predisposed an individual to a significant odds ratio of 1.76 to contract NPC. Similarly, 20-40 smoking pack-years was associated with a significantly increased risk of NPC, with an odds ratio of 1.52.

Histological and geographical similarities between NPC and PPLELC have prompted increasing comparisons between the two. Even though epidemiological studies on PPLELC are sparse, a recent study in Singapore revealed racial similarities, in which the Chinese race is more frequently affected (79). While still unexplored, a Chinese predominance may suggest that risk factors common in the traditional diet or environment of endemic regions could also be associated with PPLELC. While race is a similarity between PPLELC and NPC, there are some differences. For example, PPLELC tends to occur in non-smokers (43) and females (79). More studies are warranted to better describe the environmental risk factors associated with PPLELC.

Apart from environmental and epidemiological risk factors, certain genomic variants increase susceptibility to NPC. Several HLA alleles have been associated with increased risks of NPC, such as HLA-A1, HLA-A2, HLA-A*0207, HLA-A*33:03, HLA-B14, HLA-B*38:02, and HLA-B46 (80, 81). On the other hand, HLA-A11, HLA-A*11:01, HLA-A23, HLA-A*31:01, HLA-B*13:01, HLA-B14, HLA-B22, HLA-B27, HLA-B55, HLA-B*55:02, and HLA-DR4 were found to be protective factors against NPC carcinogenesis (80, 81). Variants in expressions and polymorphisms in toll-like receptors (TLR), a mediator of the immune system, were also found to increase the risk of NPC. Sequence variants in TLR10 and a functional variant in the 3'UTR of TLR4 were associated with increased NPC risk (82, 83). Genetic polymorphisms of TLR3 (84), TLR9 (85), and TLR4 (86, 87) were also linked to increased risks of NPC.

Compared to NPC, little is known about HLA and TLR variants in EBVaGC and PPLELC. While one study with 52 EBVaGC patients found no interactions between EBV and TLR polymorphisms (88), other recent studies have shown a reduced level of TLR9 in EBVaGC, suggesting its potential involvement in EBV reactivation (89). In PPLELC, TLR variants and HLA haplotypes conferring increased susceptibility have yet to be investigated, and is an avenue for future research.

6 Genetic and epigenetic characteristics in NPC, PPLELC, and EBVaGC

In NPC, early events such as genetic susceptibility, environmental carcinogens and epigenetic alterations drive normal epithelium to dysplasia through chromosomal loss and EBV-related malignant transformation (4). Subsequently, further somatic mutations and/or epigenetic inactivation events (cell cycle checkpoint, TP53 and other TSG inactivation) lead to tumor progression (Figure 2). This process is

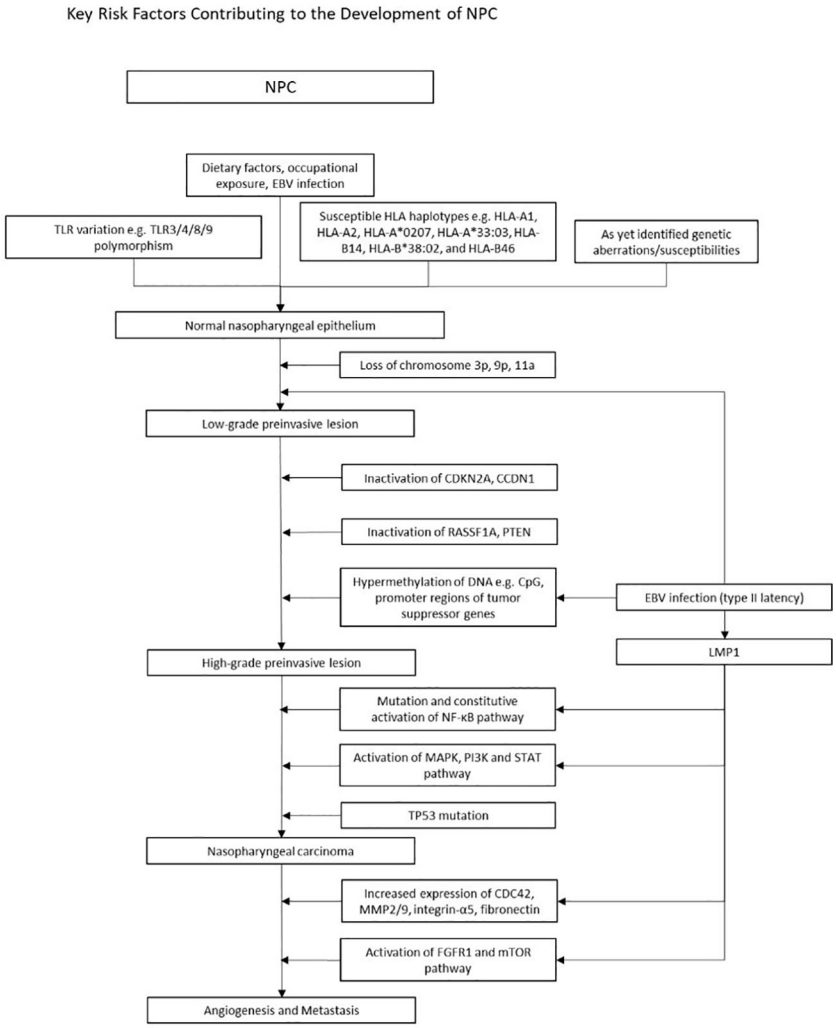

FIGURE 2
Figure 2 illustrates the multistep model of the pathogenesis of EBV positive NPC. A complex interplay of known genetic variations with environmental risk factors increases susceptibility to NPC. Subsequent chromosomal loss (3p, 9p, 11a), loss of function mutations (CDKN2A, cCDN1, RASSF1A, PTEN), and widespread hypermethylation lead to high-grade preinvasive lesions. Mutations in the NF-kB, MAPK/PI3K/STAT and TP53 pathways further drive tumorigenesis. Concurrently, EBV oncogenes upregulate the expression of various genes, perpetuating tumorigenesis.

Early events in NPC pathogenesis and epigenetic changes

Numerous environmental factors have stood out in its relation to NPC. Dietary habits, such as preserved fish and foods, as well as occupational exposures to wood dust, formaldehyde and cigarette smoke emerged as significant contributors which heightened risk of NPC. In early stages of NPC, in addition to various TLR and HLA polymorphisms, epigenetic alterations such as CpG Island hypermethylation, and chromosomal loss (3p, 9p21) play a pivotal role in silencing tumor suppressor genes, contributing to disease initiation and progression.

Abnormalities in signaling pathways

Dysregulation of the NF-KB, MAPK PI3K and STAT pathways are well known in NPC. LMP1 activates NF-KB by engaging both the canonical and non-canonical pathway, facilitating apoptotic evasion and immune escape. The MAPK, PI3K and STAT pathways are also activated via the TNF receptor, upregulating anti-apoptotic genes and pro-survival signals.

LMP1 and tumorigenic properties

LMP1 is one of the key players of tumor progression, orchestrating various mechanisms that drive malignancy. Through activation of the FGFR1, mTOR and, the NF-KB pathway, upregulation of MMP, fibronectin and integrin-a5, and enhanced VEGF expression, LMP1 stimulates new vessel formation, a critical factor facilitating tumor proliferation and metastasis. Simultaneously, LMP1 disrupts immune surveillance by hampering antigen presentation and amplifying anti-apoptotic signals, allowing it to evade immune detection and circumvent cell cycle checkpoints. EBV fosters a tumor suppressive microenvironment and this is enabled by LMP1 upregulation of IL-10 and upregulation of T helper cells. Uncontrolled cell proliferation is attained through LMP1 hyperphosphorylation of DK2 and Rb thus promoting G1/S progression, and LMP1 regulation of telomerases resulting in cell immortality. Other prominent EBV products include LMP2 and EBNA1, which are further detailed under Figure 3.

An overview of the development of NPC

In summary, Figure 2 provides a brief overview of the multi step model of NPC pathogenesis. This model posits that the development of NPC is a highly complex, multistage process of cumulative environmental exposures and genetic changes, spanning from initial infection and environmental encounters leading to epithelial dysplasia, and subsequent genetic and epigenetic alterations over time activating oncogenesis. Products of type II latency program then confer tumorigenic properties to NPC cells, enabling growth and aggressive invasion.

accompanied by angiogenesis, metabolic dysregulation and increased invasiveness, resulting in metastasis and immune evasion.

Early studies suggest chromosomal aberrations that are detected in the dysplastic nasopharynx, including the loss of chromosomes 3p, 9p, 11q, and 14q (90–93), with the most widely known being 3p (95%) and 9p21 (85%) (4). Interestingly, these genetic deletions occurred even in the epithelium of people with no NPC in endemic regions, suggesting that these epigenetic changes occur early in disease, and may predispose individuals to EBV infection (91, 93, 94). Significant hypermethylation at chromosome 6p21.3 were found in both NPC and EBVaGC (95). This region contains numerous genes encoding for tumor suppressors and HLA genes that are known to be important risk factors in NPC (95, 96). Apart from the epigenetic alterations in tumor suppressor genes (TSGs), amplification of several oncogenes were also found in 8-20% of NPC. These include phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (PIK3CA) and CCND1 on 3q26.3 and 11q13, respectively. The lymphotoxin-β receptor, a NF-κB signaller found on 12p13, was also amplified. Augmentations in PI3K-Akt and NF-κB signaling contribute to the tumorigenesis of NPC (97–99). Similar to NPC, mutations in PIK3CA are widely seen in EBVaGCs.

Genome sequencing and array-based methylomes have revealed a viral-mediated CpG Island hypermethylation Phenotype (CIMP) in NPC (95, 100, 101). EBV is known to induce a distinctive hypermethylated epigenotype in EBVaGC, NPC and LELC (63, 102–106), where important TSGs are inactivated by extensive methylation at the promoter region (107), in line with oncogenic behavior. Hypermethylation alters the balance between DNA methyltransferases and demethylases, maintaining type II latency. LMP1 and LMP2 are key players in promoting CIMP hypermethylation, which subsequently downregulates several TSGs such as Ras association domain-containing protein 2 (RASSF2A) (108), follistatin-like 1 (109), cyclin-dependent kinase inhibitor 2A (CDKN2A) (110), p16 (111), phosphatase and tensin homolog (PTEN) (112) and cadherin-1 (CDH1) (113), facilitating tumorigenesis and subsequent metastasis (94, 114). Although the pathogenesis of EBVaGC is not yet well defined, some have suggested that when EBV enters and establishes latency in the gastric epithelium, it undergoes genome wide methylation, a process similar to that in NPC (22, 115). The methylation of TSG (APC, PTEN and RASSF1A), cell adhesion molecules (THBS1 and E-cadherin), and CDKN2A are also widely found in EBVaGC (20, 116–119). Other methylated genes include MLH1, CXXC4, TIMP2 and PLXND1 (20).

Several molecular commonalities underlie PPLELC and NPC. Molecular profiling revealed similar LMP1 (120) and BART miRNA profiles between EBV positive PPLELC and NPC (121). Due to the expression of similar latent genes, it has been postulated that PPLELC and NPC share a similar pathogenesis involving a type II or III EBV latency programme, activating similar downstream pathways (122). Whole exome sequencing data from 30 patients with PPLELC showed that the mutational landscape of PPLELC closely resembled NPC, instead of other lung cancers or NKT cell lymphoma (123). NF-κB pathway genes, such as silencing mutations of negative regulators of NF-κB including TRAF3, were found in PPLELC, suggesting a similar oncogenesis pathway to NPC (122, 123). Likewise, mutations in TP53, JAK/STAT, and cell cycle genes such as CDKN2A and CCND1 were found in both NPC and PPLELC (116, 123, 124). High activation of AID/APOBEC genes (type 2 mutation signature), indicating a secondary immune response to EBV, is associated with the carcinogenesis of PPLELC (123), again pointing to the importance of EBV in shaping the somatic mutational landscape. While the tumor mutation load of PPLELC is low, large amounts of gene copy number changes, such as 11q13.3 amplification and 9p21.3 deletion were found, similar to the copy number variation in NPC (123).

EBV positive NPC has several unique traits which are distinct from other cancers, and these characteristics are reflected in PPLELC. Although a loss of function mutation of TSG p53 is present in many cancers, EBV associated NPC is known to lack this aberrance and usually have a normal profile of p53. Similarly, p53 levels were also found to be normal in EBV positive PPLELC tissues and with low levels of mutated p53 in PPLELC (122), suggesting a similar mechanism could be driving both NPC and PPLELC. This is similar to EBVaGC, where p53 mutations are rarely seen (125, 126).. EGFR activating mutations, ALK gene arrangement and ROS1 gene arrangement were also rarely found in PPLELC (127). Microsatellite instability is exceedingly rare in NPC (128), and this has been reflected in PPLELC (129). Also, the loss of heterozygosity at D5S346 (5q23) is common, suggesting that the pathogenesis of PPLELC is associated with a TSG in that region (129).

Existing models in NPC postulate a multistep pathogenesis process where early environmental exposures as well as distinct genetic profiles predispose an individual to EBV, and acquired epigenetic changes may result in susceptibility to EBV infection (Figure 2). Upon infection by EBV, latent genes expressed upregulate certain gene products which facilitate cell survival and malignant transformation, resulting in the further development of NPC (91). In view of its similarities to NPC as well as a similar model described for EBV related lung cancers (130), we suggest that PPLELC may adopt a similar multistep pathogenesis pathway (Figure 1). We hypothesize that EBV infection is an early transforming event, and that along with predisposing environmental risk factors, normal airway epithelial cells are transformed into poorly differentiated carcinoma.

7 EBV Latent Infection in NPC, PPLELC, and EBVaGC

EBV (human herpes virus type 4) is a double-stranded DNA (dsDNA) of approximately 170 KB. EBV is usually transmitted through the saliva (131) and infects both lymphoid and epithelial cells. Primary infection of EBV occurs as the virus enters the mucosal epithelium from the oropharynx and spreads to lymphoid tissues where it gains entry into the nasopharyngeal epithelial cells, facilitated by Ephrin A2 receptor and the vitronectin receptors αvβ5, αvβ6 and αvβ8 (132–134). Unlike in the latent phase where the EBV genome is only replicated once throughout the cycle, in the lytic phase, the EBV genome can undergo large numbers of amplification to generate numerous viral

genomes which can infect other cells (81). While recent studies of the lytic phase and its involvement in tumorigenesis (135, 136) suggest that both phases might be involved in oncogenesis, the role of the latent phase in driving both lymphoid and epithelial tumors has been well established (91).

EBV enters a latent phase where the virus remains dormant in the nucleus and the carrier remains asymptomatic. EBV establishes latency, ranging from type I to III. In type I latency, latent genes such as EBNA1, EBER, and BART RNAs are expressed (137). In type III latency, nine proteins, namely (EBNA)-1, -2, -3a, -3b, -3c, and -LP, and (LMP)-1, -2a, and -2b are expressed. Of note, latency II gene expression is known to play a primary role in the pathogenesis of NPC (138), resulting in the expression of latent genes such as EBV nuclear antigen 1 (EBNA1), LMP1, LMP2, EBV-encoded small RNAs (EBER), Bam-HI A right frame (BARF) proteins, BARTs, and BART microRNAs (miR-BARTs). EBV effectively transforms and immortalizes B cells through various latent gene expressions such as LMP1, LMP2A (139) EBNA, BART and mir-BARTs (140), and a similar type II latency programme is suggested to exist in PPLELC (122). LMP1, LMP2a, and the NF-κB pathways were found to be involved in the carcinogenesis of PPLELC (122), suggesting that the pathway of tumorigenesis is likely to be highly similar (Figure 1).

7.1 Mechanisms of latent gene products in type II latency programme

The involvement of latent gene products in NPC pathogenesis has been extensively documented and understanding its oncogenic properties and pathways in NPC allow us to hypothesize the pathogenesis and characteristics of other EBV positive tumors with a similar molecular profile, such as LELC. Figure 3 summarizes the EBV gene products and molecular pathways described below which are known to be involved in NPC.

Unlike EBVaGC where mutations in the NF-κB pathway are uncommon (3), NF-κB pathway dysregulation is known to occur in EBV positive NPC (141). LMP1 is a key oncoprotein in NPC tumorigenesis and is known to establish multiple oncogenic hallmarks in the dysplasia-carcinoma model. It is a transmembrane protein that activates NF-κB through its C-terminal activation regions (CTAR1 and CTAR2) via the canonical and non-canonical pathways. These signaling domains mimic CD40, constitutively activating NF-κB. NF-κB transcription is upregulated through the canonical and non-canonical pathway. In the canonical pathway, CTAR2 recruits TRAF6/Transforming growth factor-β-activated kinase 1 (TAK1)/inhibitor of nuclear factor kappa-B kinase subunit β (IKK-β), and CTAR1 activates NF-κB through the non-canonical pathway, by recruiting TRAF1, 2, 3, 5, NF-κB-inducing kinase (NIK), and IKK-α (3, 142–146). CTAR1 also independently activates the canonical pathway of NF-κB (147). The LMP1 mediated canonical and non-canonical mediation of NF-κB pathways confer a survival advantage to tumor cells by enabling apoptosis evasion, B-cell activation, and survival (142), and is important for tumorigenesis via many pathways. Recent studies have also elucidated a role of NF-κB in regulating viral gene transcription of BART miRNAs and long noncoding RNAs, modulating the shift between latent and lytic states (124, 148), as well as promoting somatostatin receptor 2 (SSTR2) induction (149).

Apart from modulating the NF-κB pathway, LMP1 also functions as a constitutively active tumor necrosis factor (TNF) receptor, activating several other signaling pathways, including mitogen-activated protein kinase (MAPK), PI3K, and signal transducer and activator of transcription (STAT), resulting in the upregulation of anti-apoptotic genes (150). LMP1 also promotes stem-cell like properties, which are further maintained by the upregulation of oncogenes and anti-apoptotic genes like BCL2 and LEF1 (141).

LMP2A is a viral-encoded mimic of the B-cell receptor (BCR), and constitutively activates BCR-like signaling through recruitment of the Lyn and Syk kinases (151). It also provides a counteractive effect by depriving the BCR-signaling complex of its components. These two counterbalancing activities help EBV stay latent in B cells, evading detection by the immune system (152).

EBNA1 helps maintain the presence of the EBV genome in NPC as circular DNA episomes by binding to viral DNA. EBNA1 was found to be critical for the immortalization of B cells (153, 154). Survivin, an inhibitor of apoptosis, was also found to be activated in B lymphocytes by EBNA1 (155). In a recent study, it was demonstrated that EBNA1 results in a dose-dependent breakage at chromosome 11q23 (156). This is consistent with the fact that latent EBV reactivation has been associated with tumorigenesis, especially NPC (157). Similar to NPC, EBNA1 is also consistently expressed in EBVaGC (158) where it induces the loss of promyelocytic leukemia (PML) nuclear bodies (NBs) (61). This decreases p53 activation and apoptosis in response to DNA damage, allowing for EBVaGC carcinogenesis.

BART transcripts are abundant in NPC cells (121, 159), and are subsequently processed to miR-BARTs. miR-BARTS stabilize latent infection, suppress lytic replication by targeting BZLF1 and BRLF1 (159), downregulate apoptosis (160), and activate pathways promoting tumorigenesis (161).

While non-exhaustive, EBV latent gene products also trigger downstream pathways known to be involved in malignant transformation. For instance, aberrance in Wnt signaling regulation in NPC has been demonstrated by gene expression profiling studies (162). While studies have found that LMP1 does not play a critical role in direct activation of the WNT pathway (163), other latent gene products such as LMP2A activates PI3K/AKt to inhibit the downstream GSK-3b of Wnt, sequestering b-catenin in the nucleus and inducing EMT (164). Both LMP1 and LMP2A were also found to induce stem cell-like properties in NPC cells through Hedgehog signaling (165, 166). TGF-B1, a growth suppressor, is also inhibited by LMP1 (167). LMP1 also induces the expression of IL-10 and vIL-10, an immunosuppressive cytokine whose levels are raised in many cancers (168). Not only do the EBV latent gene products independently activate several downstream pathways beneficial for tumorigenesis, they also positively regulate one another leading to further upregulation (169, 170).

Interestingly, NPC and PPLELC have highly similar molecular landscapes. Like NPC, PPLELC also expresses high levels of LMP1,

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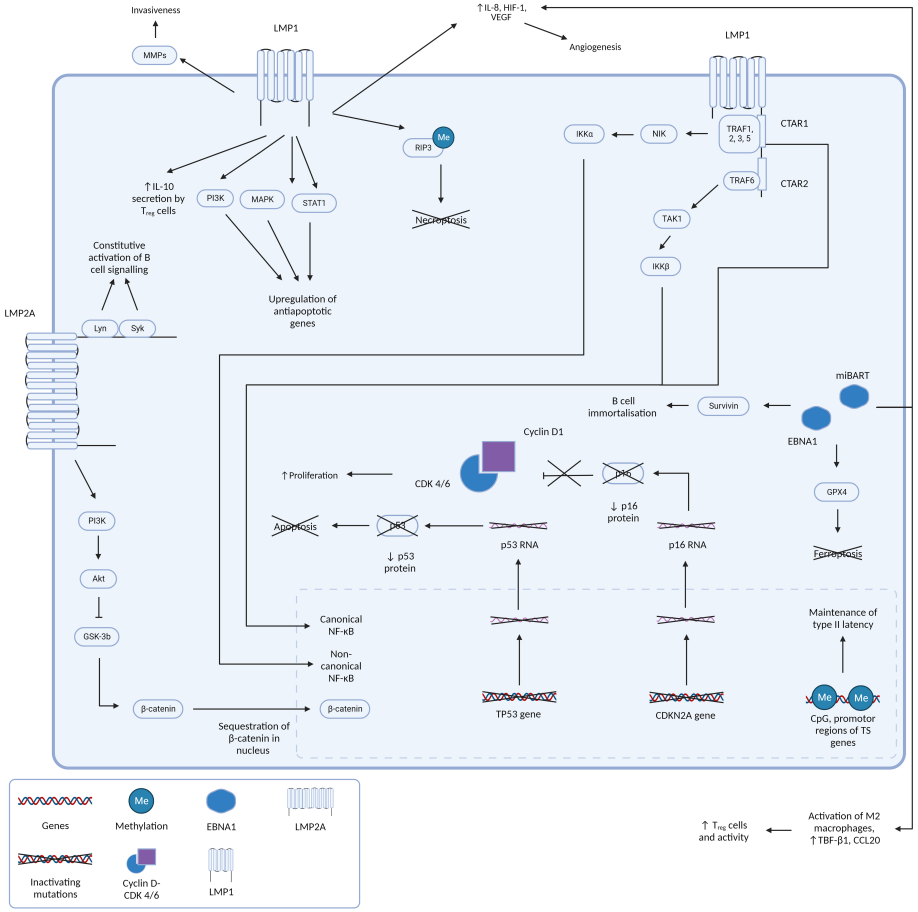

FIGURE 3
Figure 3 presents a comprehensive and intricate depiction of the pivotal roles played by type II latency Epstein-Barr virus (EBV) oncoproteins, specifically Latent Membrane Proteins 1 and 2A (LMP1 and LMP2A), in conjunction with the crucial gene product EBNA1. It sheds light on how their orchestrated actions contribute to the initiation and progression of tumorigenesis by silencing of key tumor suppressor genes and driving cell proliferation and survival.

LMP1 and LMP2A: masters of oncogenic signaling

At the forefront of this dynamic interplay are the oncoproteins LMP1 and LMP2A. LMP1 is a multifunctional transmembrane protein. By emulating the constitutive activation of CD40, a pivotal B-cell receptor, LMP1 engages the TNF receptor-associated factors (TRAFs) and orchestrates the activation of the canonical NF-kB pathway. This activation culminates in the creation of a proinflammatory milieu that is conducive to cell survival and unbridled proliferation. Furthermore, LMP1's capacity to activate the non-canonical NF-kB pathway further amplifies these signals, resulting in a sustained NF-kB activity that effectively shields cells from apoptotic cues. LMP1's influence isn't confined to NF-kB signaling alone. It interfaces with a multitude of molecules, including Janus kinases (JAKs), STATs, and PI3K, thereby activating pathways that foster cell growth and survival. Moreover, LMP1's impact on various microRNAs and transcription factors significantly influences gene expression and cellular behavior. Beyond direct oncogenic signaling, LMP1 also modulates the tumor microenvironment, augment cellular invasiveness, and promotes angiogenesis. These help create a supportive niche for tumor progression and metastasis. Complementing LMP1, LMP2A emerges as a critical player in EBV-associated oncogenesis. This transmembrane protein mimics the B-cell receptor (BCR), a critical component in B-cell signaling. By recruiting protein tyrosine kinases, including Lyn and Syk, LMP2A effectively dampens BCR signaling. Activation of the PI3-kinase/Akt axis stimulates GSK3 which culminates in the accumulation of betacatenin, thereby promoting WNT signaling. This strategic inhibition aids in evading the regulatory mechanisms that would otherwise prompt apoptosis of autoreactive B cells. This evasion, in turn, promotes the survival of EBV-infected cells, and similar to LMP1, fosters a cellular milieu that supports enhanced proliferation and survival.

EBNA1: guardian of viral persistence and cellular transformation

Central to this network of interactions is the gene product EBNA1. This multifaceted protein stands as a linchpin in ensuring the persistence of EBV within the host cell. In addition to its role in maintaining viral genome replication, EBNA1 actively engages cellular machinery to promote cell proliferation and survival. Importantly, EBNA1 orchestrates the silencing of key tumor suppressor genes, notably CDKN2A and TP53. This results in an imbalance that shifts the cellular equilibrium toward unchecked cell growth and survival, fueling the malignant transformation of host cells.

A holistic view of tumorigenesis

Collectively, Figure 3 encapsulates the multifaceted orchestration of type II latency EBV oncoproteins, LMP1, LMP2A, and EBNA1, in driving the molecular intricacies of tumorigenesis. Their intricate molecular interplay, ranging from direct oncogenic signaling to the modulation of the tumor microenvironment, lays the foundation for cell transformation and the subsequent development of EBV-associated malignancies.

LMP2a, and NF-κB (122), with LMP1 detected in 42% of PPLELC (122). While little is currently known about how these gene products promote oncogenesis in PPLELC, these pathways have been well documented in NPC. Understanding how these common pathways foster angiogenesis and metastasis, cell cycle survival, and immune evasion in NPC, and also in other epithelial cancers such as EBVaGC, may guide our understanding in how these gene products establish tumor hallmarks in PPLELC.

7.2 Angiogenesis and Metastasis in NPC and EBVaGC

Angiogenesis is a major contributor to oncogenesis and metastasis as new blood vessel formation enhances nutrient and oxygen delivery to rapidly proliferating cells. In NPC, EBV latent proteins (EBNA1 and LMP1) promote angiogenesis through upregulation of angiogenic cytokines IL-8, hypoxia inducible factor-1 (HIF-1), and VEGF (171–173). LMP1 activation of NF-κB leads to the activation of the STAT pathway via increased levels of IL-6, and IL-8, promoting cell proliferation (174, 175), and angiogenesis (176) respectively. The role of EBNA1 in B cell transformation was also demonstrated in mice models where EBNA1 expression was associated with increased primary tumor formation and metastasis (177). The enhanced angiogenic ability can also be induced in neighboring endothelial cells promoting angiogenesis by the transferring of EBV encoded small RNAs and subsequent induction of vascular cell adhesion molecule 1 (VCAM-1) expression in endothelial cells (178). In EBVaGC, hypoxia-induced ebv-cir LMP2A promotes angiogenesis through the KHSRP/VHL/HIFa/VEGFA pathway (179). EBVaGC also upregulates IHH, a gene which increases metastatic potential through angiogenesis, Snail protein expression, and decreases e-cadherin and tight junctions (115). A loss of PTEN in EBVaGC activates PI3K/Akt pathway, resulting in increased angiogenesis, migration and loss of cell cycle adhesion (180). Downregulation of e-cadherin through ARID1A loss also leads to enhanced tumor migration and lymphovascular invasion (180). The WnT pathway is also downregulated by LMP2A in EBVaGC, enhancing cell migration and invasion (181). In both EBVaGC and NPC, EBV was found to promote vasculogenic mimicry formation through the PI3K/AKT/mTOR/HIF-1α pathway (182).

Matrix metalloproteinases (MMPs), key proteins responsible for the degradation of collagen IV, enabling basement membrane invasion, are also increased in EBV positive NPC and EBVaGC (183–186). LMP1 modifies its extracellular vesicles by increasing gene expression of MMP9 and MMP2 (187), and enhances cell attachment through the upregulation of integrin-α5 and fibronectin (187). Apart from directly modulating MMP expression, LMP1 also mediates other pathways such as cell division cycle 42 (CDC42), a protein known to regulate invadopodia formation (188, 189), actin cytoskeleton reorganization, and increase motility (190). Crucial cell adhesion molecules such as E-cadherin were also found to be downregulated by miR-200, miR-BART9 and LMP2A, BARF0, EBER and EBNA1 (191–193). The converse was found to be true, whereby a deletion in miR-BART9 results in elevated levels of E-cadherin expression, and a reduction in the proliferative and invasive potential of EBVaGCs (194).

A switch from oxidative phosphorylation to aerobic glycolysis is also beneficial for cell proliferation and invasion by providing intermediary metabolites (195). LMP1 activates glycolysis through the fibroblast growth factor receptor 1 (FGFR1) signaling pathway (196) and the mTOR pathway through auto-secretion of insulin-like growth factor 1 (IGF-1) (197). The predilection for aerobic glycolysis was evidenced by increased lactate levels, extracellular acidification ratio and oxygen consumption ratio suggestive of enhanced glycolysis, as well as reduced oxidative phosphorylation enzymes (197, 198). Glycolysis has also been linked with higher invasiveness as glycolytic enzyme expression was increased in cells with higher invasive properties than those without (197). Furthermore, the acidic tumor microenvironment due to lactate production also favors an environment for tumor cell migration (196, 199).

7.3 Cell cycle survival in NPC and EBVaGC

Tumour cells gain proliferative advantage by expressing aberrant genes which hijack regulation checkpoints, enabling hyperproliferation and apoptotic escape. In EBV-associated NPC, the CTAR1 domain of LMP1 hyperphosphorylates CDK2 and Rb, promoting G1/S cell cycle progression and protecting the cell from apoptosis (200, 201). Furthermore, LMP1 also regulates telomerase activity through the $p16^{INK4a}$/Rb/E2F1, PI3K-AKT and c-Jun N-terminal kinase (JNK), promoting immortalisation (202). In fibroblasts, LMP1 suppresses $p16^{INK4a}$ and p21WAF1 by H-ras61L, suppressing cell senescence (203). In EBVaGC, there is an upregulation of Bcl-2 and CyclinD1, allowing apoptotic evasion and progression of cell cycle through the G1 phase respectively (204). LMP2A plays a regulatory role in modulating the expression of cyclin E and the ratio of cells in the S phase (205) and inhibits TGF-β1 induced apoptosis (206, 207) miR-BART9, 11, 12 also reduced Bim expression, a pro-apoptotic protein (208).

Similar to other cancers, multiple cell cycle checkpoints (e.g. p16 and p21) are compromised by EBV. p16 is a TSG (CDKN2A) which inhibits cyclin D1/CDK4, and is commonly hypermethylated and silenced in NPC and EBVaGC (111, 114). Knocking out p16 expression, or over expression of its negatively regulated target cyclin D1/CDK4, promoted expansion of EBV infected cells (209). LMP1 also inactivates p16 by inducing sequestration of E2F4/5 and ETS-2 in the cytoplasm, rendering p16 dysfunctional in these cells (210). p21, another cell cycle inhibitor, was also downregulated in NPC cell lines by the binding of miR-17-5p to its 3' untranslated (3'UTR) region (211). EBV positive NPC tumors are also more resistant to ferroptosis through EBNA1 upregulation of glutathione peroxidase 4 (GPX4) (212), and LMP1 inhibited necroptosis through RIP3 hypermethylation (213).

Interestingly, while p53 mutations, like other TSGs, are classically present in most other cancers, NPC has a unique profile of p53. Unlike other cancers which usually have mutations or loss of p53, there is little association between p53 mutations with NPC, and normal or elevated levels of p53 are often present (214–217). LMP1 levels positively correlate with p53 levels, and can induce p53 expression via the H19/miR-675-5p axis (218). Despite normal or elevated levels of p53, rapid proliferation suggests that EBV encoded products suppress the function of p53 instead (219). For instance, p53 expression can be inhibited by EBV encoded miR-BART5-3p, a loss of p14, as well as excess p63 (219–221). A similar pattern is also seen in EBVaGC where p53 mutations are rare (125, 126, 222), again suggesting that EBV encoded products suppress p53 function instead. For instance, BART3-3p and BART 5-3p bind TP53 to reduce senescence of EBVaGC, accelerate cell cycle

progress, and prevent apoptosis (219, 223). It has also been suggested that normal or high p53 levels may confer a survival benefit as these cells will not be susceptible to JNK and Rapamycin-induced apoptosis (224, 225). Taken together, these suggest that p53 mutation does not have a major role in early pathogenesis (226), and subsequent p53 loss may occur as an independent event.

7.4 Immune modulation and evasion in NPC and EBVaGC

EBV is known to persist and escape immune clearance, and its presence induces an immunosuppressed tumor microenvironment (TME). Even though inflammation occurs in NPC (227), much of the TME remains immunosuppressed. While the latent phase was thought to be responsible for NPC pathogenesis, recent studies have also elucidated a role of the lytic phase in pathogenesis (135, 136).

In LMP1 positive NPC cells, IL-10, an immunosuppressive cytokine which reduces antigen presenting cells and T helper cell activity, was also found to be fourfold higher (228). LMP1 also plays an important role in creating an immune-suppressed TME by inducing interleukin-10 (IL-10) secretion in regulatory T cells. EBNA1 stimulated regulatory T cell (Treg) chemotaxis towards the NPC TME, through the upregulation of the transforming growth factor-β1 (TGF-β1)-SMAD3-PI3K-AKT-c-JUN-CXCL12-CXCR4 axis and downregulation of miR-200a (229). EBNA1 also upregulated TGF-B1 and CCL20 which converted naive T cells into Treg cells and increased Treg cells migration, respectively (230). Additionally, EBNA1 also activates M2 macrophages which encourages conversion of naive T cells into Treg cells (230). These are congruent with the presence of elevated levels of Treg cells found in the TME of NPCs (231) as well as its positive correlation to plasma EBV levels of NPC patients (230).

In EBVaGC, increased levels of Tregs, IL-1B, and IL-10 in EBVaGCs suggest an immunosuppressive environment (232, 233). This is evidenced by the impaired function of cytotoxic T lymphocytes (CTLs) and natural killer cells in EBVaGC. Elevated levels of IFN-γ depletes tryptophan and suppresses tryptophan sensitive cytotoxic T lymphocytes (CTLs) and natural killer cells (234). Additionally, LMP2A mutations on exons 1-8 (30) and PD-L1 upregulation in both EBVaGC and NPC (235–237) limit CTL detection and promote differentiation of CD4+ cells into Treg cells (238). Other molecules contributing to immunosuppression include CCL22 which increases Treg recruitment, and indoleamine 2,3-dioxygenase (IDO1) (234, 239, 240). Antigen processing in EBVaGC is also inhibited by EBNA1 repeats and early lytic gene BNLF1a (234, 241), perpetuating immune evasion.

8 Treatment options for NPC, PPLELC, and EBVaGC

Stage 0 and I NPC are usually treated with radiotherapy to the site of disease, with prophylactic radiation to cervical lymph nodes (242). Patients with stage II, III, and IV NPC are treated with concurrent chemoradiation (chemoRT), with stage III and IV NPC requiring either induction or adjuvant chemotherapy on top of chemoRT, usually cisplatin or cisplatin with fluorouracil (5-FU) or adjuvant oral capecitabine (243). Recurrent or metastatic NPC can be treated with palliative radiotherapy, chemotherapy, targeted therapy and/or immunotherapy. On the other hand, no clear treatment guidelines for PPLELC have been outlined at present (34). In general, surgical treatment is the mainstay of treatment for early PPLELC, and treatment options like chemotherapy, radiotherapy are usually reserved for more advanced stages of PPLELC (45). Because PPLELC is uncommon, the adoption of standardized treatments has been challenging. Existing treatment regimes are adapted from those used for stage IV NPC, which involves gemcitabine-based chemotherapy (45), taxanes (244), anti-angiogenic therapy (245), and anti-PD1/PD-L1 therapy (246). The favorable response of PPLELC to NPC treatments is evident of the therapeutic implications of molecular similarities between the two cancer types, and the substantial overlap in their molecular characteristics provides an explanation for the effective response of PPLELC to chemotherapies typically used in NPC. For EBVaGCs, early stage cancers are treated surgically via endoscopic mucosal resection or gastrectomy. The treatment options for locally advanced EBVaGC is similar to that of other gastric cancers. This includes perioperative chemotherapy with primary resection. Advanced EBVaGCs are treated with palliative chemotherapy (taxanes, irinotecan, platinums, and fluoropyrimidines) and VEGF-R antagonist ramucirumab (247).

The role of immunotherapy in the treatment of NPC has been increasingly explored and this is especially so because the tumor microenvironment of EBV-driven NPC creates conditions favorable for immunotherapy. Much attention has been placed on immune-checkpoint inhibitors (248). In 44 patients with recurrent or pretreated metastatic NPC, patients receiving nivolumab achieved a 1-year overall survival rate of 95% and 1-year-progression free survival of 19.3% (249). In the KEYNOTE-028 study (250), 27 patients with PD-L1 positive unresectable or metastatic NPC achieved a 25.9% objective response rate of partial response and stable disease. The more recent JUPITER-02 study demonstrated that the addition of toripalimib to the standard first line chemotherapy treatment of gemcitabine and cisplatin yielded a significant increase in progression-free survival (HR = 0.52, 11.7 v.s 8.0 months) and overall response rate (77.4 v.s 66.4%) (251). The addition of camrelizumab to the standard gemcitabine and cisplatin chemotherapeutic agents in the CAPTAIN-1st trial also demonstrated a significantly increased progression-free survival (HR = 0.54, 9.7 v.s 6.9 months) (252). In RATIONALE-309, the addition of tislelizumab to standard chemotherapy resulted in a significantly improved progression-free survival (HR = 0.50, 9.6 v.s 7.4 months) (253). However, the efficacy of immunotherapy does not extend when it is used alone as KEYNOTE-122 showed no improvement in overall survival when pembrolizumab was used as a monotherapy as compared to the standard chemotherapy agents used for recurrent or metastatic NPC (254).

Similar to NPC, PD-1/PD-L1 inhibitors are potential therapeutic options in PPLELC (255).. A recent study demonstrated that the use of sintilimab, pembrolizumab, and nivolumab in patients with advanced PPLELC helped achieve

stable disease in 60% of the patients (255). The use of nivolumab resulted in partial remission in 36.4% of the patients, and stable disease in 54.5% of the patients. While the predictive value of PD-L1 as a biomarker of immune checkpoint inhibitor remains debatable (256, 257), a recent study by Zhong et al. demonstrated that higher PD-1/PD-L1 and LAG3 levels were indicative of a significantly better progression-free survival in a retrospective study (19 months v.s 3.9 months) (257).

Immunotherapy such as pembrolizumab have also demonstrated efficacy in EBVaGC as one study demonstrated a 100% (6/6) response to pembrolizumab in patients with EBVaGC (258). However, given the small sample size of that study, it is necessary to validate this finding with larger studies. The positive response of immunotherapy could suggest a potentially synergistic effect when administered alongside chemotherapy in EBVaGC. Though not shown yet, this is currently seen in advanced gastric cancer patients where the combination of chemotherapy and immunotherapy such as pembrolizumab and nivolumab was superior to using chemotherapy as a single agent (259), as well as in NPC as seen in the JUPITER-02 study.

Apart from immunotherapy, another treatment option is to employ cytotoxic T lymphocytes (CTLs). EBV-CTL is an autologous T-cell therapy generated through irradiated EBV-transformed lymphoblastoid cell lines (LCL). It was found that infusion of autologous EBV-CTL post lymphodepletion had efficacy as an anti-tumor agent for patients with locoregional NPC. In a recent phase III trial (VANCE trial, ESMO 2022 Oral presentation) in patients with recurrent or metastatic NPC, EBV-CTL administration following gemcitabine and carboplatin (GC) treatment also did not reveal superior efficacy over first line standard of care GC. Still, in the VANCE trial, improved survival trends were seen in a subset country specific analysis of overall survival and progression free survival for Taiwan, United States, and Singapore (unpublished). EBV-CTL may still be a viable treatment option as CTLs specific for LMP2 were shown to be associated with a significantly higher overall survival in a phase II study (260). EBV-CTL also demonstrated a very favorable safety profile (261). Like NPC, EBV-CTL also displayed efficacy in the treatment of PPLELC. In an unpublished study, our group was able to demonstrate complete response to EBV-CTL in a patient with heavily pre-treated advanced PPLELC in combination with an immune checkpoint inhibitor after previous progression on immune checkpoint inhibitor after just a few months.

A phase II trial in patients with locally recurrent NPC found that patients with Endostatin, an inhibitor of angiogenesis, administered in addition to cisplatin and paclitaxel had longer progression free survival with increased disease control rates (262). It was also found that anti-angiogenic therapy on top of radiotherapy improved clinical outcomes (263). Similarly, angiogenesis inhibition was also efficacious in PPLELC. In one study, small molecule multi-targeted TKI, inhibiting VEGFR, platelet derived growth factor (PDGFR), FGFR, and c-KIT was used to treat a patient with FGFR3-TACC3 fusion, and response was favorable. While studies are limited, this suggests that anti-angiogenic therapy can potentially be employed in the treatment of PPLELC (245).

Targeting EBV latent proteins has also shown potential as a viable therapeutic option. Not only is EBNA1 the only viral protein expressed in all EBV infected cells regardless of latency type, its vital role in maintaining EBV episomal genome and its consistent expression of EBNA1 in all tumor cells makes it a promising therapeutic target (47). Administration of conditionally replicating adenovirus expressed in an EBNA-1-dependent manner (adv.oriP.E1A) in EBV-positive NPC cells demonstrated cytotoxic effects and tumor regression in xenografts models, albeit limited clinical potential due to liver sequestration (264, 265). Preventing DNA binding of EBNA1 has also been an area of study. In a high-throughput virtual screen, several small molecule inhibitors, such as LB2, LB3, LB7 and LC7, SC7, SC11, SC19, SC27) (266) have been identified to selectively inhibit EBNA1 DNA binding (267). Others have also developed molecules that could inhibit EBNA1 DNA binding in xenografts, such as 2,3-disubstituted benzoic acid (268), of which one of its inhibitors is in a Phase/I/IIa clinical trial (269) (NCT03682055) in EBV positive NPC (47). Apart from targeting EBNA1, inhibition of LMP1 targeting DNAzyme found lower short-term tumor regression rate in a clinical trial of 40 patients with NPC (270).

Lytic inducers have the benefit of sensitizing tumor cells to anti-virals via the expression of viral kinases (271) during EBV reactivation. During reactivation, highly immunogenic viral replication proteins and virions are expressed which results in a strong immune response against EBV reactivated tumor cells (272). Because of this, oncogenesis is largely dependent on latency, and therapeutic strategies have been devised to inhibit latent genes or activate lytic genes with the aim of inducing oncolysis. For instance, it was found that transfection of EBNA-driven CMV BZLF1 plasmid induced BZLF1 reactivating EBV, resulting in cell death (273). Other compounds, such as histone deacetylase inhibitors (HDACis) or protein kinase C (PKC) activators have also been used to activate EBV lytic genes, albeit with varying effectiveness (274–277). This variability is likely attributed to the highly cell-dependent sensitivity towards lytic inducers, which also explains the diverse responses observed in oncolysis following the administration of gemcitabine and valproic acid in Phase I/II trials (278). Somatic mutations in TP53 and TGFBR2 were also found to maintain EBV latency in NPC (122, 279, 280), and may affect a patient's response to lytic-inducer treatment.

The goal of understanding the intricacies of cancer pathogenesis is to intervene as upstream as possible, thereby thwarting the progression into dysplastic changes. Mitigating dysplasia and minimizing exposure to risk factors play pivotal roles in substantially lowering cancer risk. Consequently, there has been increasing focus on advancing vaccine development, implementing nuanced screening methodologies, and cancer biomarker development as part of a comprehensive preventative approach. Patients with NPC were found to have persistently elevated plasma EBV DNA (281) and plasma EBV is increasingly used as a screening tool for NPC (9) and a surveillance biomarker for patients in remission (282). Posttreatment plasma EBV DNA is the strongest independent predictor for cancer recurrence and long term survival, with high EBV DNA levels strongly correlating with disease progression, and low plasma EBV DNA posttreatment correlating

with high progression-free survival rate at 2 years (283). Because EBV insidiously results in disease, therapeutic EBV vaccines can be highly efficacious and cost effective in lowering disease recurrence (284). The majority of current vaccines currently target EBV latent proteins such as EBNA1, LMP1, LMP2 (284). Several trials have also explored the potential of chimeric antigen receptor T cell (CAR-T) and T cell receptor-engineered T cell therapy (TCR-T) immunotherapy, with the latter more effective for solid tumors (284).

With EBV being a commonality behind the pathogenesis of EBV related epithelial cancers, we speculate the possibility of a universal off-the-shelf cell therapies targeting EBV-driven cancers and diseases, and this is an avenue for future research. This is especially relevant since recent studies noted clinical improvement in EBV-CTL treated EBV-driven MS, in which B-cells are proposed to be involved (285). With PPLELC and NPC sharing similar biological and molecular characteristics, it is unsurprising that treatments involving the same therapeutic targets have shown efficacy.

9 Discussion

Of the epithelial cancers associated with EBV, NPC and PPLELC have a predominant distribution in South China and Southeast Asia compared to the western population. On the other hand, EBVaGC is more prevalent in the western countries such as Germany and the United States (15-18%) (22). The oncogenic properties of EBV in NPC are well documented, and its latent genes LMP1, LMP2A, EBNA1, and BART transcripts, as well as its lytic genes, such as BZLF1, are known to participate in multiple pathways promoting tumor growth and metastasis, as well as imparting immuno-evasive characteristics. An overview of the characteristics of NPC, EBVaGC and PPLELC is shown in Table 1. While the connection between EBV and NPC has been well documented, its precise mechanisms remain elusive.

EBV infection is the common oncogenic pathogen underlying several seemingly anatomically unrelated cancers, in particular, NPC, EBVaGC and PPLELC. Even though EBV is spread through droplets, it is often absent in nasopharyngeal epithelium even though EBV titres are high (48). This led to hypotheses that EBV could remain latent in other anatomical sites (48). This postulation is bolstered by the suggestion that one of the variants of NPC originates from the basal cells of the respiratory epithelium despite NPC's squamous origin (286).

Although EBV is involved in NPC, EBVaGC and PPLELC, it enters different latency programs as evidenced by their distinct molecular profiles. EBVaGCs express LMP2A, BARTs, miR-BARTs, EBNA1, and EBER (57, 58), however, they express significantly lower levels of EBNA2 and LMP1 compared to NPC. This suggests that EBVaGC exhibits a type I latency program (287), and is more similar to burkitt's lymphoma and distinct from NPC (30, 287, 288). On the other hand, EBV latent genes expressed in NPC include EBNA1, LMP1, LMP2, EBER, BARF, BARTS and miR-BARTs, characteristic of a type II latency program. While the type of EBV latency exhibited in LELC still remains to be uncovered, expressions of LMP1 and LMP2 strongly suggest a Type II or III EBV latency program, akin to that of NPC (122). Understanding the pathogenesis of NPC may guide current understanding of PPLELC pathogenesis.

Due to the rarity of PPLELC, there is a paucity of studies examining the role of EBV in PPLELC and our understanding is still in its infancy. Histological similarities between NPC and PPLELC suggest a closely related pathogenesis, and current perspectives of the role of EBV in NPC pathogenesis can potentially guide our future understanding of EBV in PPLELC and. Despite different cell lines of origin in NPC and PPLELC, the mutational landscape in PPLELC implies similar driver mutations in NF-κB, CDKN2A,

TABLE 1 Overview of the key similarities and differences between NPC, PPLELC, and EBVaGC.

	NPC	PPLELC	EBVaGC
Etiology	EBV infection	Postulated to be associated with EBV infection	EBV infection
Epidemiology	Rare in Western Countries, and more commonly found in Guangdong province of China, Hongkong, Southeast Asia, East Asia, and the Mediterranean area Males > Females	Rare in Western countries, commonly found in Asian countries like South China Females > Males	More common in Western countries like Germany and the United States Males > Females
Risk Factors	Smoking and preserved food containing volatile nitrosamine and salted fish	Smoking is not a risk factor	Smoking, salty food, exposure to wood dust and iron filings.
EBV Latency	Type II latency	Postulated to be type II latency	Type I latency
Key Molecular and Immune Characteristics	NF-κB pathway activation, mutations in CDKN2A, CCND1, TP53, JAK/STAT, PI3K-Akt pathway, and Chromosomal instability phenotype EBV-CIMP (CpG island methylator phenotype). Upregulation of SSTR2.	NF-κB pathway activation, mutations in CDKN2A, CCND1, TP53, JAK/STAT. Upregulation of SSTR2.	Mutations in PIK3CA and mutations in CDKN2A. EBV-CIMP
Somatic Mutations	Loss of chromosomes in 3p, 9p (9p21), 11q (11q13), and 14q Hypermethylation at 6p21.3	9p21.3 deletion and 11q13.3 amplification	Hypermethylation at 6p21.3 Methylation of TSG (APC, PTEN and RASSF1A), cell adhesion molecules (THBS1 and E-cadherin), CDKN2A, MLH1, CXXC4, TIMP2 and PLXND1. Mutations in ARID1A and BCOR

JAK/STAT pathway. They also have similar p53 and PD-L1 regulatory patterns. Its expression of latent phase gene products such as LMP1 and LMP2 also suggests that it has a type II latency program similar to NPC. The parallels in genomic and molecular profiles of EBV positive NPC and PPLELC raise the possibility for common therapeutic strategies for advanced NPC and PPLELC. With its distribution mainly in Asia as well as its good prognosis, future studies investigating the mechanisms driving the PPLELC can potentially guide therapeutic targets. Given the histopathological and molecular similarities between NPC and PPLELC, it is unsurprising that therapeutics proven effective for NPC have demonstrated efficacy in treating PPLELC as well. This convergence in treatment strategies particularly between NPC and PPLELC could potentially extend to other EBV-related epithelial cancers in future. The development of a universal EBV-directed therapeutic approach in future holds promise to effectively treat a wide range of EBV-positive epithelial cancers, including but not limited to NPC and PPLELC. However, because of the limited studies in PPLELC, studies on EBV CTL or other novel EBV related therapies on PPLELC can help guide our understanding in this area.

The complex interplay between genetic mutations with local inflammation of the nasopharyngeal epithelium is thought to establish latent EBV infection, which is the primary driver of NPC pathogenesis (289). Despite the molecular similarities between NPC and PPLELC, more studies are required to better understand the distinct *in vivo* microenvironments of their respective host stromal components. The role of the *in vivo* microenvironment in pathogenesis is reinforced by the challenges in replicating the microenvironment *in vitro* when creating NPC cell lines, as well as the continuous loss of EBV episomes in NPC cells *in vitro* (289). Our group is currently investigating the spatial transcriptomic differences between NPC and PPLELC. A clearer understanding of the evolutionary advantages occurring *in vivo* in both NPC and PPLELC pathogenesis can elucidate fresh avenues for therapeutic intervention.

10 Conclusion

Though the role of EBV in the pathogenesis of NPC is well-described in existing literature, there is still much to be understood about the role of EBV in the pathogenesis of LELC. In this review, we summarized the genetic and environmental factors that influence susceptibility to NPC, and the role of EBV in the stepwise pathogenesis of NPC. We also summarized the differences and similarities of the roles of EBV in both NPC, EBVaGC, and postulated its role in LELC pathogenesis. With increasing understanding of the role of EBV in LELC pathogenesis, better therapeutics can be developed, and novel more specific drug targets can be elucidated to improve the treatment options of EBV driven cancers.

Author contributions

YL and CL contributed equally to manuscript writing and share first authorship. AC contributed to manuscript writing. DP contributed to the illustration of the manuscript. SH and HT both contributed to conception, guidance, and manuscript writing. All authors contributed to the article and approved the submitted version.

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