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Epstein–Barr virus and carcinogenesis: beyond Burkitt's lymphoma

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Abstract

Subsequent to its discovery over 45 years ago, Epstein–Barr virus (EBV) has been associated with numerous human carcinomas. Approximately 95% of the world's population sustain an asymptomatic life-long EBV infection. EBV persists in the memory B cell pool of normal healthy individuals and any disruption of this interaction results in virus-associated B cell tumours. The association of EBV with epithelial cell tumours, specifically nasopharyngeal carcinoma and EBV-positive gastric carcinoma, is less clear and is currently considered to be a consequence of the aberrant establishment of virus latency in epithelial cells displaying pre-malignant genetic changes. Although the precise role of EBV in the carcinogenic process is currently poorly understood, the presence of the virus in all tumour cells provides opportunities for the development of novel therapeutic and diagnostic approaches. The study of EBV and its role in carcinomas continues to provide insights into the carcinogenic process that are relevant to a broader understanding of tumour pathogenesis and to the development of targeted cancer therapies.

Keywords: Carcinogenesis, EBV, EBV-GC, epithelial, gastric, NPC, review

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Introduction

In 1958 Denis Burkitt, [1] a British surgeon, described a novel tumour common to children in equatorial Africa that was subsequently termed Burkitt's lymphoma (BL). Originally, it was hypothesized that BL was linked to an 'arthropod-borne' infectious agent, owing to the fact that its geographical distribution was dependent on climatic factors [2]. In 1964, Epstein *et al.* [3] successfully used electron microscopy to identify herpesvirus-like particles in a cell line established from a BL biopsy, which was later classified as Epstein–Barr virus (EBV) (also known as human herpesvirus-4). The causal link between EBV and BL was corroborated by evidence showing that BL patient sera had elevated antibodies to EBV antigens [4]. This group also established a link between primary EBV infection and infectious mononucleosis [5] and, subsequently, the association of EBV with the then-called lymphoepithelioma, nasopharyngeal carcinoma (NPC) [6,7].

The oncogenic potential of EBV was further realized through the association with numerous human malignancies. In addition to endemic BL and NPC, EBV was later found in a proportion of cases of Hodgkin's lymphoma (HL), post-transplant lymphoproliferative diseases, some T-cell lymphomas and a proportion of cases of gastric carcinomas (EBV-GC) [8]. Research is currently ongoing to determine the role of EBV-encoded gene products in these different cellular environments in an attempt to understand the role that EBV plays in the pathogenesis of these malignancies. Current knowledge about the biological properties of the individual genes has been reviewed in detail by Young and Rickinson [9].

Much of the known biology of EBV relates to its interaction with B-lymphocytes. This is mainly a result of the ability of EBV to readily infect and transform normal resting B-lymphocytes *in vitro*, which also confirms the B-lymphotropic nature of this virus. EBV latent gene expression in various EBV-associated malignancies and EBV-derived cell lines has led to the identification of three different and distinct latency programmes. These latency programmes are the result of differential promoter activity and are influenced by host cell factors.

Latency type 0: This is a controversial latency designation with a putative role in EBV persistence in B cells, where infected cells express no detectable latent mRNA or proteins.

Latency type I: As characterized by BL: the expression of the EBV-encoded RNAs (EBERs) and the BamHI-A rightward transcripts (BARTs) are observed in addition to Qp promoter-induced EBV nuclear antigen-1 (EBNA1) expression; the expression of all other EBNAs and the latent membrane proteins-1, -2A and -2B (LMP1, LMP2A and LMP2B) is not observed.

Latency type I: As characterized by NPC, EBV-GC and EBV-positive HL: in addition to the expression of the EBERs, BARTs and Qp promoter-driven EBNA1, the expression of the latent membrane proteins (LMP1, LMP2A and LMP2B) is detected to varying degrees; all other EBNAs are absent.

Latency type III: As characterized by lymphoblastoid cell lines and post-transplant lymphoproliferative disease: the full spectrum of latent gene products are expressed, which includes EBNAs 1, 2, 3A, 3B, 3C and -LP that are spliced from a single poly-cistronic transcript from the Cp/Wp promoter, the expression of all three latent membrane proteins (LMP1, LMP2A and LMP2B) and the EBER and BART RNAs.

Although these classifications of latency are useful in defining the different distinct gene expression programmes, they are by no means completely definitive [10]. In recent years, there has been increasing interest in the presence of different viral and cellular micro-RNAs in EBV-infected B cells and epithelial cells [11]. Roles for EBV-encoded micro-RNAs in the transcriptional regulation of both the viral and cellular genome have been described, but much more work is required to characterize the function of these RNAs.

Both benign and malignant conditions, which vary in severity, duration and pathology, are associated with EBV [12]. The development of specific monoclonal antibodies to viral proteins and sensitive *in situ* hybridization has allowed the detection of both latent and lytic antigens and viral DNA/RNA in these disease states. The contribution made by EBV and the individual viral genes to the pathogenesis of many of these malignancies is continuously being explored. This review will concentrate on the evidence available, supporting the association of EBV with epithelial carcinomas.

EBV and Nasopharyngeal Carcinoma: Epidemiology, Pathogenesis and Clinical Implications

The World Health Organization (WHO) has classified NPC into two main histological types: keratinizing squamous cell carcinoma (WHO1) and non-keratinizing squamous cell carcinoma (WHO2/3). The non-keratinizing type is further subdivided into differentiated non-keratinizing (WHO2) and undifferentiated carcinomas (WHO3) [13]. NPC is a tumour of the surface epithelium, often presenting as a neck mass or with symptoms of nasal obstruction and the loss of hearing. The well differentiated keratinizing NPC (WHO1) accounts for 20% of all NPC cases, whereas the remaining 80% of non-keratinizing NPC cases are split between differentiated and undifferentiated NPC. It is the WHO2 and WHO3 types that are distinct from all other squamous cell carcinomas because of their universal association with EBV. EBV exists in a latent state in this undifferentiated carcinoma, exclusively in the tumour cells, and absent from the surrounding lymphoid infiltrate [14,15]. However, the interaction between the prominent lymphoid stroma and adjacent carcinoma cells appears to be crucial for the continued growth of the malignant NPC cells.

Similar to BL, NPC has a distinctive geographical distribution. NPC is most common in southern China, where it accounts for approximately 20% of all adult cancers in this region, with 25–30 cases per 100 000 population in Canton and Hong Kong. NPC is very rare in Europe and North America, where the incidence rate is <1 per 100 000 population [16]. EBV-associated NPC have been identified in Eskimos as far afield as Alaska and Greenland. In 2000, 64 798 new cases were registered worldwide, with 80% of cases being in China, Southeast Asia and other Asian countries [17]. Interestingly, the incidence rates of NPC vary greatly within the Chinese population, decreasing from south to north, where approximately two or three cases per 100 000 population per year are observed among Chinese men in the northernmost provinces [18].

The association of EBV with NPC was suggested when serological studies identified a link between EBV and the development of NPC [19,20]. Examination of DNA extracted from undifferentiated NPC's revealed that all cases, taken from high, intermediate and low incidence areas, were consistently positive for EBV [21]. *In situ* hybridization techniques further confirmed the presence of EBV DNA in the tumour cells of virtually all low-grade differentiated or undifferentiated tumours [7].

Many epidemiological studies have been performed concerning NPC, and three well-defined aetiological factors involved in its pathogenesis have now been identified. These include a genetic susceptibility in some individuals (particular human leukocyte antigen haplotypes), an early-age exposure to chemical carcinogens (particularly of Cantonese salted fish) and an association with a latent EBV infection [22,23]. However, the incidence of NPC has begun to decline in the past 25 years and this correlates with the declining use of salted fish as part of children's diet, further demonstrating

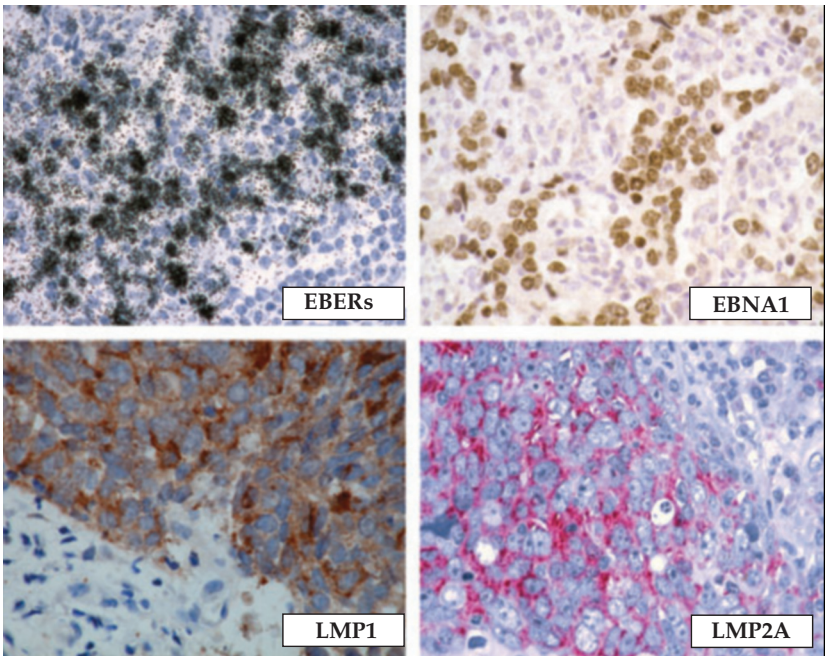

FIG. 1. Epstein–Barr virus (EBV) latent gene expression in nasopharyngeal carcinoma (NPC). *In situ* hybridization to the abundant EBV-encoded EBV-encoded RNA (EBER) transcripts (left, upper panel) is the standard approach for detecting EBV infection in cells and tissues. Immunohistochemical staining of NPC confirms EBV nuclear antigen-1 (EBNA1) expression in every tumour cell (right, upper panel). The expression of latent membrane protein (LMP)1 and LMP2A in NPC biopsies (lower panels) is more variable. Note the prominent lymphoid infiltrate in NPC, which is considered to contribute to the growth and survival of the tumour cells.

that a combination of both environmental and genetic factors contributes to the progression of NPC [16].

Analysis of EBV termini in NPC tumours has revealed the presence of clonal EBV genomes, suggesting that these carcinomas arise from the clonal expansion of a single EBV-infected progenitor cell [24]. Similar to most EBV-associated malignancies, the exact role of EBV in NPC pathogenesis remains poorly defined. The development and progression of NPC involves the accumulation of a number of genetic changes. Both genetic (e.g. gene amplification, deletion and mutation) and epigenetic (methylation) changes can affect the development of NPC by altering the functions of genes that are critical for proliferation, apoptosis, and differentiation [25].

In NPC, EBV adopts a type II latency programme, similar to that observed in EBV-positive HL (Fig. 1). Studies have confirmed the presence of LMP2A mRNA transcripts in a high proportion of NPC cases [26,27], and such findings that have been corroborated by the observation of LMP2A protein expression by immunohistochemistry in almost 50% of NPC cases [28]. The presence of LMP1 in NPC tumours is variable. Immunohistochemical and western blotting analysis have confirmed expression of LMP1 protein in 20–65% of cases, whereas the use of more sensitive methods, including nested RT-PCR, increases this number to >90% of cases [26]. Interestingly, LMP1 expression in NPC is associated with a better prognosis as a result of the ability of LMP1 to induce the host immune responses [29,30]. Although NPC tumours adopt a latent form of infection, the expression of immediate early proteins, indicative of lytic replication, has been detected in EBV-expressing tumour cells [31], suggesting that low-level lytic replication can occur in NPC tumours. Studies using real-time quantitative PCR to measure circulating tumour-derived EBV DNA in the blood of NPC patients have shown that the level of pre-treatment EBV DNA is strongly associated with overall survival, and that post-treatment EBV DNA levels predict the progression toward overall survival [32]. This approach is being applied in large-scale screening trials as an approach for early disease diagnosis.

The identification of genetic changes in pre-malignant lesions and NPC tumours has led to the proposal of a multi-step model for the pathogenesis of NPC [14,15,25]. Genome-wide analyses of genetic alterations in NPC have revealed consistent genetic losses at high frequencies on multiple chromosomal arms, including 3p, 9p, 9q, 11q, 14q and 16q. Recurrent chromosomal gains were also identified on chromosomes 1q, 3q, 8q, 12p and 12q. The most common genetic change was the loss of chromosome regions on 9p21 and 3p, which is thought to occur early during NPC pathogenesis [33,34]. More recent findings showed that the southern Chinese population in Hong Kong (a population at high risk for development of NPC) have a higher frequency of 3p/9p losses in the normal nasopharyngeal epithelium compared to the low-risk Chinese populations [16]. This leads to the conclusion that the elevated frequencies in 3p and 9q loss may predispose nasopharyngeal cells to facilitate latent EBV infection and this is a crucial event in the multi-step progression towards NPC. Although the role that these genetic alterations play in NPC pathogenesis remains

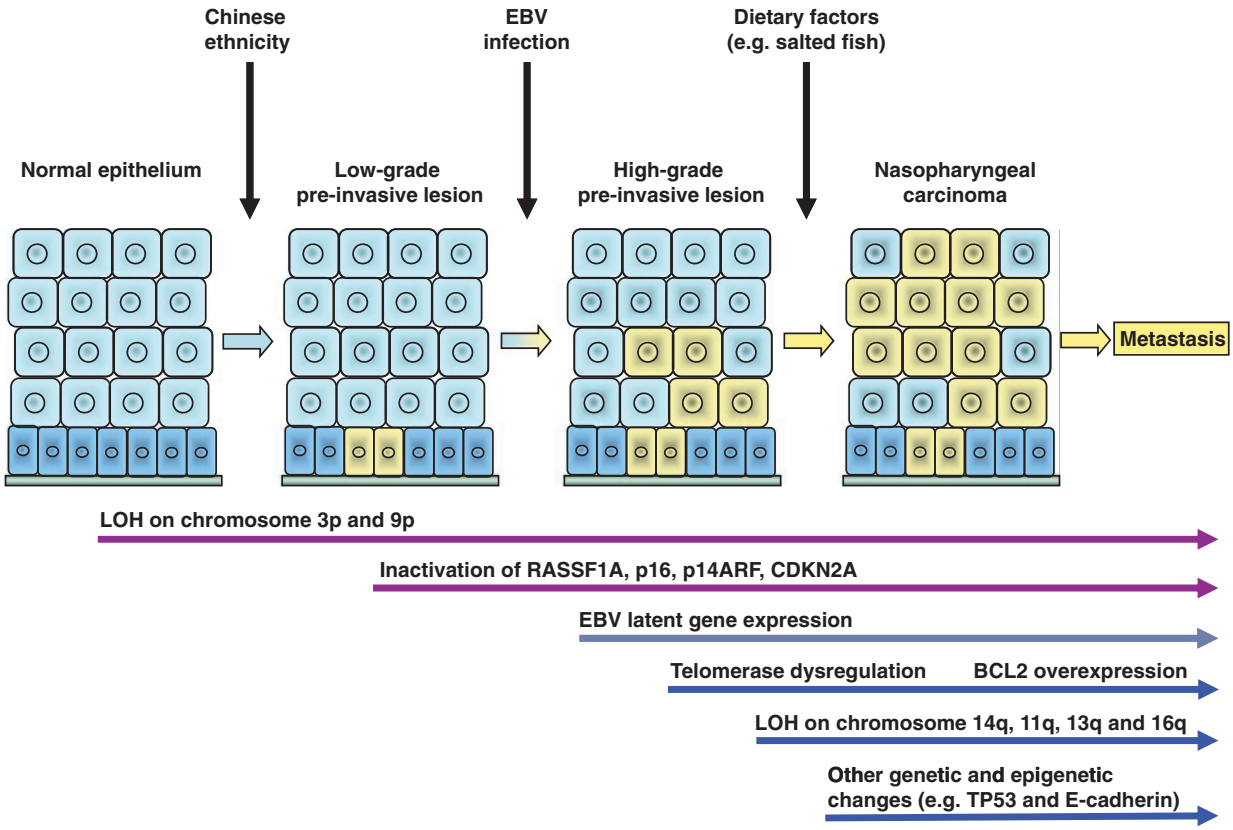

FIG. 2. Schematic representation of the pathogenesis of nasopharyngeal carcinoma (NPC). This model highlights the multi-stepped process that leads to the development of NPC. Epstein–Barr virus (EBV) infection alone cannot drive normal cells towards carcinoma development. It is thought that loss of heterozygosity (LOH), possibly as a result of inherited traits (Chinese ethnicity) as well as exposure to dietary factors (salted fish) and other environmental cofactors, is an early stage event in the pathogenesis of this disease. It is within these low-grade pre-invasive lesions, subsequent to further genetic and epigenetic alterations, where EBV infection occurs. The expression of EBV latent genes provides growth and survival advantages to these infected cells, ultimately leading to the development of NPC. Further genetic and epigenetic alterations post-NPC development can occur, which may result in a more metastatic disease.

to be identified, the role that certain genes play has been confirmed. The introduction of either p16 or RASSF1A (which are important in cell growth regulation and are located on 9p and 3p, respectively) in the C666.1 NPC cell line resulted in inhibition of cell growth, a marked reduction in soft-agar colony formation, and, more importantly, a reduction in the tumourigenic potential of cells in athymic nude mice [35].

Taken together, these data suggest that, unlike EBV-associated B cell tumours, where the virus is considered to be an initiating factor in the oncogenic process, virus infection in the context of NPC pathogenesis behaves as a tumour-promoting agent (Fig. 2). It is possible that EBV infection of normal differentiating epithelial cells results in virus replication, whereas, in epithelial cells that are unable to differentiate (perhaps as a consequence of genetic and epigenetic alterations), EBV is able to establish a latent infection that contributes to malignant progression.

EBV and Gastric Carcinoma: Epidemiology, Pathogenesis and Clinical Implications

Gastric carcinoma is the second most common carcinoma worldwide [36] and is divided into two main types: gastric cardia cancer, a cancer of the top inch of the stomach where it meets the esophagus; and noncardia gastric cancer, a cancer in all other areas of the stomach. Overall gastric cancer incidence rates are decreasing, however; this decline is mainly in noncardia gastric cancer rates. By contrast, gastric cardia cancer rates are increasing, particularly in Western countries such as the USA and many parts of Europe. The large variations in incidence and mortality suggest an important role of environmental factors in gastric cancer risk.

The WHO reports that almost half of the world population is infected with *Helicobacter pylori*, a bacterium that

establishes long-term infection of the gastric mucosa. Subsequent to its discovery in 1982 by Warren and Marshall (who were awarded the 2005 Nobel Prize in Medicine), *H. pylori* has been associated with gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma and gastric cancer. Almost 63% of noncardia gastric cancer worldwide is a result of *H. pylori* infection. Despite the strong association of *H. pylori* with gastric cancer, the majority of infected individuals do not develop gastric cancer. This has prompted the search for bacterial, host and environmental co-factors that explain why some infections progress to gastric cancer.

EBV is associated with approximately 10% of more typical gastric adenocarcinomas (GC), accounting for up to 90 000 new cases worldwide per year [37]. EBV-GC presents as two histomorphologically distinct forms: a rare lymphoepithelioma-like carcinoma, similar in appearance to NPC, and a common gastric carcinoma type (glandular adenocarcinoma). The relative ratio of the two types is 1 : 4 respectively, and, similar to BL and NPC, is more frequent among males. RT-PCR and *in situ* hybridization techniques were used to confirm the presence of EBV in almost 90% of gastric lymphoepithelioma-like carcinoma cases, ranging in morphology from the poorly and moderately differentiated tumours to the well differentiated tumours [37]. EBV infection is observed to occur mostly in the upper middle portions of the stomach rather than the lower part of the stomach [38]. EBV infection is also associated with primary gastric carcinoma of the lymphoepithelioma type [39,40].

There is significant geographical variation in the association of EBV with GC, which may be attributed to ethnic and genetic differences. Gastric carcinoma is one of the most common cancers in Japan, with approximately 7% being EBV-positive gastric carcinomas. Epidemiological studies have suggested that EBV-GC is related to birth order, high salt intake, and exposure to metal dust, although these factors may vary geographically (e.g. between Japan and Colombia), supporting the need for more detailed investigation [41].

Similar to NPC, EBV-GC tumours display a type II latency program of EBV latent gene expression [42]. EBV-GCs have distinct phenotypic and clinical characteristics compared to EBV-negative GC, including the loss of p16 expression, p73 promoter methylation, wild-type p53, a different pattern of allelic loss, and improved patient survival [43–46]. As in NPC, the precise role of EBV in the pathogenesis of gastric carcinoma remains to be determined, although the absence of EBV infection in premalignant gastric lesions supports the contention that virus infection is a relatively late event in gastric carcinogenesis [47].

Is EBV Associated with Other Common Epithelial Malignancies?

A number of other more common carcinomas, such as breast cancer [48] and liver cancer [49], have been reported to be infected with EBV. Difficulties in confirming these associations have raised concerns about the use of PCR analysis alone to define EBV association and about the specificity of certain monoclonal antibody reagents. Definitive designation of a tumour as 'EBV-associated' should require unequivocal demonstration of the EBV genome or virus gene products within the majority of the tumour cell population. This is not the case with breast cancer, where it is clear that a small and extremely variable proportion of tumour cells are susceptible to EBV infection *in vivo*, resulting in a low level lytic EBV infection [50]. A subset of EBV-infected breast carcinoma cells undergoing the virus lytic cycle may produce soluble factors that are able to influence the growth and survival of surrounding EBV-negative tumour cells, but this remains to be demonstrated. The association of EBV with liver cancer, which was originally described in Japanese cases, has not been confirmed in cases from Europe and the USA, raising the possibility of geographical variation [51].

Conclusions

EBV was discovered over 45 years ago and its DNA was fully sequenced in 1984. It remains the most common persistent virus infection in humans, with over 95% of the population sustaining an asymptomatic life-long infection, which is testimony to the intimate interaction between EBV and the immune host. This relationship relies on the ability of EBV to persist in the memory B cell pool of normal healthy individuals and perturbation of this interaction results in virus-associated B cell tumours. The association of EBV with NPC and EBV-GC is less clear and may be a consequence of the aberrant establishment of virus latency in epithelial cells that have already undergone pre-malignant genetic changes.

In striking contrast to EBV-infected B cells, epithelial cells (of either primary or transformed origin) infected with EBV are difficult to maintain in continuous passage *in vitro*. Even when successfully infected, transformed epithelial cell lines tend to lose the EBV genome on serial passage. Therefore, *in vitro* epithelial cell model systems have been generated using recombinant strains of EBV with drug selectable markers to ascertain the impact of cellular and viral factors to the persistence and stability of virus infection [52].

Whatever the nature of these interactions and the precise role of EBV in the carcinogenic process, there is clearly an opportunity to exploit this association for the clinical benefit of patients. NPC is highly radiosensitive and there is a high cure rate for those patients who are diagnosed early; therefore, mass screening programmes are underway in Hong Kong to identify patients with early stages of NPC [53]. Alternative novel therapeutic approaches are currently being explored with gene therapy [54] or therapeutic vaccinations [55], showing promise for the ability to effectively target EBV-associated carcinomas.

The strategy of choice when considering novel treatments for EBV-associated epithelial carcinomas is to use an epitope-based vaccination approach, which aims to boost EBV specific cytotoxic T-lymphocyte responses to the infected cells. This approach has already shown some promise using LMP1 and LMP2 epitopes, as a polyepitope vaccine [56] or using pulsed dendritic cells to boost the cytotoxic T-lymphocyte response [57]. These studies are paradigms for the development of targeted cancer therapies and diagnostics, and they further confirm the far-reaching value of tumour virology to the entire field of cancer.

The study of EBV and its role in carcinomas continues to provide insights into the carcinogenic process that are relevant to a broader understanding of tumour pathogenesis and the development of targeted cancer therapies.

Transparency Declaration

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